THE DETECTION OF BACTERIAL POPULATIONS BY FLUORESCENCE

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POPULATIONS BY

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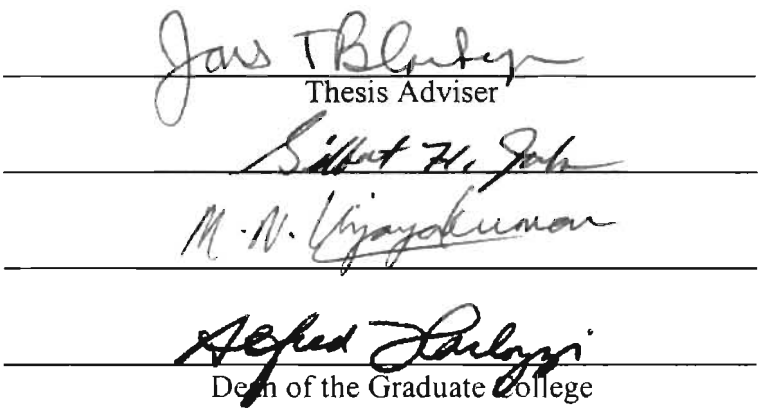

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PREFACE

This study was conducted to characterize the autofluorescence of *Escherichia coli*, *Salmonella typhimurium*, and *Staphylococcus aureus*. This study also compares the autofluorescence of *E. coli* to current fluorescent dye techniques.

I would like to thank my major advisor, Dr. James T. Blankemeyer, for superior guidance and supervision. I would also like to thank my other committee members, Dr. Moses Vijayakumar and Dr. Gilbert John, for advice and critical reading of manuscripts. I would like to extend my appreciation to the Oklahoma Center for the Advancement of Science and Technology (OCAST) and The Center for Sensors and Sensor Technology for their financial support. I would also like to thank Sandy Peterson for her technical assistance. A very special thanks goes to two of the best lab technologists I've ever had the privilege of working with, Shelley Theiss and Wambui Machua.

TABLE OF CONTENTS

Chapter		Page
1.	INTRODUCTION AND REVIEW OF THE LITERATURE	1
	Scope and purpose	1
	Enumeration techniques	5
	Optics in Biology	7
	Fluorescent techniques	9
	Autofluorescence	11
2.	AUTOFLUORESCENCE OF ESCHERICHIA COLI, SALMONELLA TYPHIMURIUM, AND STAPHYLOCOCCUS AUREUS	15
	Introduction	15
	Methods and Materials	17
	Results	19
	Discussion	23
3.	COMPARISON OF AUTOFLUORESCENCE OF E.COLI TO DILUTIONS AND FLUORESCENT DYE TECHNIQUES	31
	Introduction	31
	Methods and Materials	32
	Results	35
	Discussion	37
4.	CONCLUSION	43
5.	REFERENCES	45

LIST OF FIGURES

Figure — Page

1. Form of a Jablonski Diagram 14

2. Schematic Diagram of the Spectrofluorimeter 25

3. Fluorescent Excitation Spectra of *E. coli*, *S. typhimurium*, and *S. aureus* with Emission Wavelength At 425nm 26

4. Fluorescent Excitation Spectra of *E. coli*, *S. typhimurium*, and *S. aureus* with Emission Wavelength At 550nm 27

5. Fluorescent Excitation Spectra of *E. coli*, *S. typhimurium*, and *S. aureus* with Emission Wavelength At 650nm 28

6. Fluorescent Excitation Spectra of *E. coli* Grown in Different Media 29

7. Fluorescent Excitation Spectra of E. coli At different Growth Stages 30

8. Dilution Response Curve for *E. coli* 38

9. Fluorescent Emission Spectra of *E. coli* 39

10. Fluorescent Emission Spectra of *E. coli* Treated with Di-8-ANEPPS 40

11. Fluorescent Emission Spectra of *E. coli* 41

12. Fluorescent Emission Spectra of *E.coli* Treated with the BacLight Viability Kit 42

Chapter 1

Introduction and Review of Literature

Scope and Purpose

The detection of bacteria is a problem of both medical and economic importance. Food related pathogens such as *Staphylococcus* and *Salmonella* can cause serious illnesses while recent outbreaks of *Escherichia coli* and *Listeria* occuring in commonly eaten foods such as hot-dogs and lunch meats, show there is a need for quickly, efficiently, and economically detecting bacteria (16). Increased government regulations of the food industry have prompted a need for better tests for microbiological contamination.

Escherichia coli and *Salmonella typhimurium* are two of the serious food related pathogens chosen for this study. Food poisoning by *E. coli* involves the ingestion of contaminated water, raw vegetables, cheeses, and raw or rare beef (36). Recently, there have been reports of *E. coli* outbreaks due to contaminated, unpasteurized apple cider. *S. typhimurium* can be a contaminant of water, poultry, eggs, dairy products, and meats(36).

Both *E. coli* and *S. typhimurium* are members of the *Enterobacteriaceae* family and have many common characteristics. Both are gram-negative, nonspore-forming facultative anaerobes that are motile with peritrichous flagella. They vary in length from 1 to 8 μm and ferment glucose, but do not produce oxidase (23). Pathogenicity of the *Enterobacteriaceae* family comes from complex surface antigens and on the basis of immune responses. Antigens are classified H for the flagellar antigen, K for the capsule and/or fimbrial antigen, and O for the cell wall antigen (36). *E. coli* classification by

serotype is difficult because there are over 160 O antigens, over 80 K antigens and over 55 H antigens. Pathogenicity can also be due to endotoxins. *E. coli* produces two endotoxins: heat-labile toxin (LT) and heat-stable toxin (ST). These two endotoxins initiate severe diarrheal illnesses by stimulating secretion and fluid loss. *E. coli* can be divided into four groups based on pathogenicity: enterotoxigenic, enteroinvasive, enteropathogenic, and enterohemorrhagic. All of these groups are transmitted through the ingestion of contaminated food or water. Enterotoxigenic *E. coli* is the major cause of infant diarrhea and traveler's diarrhea. Virulence factors assocoated with this group are LT and ST endotoxins, and fimbrial adhesion called colonization factor (CFA). Enteroinvasive *E. coli* causes diarrhea in adults and infants and resembles Shigella in its pathogenicity because it causes epithelial cell death. Virulence factors associated to this group are several outer proteins that are similar to Shigella and the production of a Shiga-like toxin. Enteropathogenic *E.coli* is not well defined. It is the most important cause of infant diarrhea in some countries. This group does not produce any toxins and the adherence factor seems to be responsible for the illness. Enterohemorrhagic *E. coli* (EHEC) is the most virulent of the four groups. It is the major cause of gastrointestinal illness in the United States, Canada, and Great Britain. All of the serotypes for this group produce a Shiga-like toxin (6).

Salmonella can lead to gastroenteritis, enteric fever, and bacteremia. *S. typhimurium*, which is one of the more invasive species, is involved in bacteremia, which can lead to metatastic infections that involve the bones and joints, the cardiovascular system, and the meninges(6).

Staphylococcus aureus is the third bacterium used in this study. Some strains of *S. aureus* produce an enterotoxin that is associated with food poisoning. This toxin is excreted into foods and is extremely heat stabile; the enterotoxin can withstand 30 minutes of boiling. Food poisoning from *S. aureus* occurs through ingestion of contaminated foods, especially in foods high in protein (6).

S. aureus is a member of the *Micrococcaceae* family. The genus *Staphylococcus* is a gram-positive, non-sporeforming, facultative anaerobe (36). Morphology of Staphylococcus consists of spherical cells ranging from 0.5 to 1.5μm in diameter occurring singly, in pairs or tetrads. They lack flagella and are sometimes encapsulated. They are usually catalase-positive and can grow in temperatures between 18 and 40°C (35). Most of the members of this genus are considered to be part of the normal human microflora. Three species are considered pathogenic: *S. aureus*, *S. epidermidis*, and *S. saprophyticus* (36).

Pathogenicity of *Staphylococcus* is dependent on coagulase, hemolysins, leukocidin and protein A. Coagulase is a plasma clotting protein that coats staphylococci with fibrin and may prevent phagocytosis (6). *S. aureus* is the only coagulase-positive species of *Staphylococcus* (36). Hemolysins or exotoxins are cytotoxic to red blood cells and are classified alpha, beta, gamma, and delta. Only alpha and delta toxins are of major importance in human infections. Leukocidin differs from hemolysin by increasing the permeability of leukocytes to cations. This results in the release of cyotplasmic granuals and cell disruption. Protein A is a surface toxin linked to the peptidoglycan layer of the cell wall. It is believed that protein A acts as an antiphagocytic component for *S. aureus* because it competes with neutrophils by binding immunoglobin.

E. coli, *S. typhimurium*, and *S. aureus* were chosen for this study not only because of their pathogenic features, but because they are also available as fairly noninfectious strains. These three bacteria are also easy to grow and maintain in a laboratory setting.

Enumeration Techniques

There are several methods for enumerating bacteria, but plate counts remain the most common method for estimating viable cells (20). In this method, dilutions of bacteria are plated on agar plates, incubated for at least 24 hours, and then colonies are counted. The original concentration can then be determined by calculating the original cell density. Although there are improved plate-counting techniques, this procedure is still time consuming and expensive (30). Also, bacteria must compete for nutrients, thus inhibiting colony formation and growth (20). Besides these limitations, plate counting is also very labor intensive due to preparation of media and the allotted time needed for cell growth (3, 20, 21, 30). One alternative method that shows promise is the use of a fluorescence to identify and count bacteria.

Two things can occur when a wavelength of light strikes a molecule: it can be scattered or absorbed. A molecule can only absorb a certain wavelength of light. When a particular wavelength of light cannot be absorbed, the molecule re-radiates or scatters that wavelength. The light can be scattered in any direction. The light scattered by a molecule is inversely dependent on the fourth power of the wavelength of light. Turbidimetry and nephelometry are two methods that utilize scatter for the detection of bacterial concentrations.

Turbidimetry measures forward scatter from the bacteria. The larger the concentration of the bacterial suspension, the more light is scattered. Most instruments that measure turbidity use absorbance scales or optical density (OD) and is proportional to the log of the percent transmittance(28). Nephelometry measures the light scattered at

a 90° angle. This process can use a spectrofluorimeter set so that the excitation and emission monochromator are set to the same wavelength.

Another instrument that utilizes scatter for detection purposes is a Klett-Summerson colorimeter. The Klett-Summerson colorimeter was one of the first photoelectric instruments used for the detection of bacteria and measures the forward scatter of light. This instrument differs from turbidimetry instruments in that it does not use an absorbance scale, but rather a Klett scale.

Flow cytometry utilizes both scatter and fluorescence to detect and count bacteria. This method involves the use of fluorescent rRNA-targeted probes, fluorescent antibodies, or the *gfp* marker gene (20). Flow cytometry detects the bacteria based on its fluorescent intensity and scatter (38). In Flow cytometry, the fluorescent probe attached to the bacteria is excited and then side scatter and forward scatter are recorded (20).

Optics in Biology

The amount of light a molecule can absorb at a particular wavelength can be expressed by the Beer-Lambert law, where absorbance (A) equals the log of incident light (I_o) divided by transmitted light (I): $A=\log I_o/I$. Once a molecule absorbs a wavelength of light, it becomes promoted to an excited state. This means that an electron in the atom has been moved to an outer orbital. This is a very unstable state for a molecule and therefore the molecule must dissipate the excitation energy (39).

A Jablonski diagram is the best way to illustrate the process that occurs between absorption and emission (Figure 1) (24). Internal conversion is a process by which the excitation energy is dissipated as vibrational energy or heat. Luminescence occurs when a molecule emits light after becoming electrically excited and can be divided into fluorescence and phosphorescence. Light emitted from a triplet excited state is referred to as phosphorescence. Phosphorescence emission rates are very slow and lifetimes can be milliseconds to seconds. Fluorescence occurs when light is emitted from a singlet excited state molecule. Fluorescence emission rates occur much faster and have lifetimes of about 10ns. It was observed by Sir G.G. Stokes that the emission energy is typically less than the energy absorbed by the molecule. Therefore, fluorescence emission is always of lower energy or longer wavelength than the absorbed wavelength. This difference is known as Stokes' Shift (24).

There are several problems in the detection of fluorescence from a molecule. One of these is predicting the wavelengths at which the molecule is going to fluoresce. Quenching is another problem that can arise in detecting fluorescence. Fluorescence quenching is any process that decreases fluorescent intensity and can occur by several

different mechanisms. Molecular interactions such as excited-state reactions, molecular rearrangement, or energy transfer can result in quenching. Quenching can also occur due to optical properties of the sample such as turbidity. This occurs when unexcited molecules absorb the fluorescent emission from an excited molecule, thus decreasing fluorescent intensity (24).

Fluorescent Techniques

Fluorescence-based detection of cells typically has a high signal to noise ratio (8, 9). That is, bacteria could be detected at very low concentrations. This is because measurements are taken at a 90° angle and only scattered light and fluorescence emission from the molecule at a selected wavelength are detected. In current fluorescent enumeration techniques, a dye is added to the bacteria. The dye is excited and fluorescence of the dye and attached bacterium is detected (41). Three dyes that have been used are the BacLight Viability Kit, Di-8-ANEPPS and CTC dye.

The BacLight Viability Kit allows for the detection of both live and dead cells by staining dead cells red and live cells green (19). At an excitation wavelength of 480nm, dead cells fluoresce between emission wavelengths of 600 and 650nm while live cells fluoresce between emission wavelengths of 500 and 550nm. Because this dye does not penetrate intact cell membranes, it is commonly used in flow cytometry to measure complement activity. Terzieva et al. found that the BacLight dye underestimates the actual viable cell concentrations. Cells with compromised membranes that can still reproduce are often stained red (37).

The Di-8-ANEPPS is often used in studying changes in membrane potentials, and is routinely applied in vertebrate studies (10, 11, 19). It is an electrochromic probe that is suitable for monitoring membrane potential changes by an electrochromic shift and has spectral properties that are largely dependent on its environment (9, 11). There are two major drawbacks of Di-8-ANEPPS. First, there can be fluctuations in staining of the cell membrane (10, 11). Secondly, the addition of the dye can result in the production of reactive oxygen species that can damage the cell membrane (11).

The CTC dye (5-cyano-2,3-ditolyl tetrazolium chloride) is used in the enumeration of active cells by measuring electron transport chain activity. Active cells are detected by the intracellular formation of insoluble formazan crystals. The CTC dye is a class of monotetrazolium redox dyes that produces fluorescent formazan when reduced. It gives a bright red fluorescence when illuminated by long-wave UV light. Unfortunately, CTC dye has the tendency to change colors depending on the oxidative state of the cell. It is also an irritant and very toxic if ingested (19).

Fluorescing dyes can be expensive, somewhat toxic, and at times, give inaccurate results. An ideal method for detecting and quantifying bacteria would be one that does not require the use of fluorescing dyes. Self-fluorescence or autofluorescence would be detected as opposed to a dye.

Autofluorescence

Autofluorescence is the fluorescence of a molecule without the addition of a dye. The first recorded observation of fluorescence was made in 1565 by Nicolas Monardes. Monardes described an unusual wood that when shredded and put into a solution of water emitted a blue tinge when exposed to room lighting (18). This solution was called "Lignum Nephriticum", the same solution described by Robert Boyl. Neither Boyl nor Monardes understood the phenomenon because manmade excitation sources were unavailable for the observations of the blue fluorescence in the dark (5, 32).

Equipment for such observations became available in 1852. The equipment clearly established that radiation was first absorbed and then reemitted at a wavelength of lower energy. Since then many organic compounds have been found to be autofluorescent.

Aromatic amino acids, lysosomes, and mitochondria are examples of fluorescing organic compounds (2, 8). Most fluorescent organic molecules emit in the 300 to 500nm region and appear violet or blue. The aromatic amino acid tryptophan is one of the more dominant fluorophores, emitting near the wavelength of 340nm (24). Proteins containing anyone of the aromatic amino acids, such as tryptophan, tyrosine or phenylalanine, fluoresce in the region of 340nm (32).

The autofluorescence of lysosomes is due to lipofuscin, an autofluorescent age pigment. The major component of lipofuscin is malonaldehyde, which is a major product of lipid peroxidation in the lysosomes (2).

The intramitochondrial NADH pool is responsible for a majority of the autofluorescence of mitochondria, but FAD contributes some as well (2). NADH has an

absorption maximum at 340nm and an emission maximum at 460nm. FAD absorbs around 450nm and emits around 525nm (24).

The use of autofluorescence is one of the oldest techniques and still one of the most active research fields in science (18). This technique has a wide range of uses from medical detection of diseases to biochemical analyses of proteins. Britton Chance and his colleagues were the first to rely on autofluorescence as a diagnostic tool by measuring the oxidation-reduction states of respiratory carriers. The oxidized form of NADH, NAD^+, is nonfluorescent. Also, fluorescence intensity of NADH increases when it becomes bound to a protein (24). Chance et al used these spectral properties to measure the metabolic response of rat tissues to various oxygen concentrations and to the addition of respiratory inhibitors (12).

Measurement of autofluorescence in lenses is a noninvasive technique for the measurement of advanced glycation end products (AGE) levels. Levels of AGE are generally higher in diabetic patients and are related to the degree of long-term glycemic control. Increased levels of AGE in lenses of diabetic patients have higher autofluorescence than that of lenses with normal AGE levels (1). AGEs are produced by the nonenzymatic reaction of glucose and other aldoses.

Autofluorescence has been used to detect dysplasia in colonic mucosa and to identify adenomatous polyps in the early detection of colon cancer (13, 40). Autofluorescence of normal cells is much higher than for cells with abnormalities. The autofluorescence spectra can be collected during colonoscopy by small non-imaging optical fiber probes.

Another technique relying on autofluorescence is one that involves fluorophores of dental caries. Caries lesions contain long-lived fluorophores that emit in the red spectral region. Healthy tissues contain only short-lived fluorophores whose decay time is much shorter than that of carious tissues. This autofluorescence is being used in the early detection of dental caries (22).

Autofluorescence can also be used for the detection of bacteria. A number of microorganisms have been found to fluoresce. Among these are several strains of thermophilic sulfate reducing *Archaebacteria*, *Streptomyces griseus*, several other *Actinomycetes*, and *Legionella* species. *Archaebacteria*, *Streptomyces griseus*, and *Actinomycetes* all exhibit a greenish fluorescence (14, 33, 42). Bacteria that possess the coenzyme F420 also exhibit a greenish fluorescence and is a very common occurrence in methanogens (42). Three *Legionella* species exhibit a bluish white autofluorescence while two others exhibit a red autofluorescence (7).

This study shows that *E. coli*, *S. typhimurium* and *S. aureus* also have an autofluorescence and that the autofluorescence spectrum detects bacteria in water and differentiates among these three bacteria. This study also compares the autofluorescence of *E. coli* to fluorescent dye techniques.

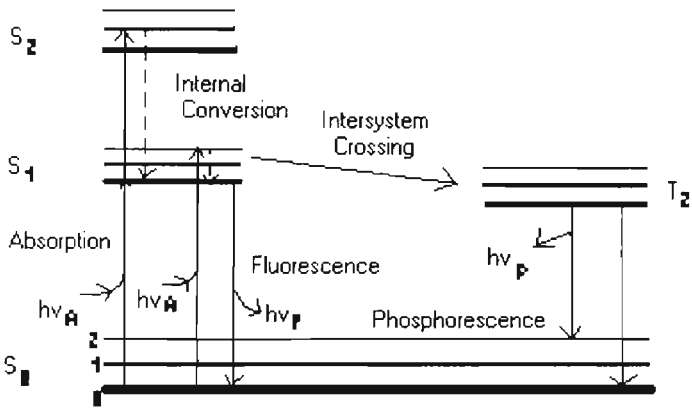

Figure 1: Form of a Jablonski diagram as described in *Principles of Fluorescence and Spectroscopy,* Second Edition, by Joseph R. Lakowics, copyright 1999.

Chapter 2

Autofluorescence of *Escherichia coli*, *Salmonella typhimurium*, And *Staphylococcus aureus*

Introduction

Detecting the presence of pathogenic bacteria is a problem with both medical and economic importance (3, 17). Plate counts are widely used for enumeration and remain the most common method for estimating viable cells, but plate counts do not detect dormant microorganisms or those with lag periods greater than incubation times. Although there are improved plate-counting techniques, this procedure is still time consuming and expensive (30). Also, bacterial colonies must compete for nutrients, thus inhibiting colony formation and growth (20). Besides these limitations, plate counting is also very labor intensive due to preparation of media and the allotted time needed for cell growth (3, 20, 21, 30).

Fluorescence-based detection of cells typically has a high signal to noise ratio (8, 9). That is, bacteria could be detected at very low concentrations. Current fluorescent-based techniques use expensive dyes to stain cells so that the cells can be easily detected. In some studies, dye-based fluorescence has resulted in inaccurate data (11, 25, 29, 34) (21, 27). Another type of fluorescence is autofluorescence occurring when a bacterium absorbs a wavelength of light and emits a photon without the addition of fluorescing dyes (41).

Autofluorescence is a useful method applied in chemistry and biochemistry, and we propose that it can also be applied to the detection of microorganisms (1, 2, 8, 13, 22, 40). Autofluorescence has been observed in many of the archaebacteria. *Archaeoglobus fulgidus* Strain Z exhibits a greenish fluorescence (42). A greenish fluorescence also

occurs in methanogens, *Streptomyces griseus* and several other *Actinomycetes* (14, 42). At least seven species of *Legionella* exhibit a bluish white fluorescence while two species of *Legionella* exhibit a red fluorescence (7). *Thermoplasma acidophilum* possess a pale yellowish-green fluorescence when viewed by UV fluorescence microscopy (33). This study shows that *E. coli*, *S. typhimurium*, and *S. aureus* have an autofluorescence and that autofluorescence differentiates among these bacterial genera.

Methods and Materials

Bacteria

E. coli C600, *S. aureus* ATCC #27697, and *S. choleraesuis* with serotype *Typhimurium* ATCC #29630 were used for this study. Tryptic soy broth was prepared from a mix provided by DIFCO laboratories; 5ml aliquots were distributed into vials and then autoclaved. Vials of prepared and autoclaved broth were inoculated with *E. coli*, *S. aureus*, and *S. typhimurium* and incubated at 37°C for 24hrs. Bacteria, in stationary phase, were centrifuged in a Fisher Scientific MICRO14 microcentrifuge by distributing 3ml of inoculated broth into three 2.0ml-microcentrifuge tubes and centrifuging for 5 min at 5×1000g. Supernatant was decanted and the cells washed with sterile reverse osmosis water (RO water). The cells were vortexed and centrifuged again for 5min at 5×1000g. Supernatant was decanted and the cells were resuspended in sterile RO water. Next, the 3ml of washed bacteria cells, along with 500ul of sterile RO water, were placed into a 4ml-quartz fluorimeter cuvette. The contents of the fluorimeter cuvette were then mixed to ensure uniformity by inverting the cuvette four to five times.

Fluorescence Characterization

To investigate autofluorescence of the bacteria, emission of the bacteria was detected at a fixed wavelength while the excitation wavelength was scanned. Each emission scan from the bacteria was compared to a negative control of sterile RO water or Tryptic Soy broth dilution by subtracting the control scan from the emission scan.

The data analysis was based on the collection of fluorescence data into data records. Each data record represented the scan of excitation wavelengths from low

wavelength to high wavelength and one specific emission wavelength. A scan number identified the data record.

In this study no fluorescing dyes or reagents were added to the bacteria. All data points represent the mean ± SEM for three independent experiments. Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparison test with GraphPad Prism version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com.

Instrumentation

Fluorescence characterization used a spectrofluorimeter (Figure 2). This spectrofluorimeter consisted of one excitation monochromator and one emission monochromator (Oriel). A tungsten halogen lamp was mounted so that it faced the entrance slits of the excitation monochromator. Both monochromators had manually controlled slits that selected the bandwidth. A sample chamber (Oriel) was mounted on the output of the excitation monochromator. This sample chamber held the 4ml cuvette and had emission ports at 90° angles to the illumination. It was also equipped with filter holders to place blocking filters in the emission/excitation light-path. The emission monochromator was connected to a photomultiplier tube (PMT - Hammamatsu R955). Emissions from the sample chamber were wavelength-selected by the emission monochromator, captured by the PMT, converted to a voltage, and collected by an attached computer. S-plus (MathSoft, Seattle, WA USA) was used to process the data and produce the graphical output.

Results

Fluorescent emission scans were performed on supernatants to rule out the autofluorescence of tryptic soy broth. Supernatant one was from the first centrifugation of bacteria, and should have only contained tryptic soy broth. There was no fluorescence detected for supernatant one. Supernatants from four washes were scanned for fluorescence. Only the fifth supernatant had detectable fluorescence.

Fluorescence emission of the bacteria was detected at a fixed emission wavelength while scanning the excitation wavelength. Emission wavelengths were scanned from 400 to 800nm in 25nm increments. Excitation wavelengths were scanned from 200 to 550nm in 1nm increments. Three emission wavelengths, 425nm, 550nm, and 650nm, were chosen for representation of bacterial fluorescence. Fig 2 represents the fluorescent excitation spectra of *E. coli*, *S. typhimurium*, and *S. aureus* at a fixed emission wavelength of 425nm. Bacteria were prepared as described in the Methods and placed in 4ml-quartz-fluorimeter cuvettes; cuvettes were then placed in the sample chamber. The x-axis of Fig 2 represents the excitation wavelength(200 to 350nm). The y-axis represents the relative fluorescence intensity. Fluorescence intensity is not an absolute metric: it is dependent on illumination, monochromators, and PMT characteristics. For Figs 2, 3, 4, 5, and 6, these variables were kept constant. For each emission and excitation wavelength scanned, a negative control of sterile RO water was also acquired. This negative control was then subtracted from the corresponding fluorescence of *E. coli*, *S. typhimurium*, or *S. aureus*. This process was repeated for all experiments. The data represents the mean ± SEM of three identical experiments for each bacterial species. Tukey's comparison of the

three bacteria revealed significant differences in fluorescence at 425nm emission wavelength among the three bacteria, with p-values less than .001 for all three. *S. aureus* had the highest peak fluorescence in all of the Figures, although at certain excitations the fluorescence values coincided for the three genera while at other wavelengths the fluorescence differed. In this Fig, *S. typhimurium* and *E. coli* appear similar even though Tukey's comparison of means show significant differences between the two; but if we reduce the y-axis to 0.5, many differences appear, particularly between the excitation wavelengths of 200 and 350nm. Fluorescence intensity is stronger in this region for *E. coli* than it is for *S. typhimurium*.

Fig 3 represents the fluorescence emission of the three bacteria with the emission wavelength at 550nm. As for Fig 2, the bacteria were treated as described in the methods and the data represents the mean ± SEM of three identical experiments for each bacteria. The x-axis represents the wavelength of excitation from 200 to 500nm. Tukey's comparison of the three bacteria showed significant differences in bacterial spectra with p-values less than 0.001 for all three comparisons. The fluorescence pattern of *S. aureus* between excitation wavelengths of 200 and 350nm differs from *E. coli* and *S. typhimurium* spectra in the same area. Fluorescence intensity gradually decreases for *S. aureus* up to an excitation wavelength of 350nm, but then increases after 350nm. Fluorescence of *E. coli* and *S. typhimurium* remains fairly constant up to 350nm excitation, and then gradually increases after 350nm excitation. Also, The fluorescence peak for *S. aureus* beginning at 400nm has an upward curve to it while those peaks for *E. coli* and *S. typhimurium* are flatter.

Fig 4 represents the fluorescence emission of the three bacteria with the emission wavelength at 650nm. Again, the bacteria were treated as described in the methods and the data represents the mean ± SEM of three identical experiments for each bacteria. The x-axis represents the wavelength of excitation from 300 to 550nm. Tuckey's comparison of the three bacteria showed significant differences among the three bacterial spectra with p-values less than 0.001. Fluorescence intensity is much lower at 650nm emission for all three bacteria than at 550nm emission. As with Fig 2, *S. typhimurium* and *E. coli* look very similar even though statistical analysis says otherwise. By reducing the y-axis to 0.5, the differences between these two become clearer. *E. coli* has a prominent peak that appears just after 450nm that is not present in *S. typhimurium*. There is also another peak between excitation wavelengths of 500 and 550nm for *E. coli* that is not present in *S. typhimurium*.

Statistical analysis was performed using GraphPad program with a one-way nonparametric ANOVA with Tukey's comparison of means in order to insure repeatability of the experiments. Tukey's comparison of the means revealed no significant differences among the three experiments for *E. coli*, with p-values greater than 0.05. The same statistical analysis was performed for three identical experiments of *S. typhimurium* and three identical experiemnts of *S. aureus*. Both revealed no significant differences among experiments.

Fluorescence of *E. coli* grown to stationary phase in Tryptic Soy broth, Lactose broth and Malt Extract was detected in order to see if fluorescence changed due to growth medium. *E. coli* cells were treated as described in the methods and suspended in a 4ml-quartz-fluorimeter cuvette. The excitation wavelength was scanned from 300 to 400nm

with the emission wavelength held constant at 420nm. Statistical analysis was performed after data had been normalized by GraphPad to account for cell concentration. The data is represented in Fig 5. The x-axis represents the excitation wavelength from 350 to 400nm. There was no significant difference in fluorescence among *E. coli* grown in the different media. Tukey's comparison of means gave a p-value greater than 0.05 for all three.

Spectral analysis was also performed on *E. coli* cells taken at different times during growth phase. *E. coli* cells collected every 30min were treated as described in the methods and suspended in a 4ml-quatrz-fluorimeter cuvette. The excitation wavelength was scanned from 300 to 420nm while holding the emission wavelength constant at 420nm. The data was then normalized to adjust for cell concentration. Fig 6 represents the fluorescence spectra of *E. coli* cells taken at120, 180 and 240min. Based on Tukey's comparison of means, the fluorescence of *E. coli* did not differ among cell growth stages with p-values greater than 0.05, with the exception of cells taken at 60min. *E. coli* cells taken at 60min were unmeasurable.

Discussion

The results of this study show that autofluorescence of bacteria is a quantifiable phenomenon and probably not due to artifact from media or other obvious sources. The three genera in this study each fluoresced at differing but easily differentiable intensities.

There were several emission and excitation wavelengths that could be used to express the differences among *E. coli*, *S. typhimurium*, and *S. aureus*. We chose the best three emission wavelengths to simplify the figures. Data expressed as a three-dimensional graph shows that the fluorescence of these three bacteria is much more complex, but three-dimensional figures are difficult to understand and analyze statistically. Figs 2, 3, and 4 showed statistically that there are differences among the bacterial autofluorescence among *E. coli*, *S. typhimurium*, and *S. aureus* and that these differences may permit identification of bacteria at the genera level. These quantifiable differences may lead to bacterial fluorescence fingerprints through which an unknown bacterium could be identified by its autofluorescence signature.

We believe that this study opens a new door into the identification of microorganisms by a fluorescent signature. We do not yet understand the source of the fluorescent spectrum nor do we understand cause of the differences among spectra. Methanogens greenish fluorescence is due to a formate dehydrogenase coenzyme known as F420. F420 has a maximal fluorescence emission peak at 455nm(31, 42). NADH also has a fluorescence peak at 460nm while FAD has a fluorescence peak at 525nm (24). *E. coli*, *S. typhimurium* and *S. aureus* all have strong fluorescence peaks in the same regions as F420 coenzyme, NADH, and FAD. We can only speculate that one or more of

these enzyme cofactors are responsible for bacterial fluorescence in this region of the spectra. Tryptophan has a fluorescence emission peak at 353nm when excited at 295nm (24). This could be responsible for the peaks present in Fig 3 just after 350nm. Further research should elucidate the causal relationship between bacteria and autofluorescence.

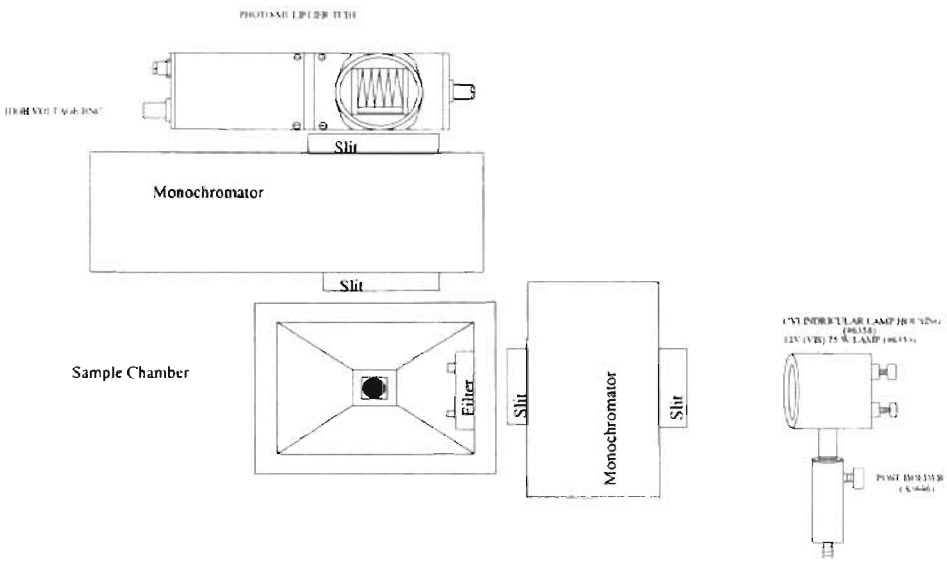

Figure 2: Syntopic diagram of spectrofluorimeter used in this study. Light from a 75W tungsten-halogen lamp enters the excitation monochromator; the selected bandwidth of the light then enters the sample chamber. Light scattered from the sample and fluorescence from the sample enter the emission monochromator. Light at the selected wavelength is then captured and amplified by the photomultiplier tube and converted to a digital signal and processed by a data acquisition computer (not shown).

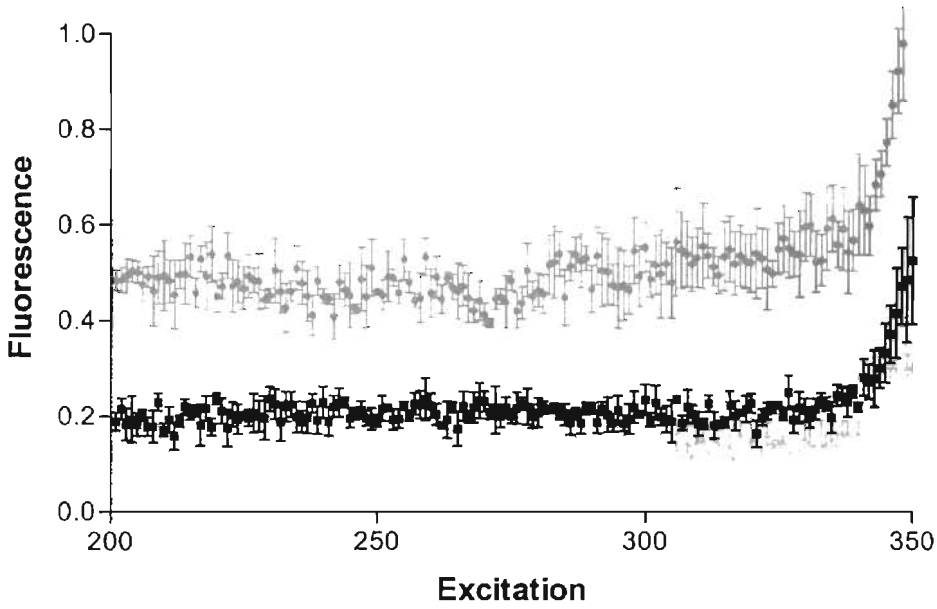

Figure 3: Fluorescent excitation spectra of *E. coli*, *S. typhimurium*, and *S. aureus* with the emission wavelength held constant at 425nm. The squares represent data from *E. coli*; the triangles represent data from *S. typhimurium*; the circles represent data from *S. aureus*. The y-axis is the relative fluorescent intensity while the x-axis is the excitation wavelength scanned from 200 to 350nm by increments of 1nm. P-value less than 0.001

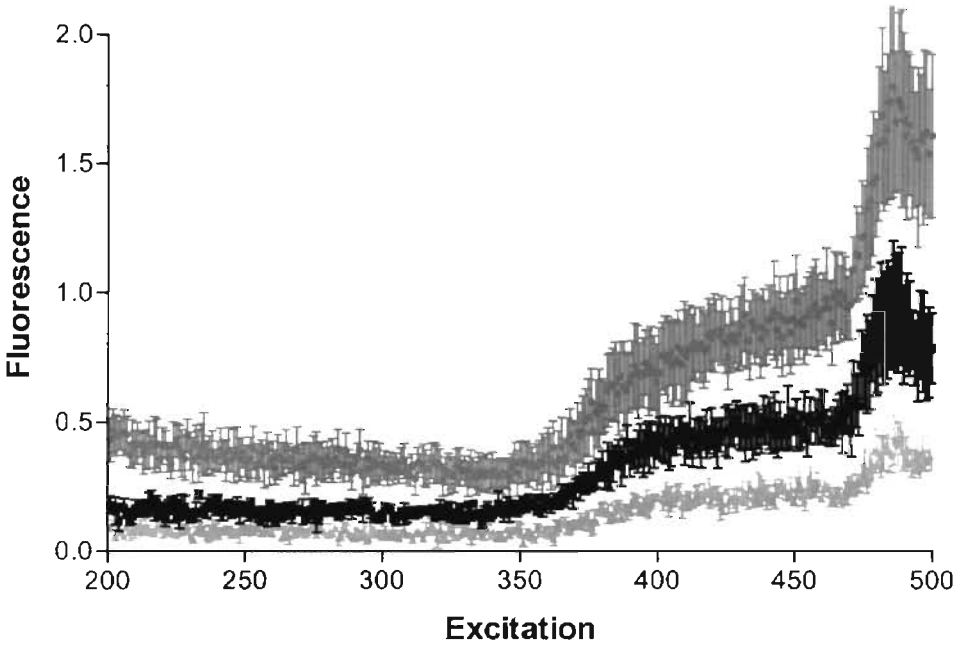

Figure 4: Fluorescent excitation spectra of *E. coli, S. typhimurium*, and *S. aureus* with emission wavelength held constant at 550nm. The y-axis represents the relative fluorescent intensity. The x-axis represents the excitation wavelength scanned from 200 to 500nm by increments of 1nm. Squares represent data from *E. coli*, triangles represent data from *S. typhimurium*, and circles represent date from *S. aureus*. P-values less than 0.001. Note that the maximum fluorescence scale differs from 3, 5, 6 and 7.

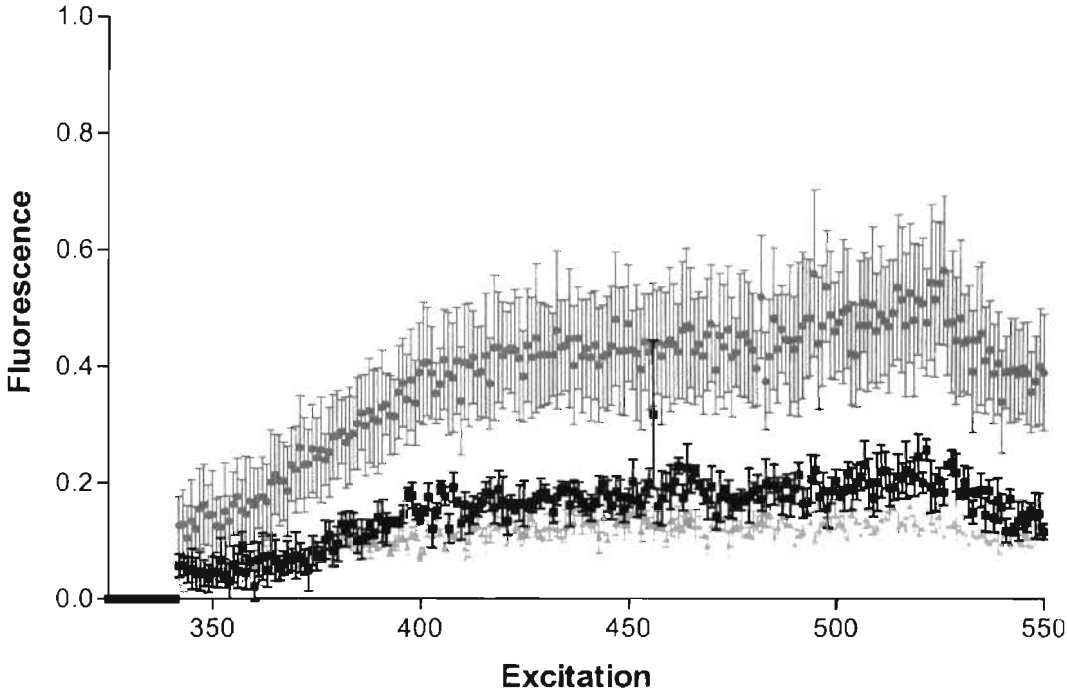

Figure 5: Fluorescent excitation spectra of *E. coli, S. typhimurium,* and *S. aureus* with emission wavelength held constant at 650nm. The y-axis represents the relative fluorescent intensity. The x-axis represents the excitation wavelength scanned from 300 to 550nm by increments of 1nm. Squares represent data from *E. coli*, triangles represent data from *S. typhimurium*, and circles represent data from *S. aureus*. P-values less than 0.001.

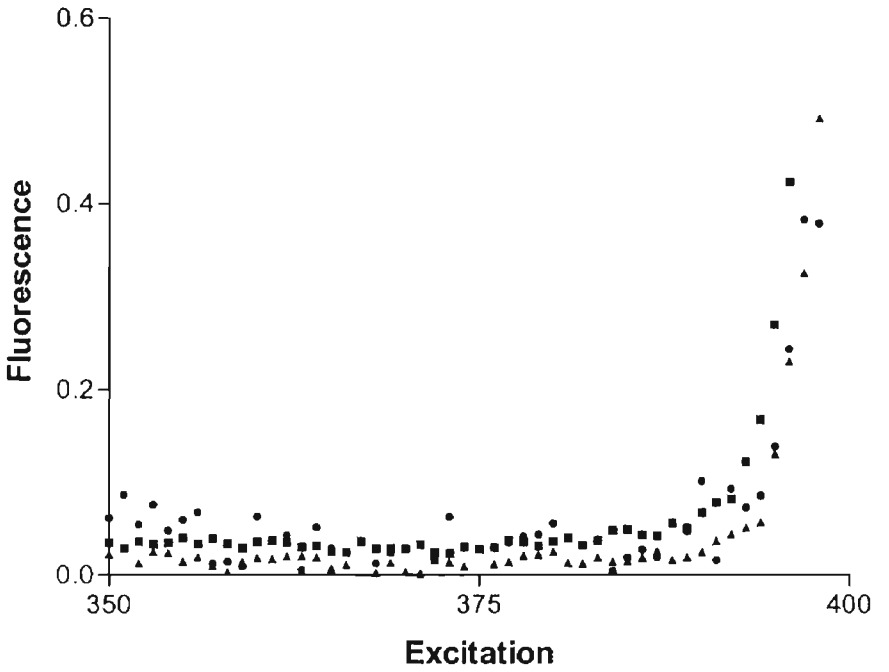

Figure 6: Fluorescent excitation spectra of *E. coli* grown in Tryptic Soy Broth, Lactose Broth and Malt Extract. The y-axis represents the relative fluorescence intensity. The x-axis represents the excitation wavelength from 350 to 400nm while holding the emission wavelength constant at 420nm. Squares represent date from *E. coli* grown in Tryptic Soy Broth. Triangles represent data from *E. coli* grown in Lactose Broth and circles represent data from *E. coli* grown in Malt Extract. Data was normalized by GraphPad before graphing. P-values greater thab 0.05.

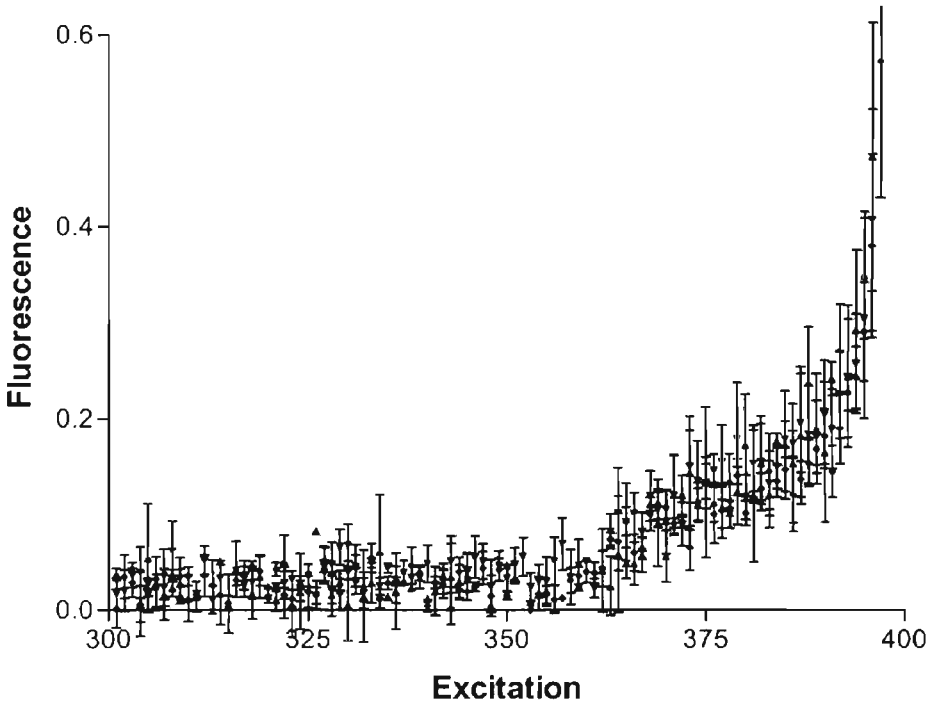

Figure 7: Fluorescent excitation spectra of *E. coli* cells taken at different stages in growth phase. The y-axis represents the relative fluorescence intensity. The x-axis represents the excitation wavelength scanned from 300 to 400nm by 1nm increments while holding the emission wavelength constant at 420nm. Triangles represent the fluorescence of cells taken at 120min, up-side-down triangles represent the fluorescence of cells taken at 180min, and circles represent cells taken at 240min. The data was normalized by GraphPad. P-values greater than 0.05.

Chapter 3

Effect of Dilutions on Fluorescent Dyes and Autofluorescence

Introduction

Detecting the presence of pathogenic bacteria has both medical and economic importance (3, 17). Although there have been improvements, current techniques such as plate counting are still time consuming and expensive (3, 20, 21, 30). Fluorescence-based detection of cells has a high signal to noise ratio (8, 9). That is, bacteria could be detected at very low concentrations. Current fluorescent-based techniques use expensive dyes to stain cells so that the cells can be easily detected. Three dyes used are CTC, Di-8-ANEPPS and the BacLight Viability Kit. In some studies, dye-based fluorescence has resulted in inaccurate data (11, 21, 25, 27, 29, 34). For example, Terzieva et al. found that the BacLight dye underestimates the actual viable cell concentrations. They noticed that cells with compromised membranes that can still reproduce are stained nonviable (37). Inaccurate data has also been obtained while using the CTC dye because this dye has a tendency to change colors depending on the oxidative state of the cell (19, 21, 34).

Another type of fluorescence is autofluorescence occurring when a molecule absorbs a wavelength of light in or on the surface and emits another wavelength of lower energy (41). Autofluorescence occurs without the addition of dye. In a previous study, we showed that *E. coli*, *S. typhimurium*, and *S. aureus* autofluoresce. We also demonstrated that autofluorescence spectra detects bacteria in water and differentiates among these three bacteria. This study shows the effect of bacterial dilutions on fluorescent intensity values of *E. coli* and compares the autofluorescence of *E. coli* to fluorescent dye techniques.

Methods and Materials

Bacteria

E. coli C600 was used for fluorescence detection. Tryptic soy broth was prepared from a mix provided by DIFCO laboratories; 5ml aliquots were distributed into vials and then autoclaved. Vials of prepared and autoclaved broth were inoculated with *E. coli* and incubated at 37°C for 24hrs. Bacteria in stationary phase, were centrifuged in a Fisher Scientific MICRO14 microcentrifuge by distributing 3ml of inoculated broth into three 2.0ml-microcentrifuge tubes and centrifuging for 5 min at 5×1000g. Supernatants were decanted and the cells washed with sterile reverse osmosis water (RO water). The cells were vortexed and centrifuged again for 5min at 5×1000g. This wash process was repeated twice. Supernatants were decanted and the cells were resuspended in sterile RO water. Next, the 3ml of washed bacteria cells, along with 500ul of sterile RO water, were placed into a 4ml-quartz fluorimeter cuvette. The contents of the fluorimeter cuvette were then mixed to ensure uniformity by inverting the cuvette four to five times. Serial dilutions were made from the original cuvette. A 50% dilution was prepared by taking 1.75ml from the original cuvette and mixing it with 1.75ml of sterile RO water into a second 4ml-quartz fluorimeter cuvette. A 75% dilution was prepared by taking 1.75ml from the 50% dilution and mixing it with 1.75ml of sterile RO water into a third 4ml-quartz fluorimeter cuvette.

Plate Counts

Plate counts were performed by making serial dilutions from the cuvette containing 3.5ml of bacteria. An aliquot of 0.1ml from each dilution was pipetted on

Tryptic Soy agar plates. The plates were then incubated for 24hrs at 37°C. Colonies were counted and the original cuvette concentration was determined by calculating the original cell density (26).

$$\text{Original cell density} = \frac{\text{number of cells counted}}{\text{ml plated}} (1/\text{dilution factor})$$

Dye Preparation

Two dyes were used for autofluorescence comparison experiments: The BacLight Viability kit (Molecular Probes L-7012) and Di-8ANEPPS (Molecular Probes D-6925). The BacLight dyes were thawed and 20ul of each dye (equal portions) were added to a microcentrifuge tube for a final volume of 40ul. An aliquot of 9.5ul of the mixed dye was added to each dilution and to the negative control of sterile RO water. Cuvettes were then incubated in the dark for 15min. The emission spectrum was measured by scanning the emission wavelengths from 500 to 675nm by increments of 1nm and holding the wavelength of the excitation monochromator at 480nm. A negative control of stained sterile RO water was subtracted from the scan of the treated *E. coli*. An aliquot of 10ul of Di-8ANEPPS (Molecular Probes D-6925) was added to a new set of dilution cuvettes. The cuvettes were incubated in the dark for 15min. Fluorescence was measured with the emission monochromator set at 630nm and excitation from 470nm to 550nm. A negative control of stained sterile RO water was subtracted from the scan of the treated *E. coli*.

Fluorescence Characterization

To investigate autofluorescence of the bacteria, emission of the bacteria was detected at a fixed wavelength while the excitation wavelength was scanned by a

spectrofluorimeter. Each emission scan from the bacteria was compared to a negative control of sterile RO water or Tryptic Soy broth dilution by subtracting the control scan from the emission scan.

The data analysis was based on the collection of fluorescence data into data records. Each data record represented the scan of excitation wavelengths from low wavelength to high wavelength and one specific emission wavelength. A scan number identified the data record.

All data points represent the mean ± SEM for three independent experiments. Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparison test with GraphPad Prism version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

We first tested the relationship between concentration and autofluorescence. Fig 8 describes the response from a dilution-response experiment for *E. coli*. Fluorescence emission was detected using a monochromator at a fixed wavelength of 420nm and scanning the excitation wavelengths from 300 to 400nm by increments of 1nm. Each data point in Figure 8 represents the mean ± SEM of three identical experiments and represents the fluorescence intensity at an excitation wavelength of 358nm. Fluorescent intensity, the vertical axis, was the difference between the experimental data and the RO water control. A one-way ANOVA with Tukey's multiple comparisons of the three dilutions revealed significant differences among mean fluorescent intensity values with the pvalues < 0.01 for all three comparisons.

Next, we compared the autofluorescence of *E. coli* to fluorescence from commercial dyes. Di-8-ANEPPS was the first fluorescent dye used in this study. Di-8-ANEPPS is often used in studying changes in membrane potentials. It is an electrochromic probe that is suitable for monitoring membrane potential changes by an electrochromic shift (9, 11). This dye has spectral properties that are largely dependent on its environment, and has also been used in vertebrate studies (4, 10, 11, 19). Dilutions of *E. coli* were prepared as described in the methods. We measured the autofluorescence of *E. coli* before treatment with Di-8-ANEPPS; this data is represented in Fig 9. The x-axis represents the wavelength of the excitation monochromator scanned from 300 to 400 by increments of 1nm while holding the emission wavelength constant at 420nm. The data represented in Fig 9 is of a solution containing 10^5 cells per ml. We then exposed the solution of *E. coli* used in Fig 9 to Di-8-ANEPPS for 15min; Fig 10 represents the

fluorescent emission of *E. coli* treated with Di-8-ANEPPS. Fluorescence emission was detected using a monochromator set to 630nm.The excitation wavelengths were scanned from 475 to 550nm. Note that the scaling of the vertical axis varies between Fig 9 and Fig 10. The autofluorescence in Figure 9 was greater than the fluorescence in Figure 10, Di-8-ANEPPS.

The BacLight Viability Kit was the second fluorescent dye used in the autofluorescence comparison study. The BacLight Viability Kit is used to detect both live and dead cells by staining dead cells red and live cells green (19). Because this dye does not penetrate intact cell membranes, it is commonly used in flow cytometry to measure complement activity (37). Fig 11 represents the fluorescent emission of *E. coli* before treatment with the BacLight Viability Kit. The horizontal axis represents the wavelength of the excitation monochromator scanned from 300 to 400nm by increments of 1nm while holding the emission wavelength constant at 420nm. As with Figs 9 and 10, the data represents a solution of 10^5 cell per ml. Fig 12 represents the fluorescence emission spectra of *E. coli* treated with BacLight dye as described in the methods. The horizontal axis represents the wavelength of the emission monochromator scanned from 500 to 675nm by increments of 1nm with the wavelength of the excitation monochromator set to 480nm. As with Fig 11, the data represents a solution of 10^5 cells per ml. Cells stained green will give a fluorescent peak between the emission wavelengths of 500 and 550nm. Cells stained red will give a fluorescent peak between the emission wavelengths of 600 and 650nm (19). All of the experiments were performed with stationary phase *E. coli*. The strongest peak present in Fig 12 is between emission wavelengths of 600 and 650nm and represents nonviable cells.

Discussion

The results of this study show that autofluorescence of bacteria is a quantifiable phenomenon probably not due to artifact. Dilution experiments showed that fluorescent intensity does decrease upon a decrease in cell concentrations.

This study also shows that the autofluorescence is a useful bacterial detection technique. Autofluorescence detects bacteria as well as, or in some cases better than, the fluorescent dyes Di-8-ANEPPS and BacLight. *E. coli* treated with Di-8-ANEPPS was not even detected by the spectrofluorimeter. That is, the fluorescence was in the noise. Autofluorescence of bacteria was actually greater than the fluorescence of the treated bacteria. In the experiments dealing with the BacLight Viability Kit, the autofluorescence of the bacteria may not have been greater than the fluorescence of the treated bacteria, but it was still detectable. Also, the largest peak present in Fig 12 is at the wavelengths that reflect dead bacteria. There should have been two peaks of equal height present if the bacteria were in stationary phase. It is possible that some of the cells that were stained red had damaged membranes due to centrifugation and wash treatments.

We do not yet know if there are differences between autofluorescent spectra of culturable bacteria and autofluorescent spectra of viable-but-nonculturable bacteria. Further research should elucidate the source or sources of autofluorescence and determine how different bacterial treatments affect autofluorescence.

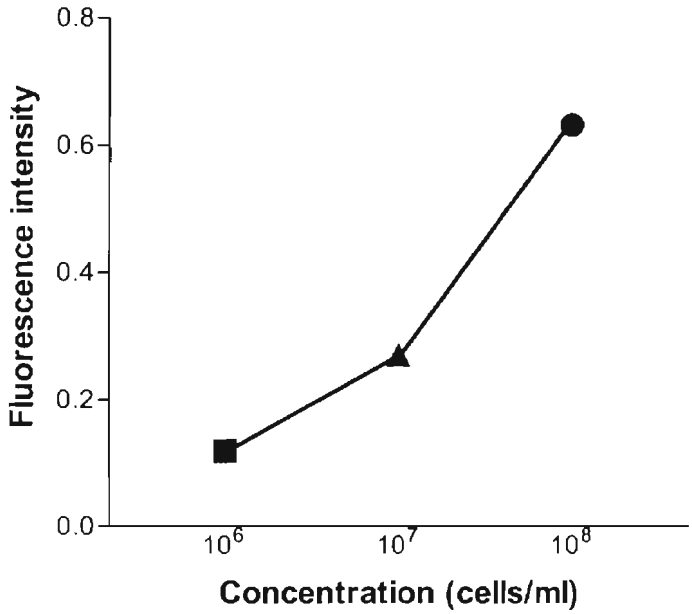

Figure 8: Fluorescent emission from dilution response study for *E. coli*. The x-axis represents bacterial concentrations while the y-axis represents the relative fluorescent intensity with emission wavelength set at 420nm and the wavelength of excitation at 358nm. The filled circle represents 5.68×10^8 cells per ml, the filled triangle represent 5.0×10^7 cells per ml, and the filled square represent 5.0×10^6 cells per ml.

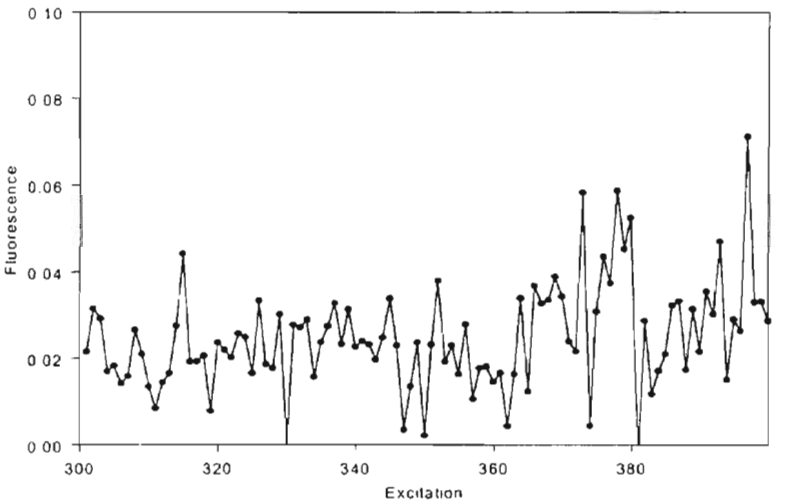

Figure 9: Fluorescence emission spectra of untreated *E. coli*. The y-axis represents the wavelength of excitation scanned from 300 to 400nm by increments of 1nm while the y-axis represent the relative fluorescent intensity with the emission wavelength set at 420nm. Filled circles represent a cell concentration of 10^5 cells per ml.

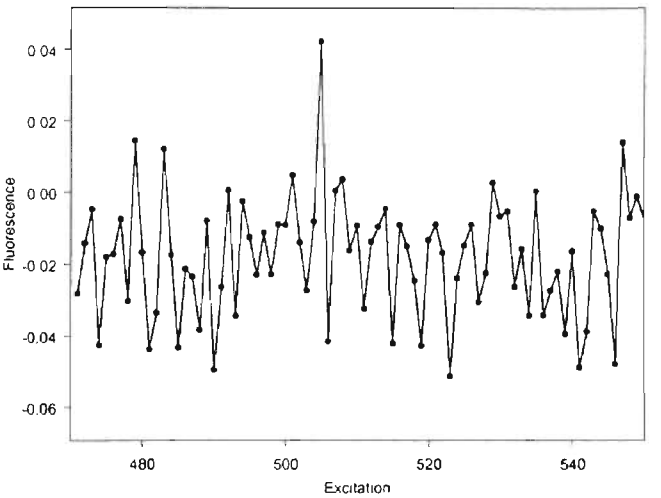

Figure 10: Fluorescence emission spectra of *E. coli* treated with Di-8-ANEPPS. The x-axis represents the excitation wavelengths scanned from 475 to 550nm by increments of 1nm while the y-axis represents the relative fluorescent intensity with the emission wavelength held constant at 630nm. Filled circles represent a cell concentration of 10^5 cells per ml.

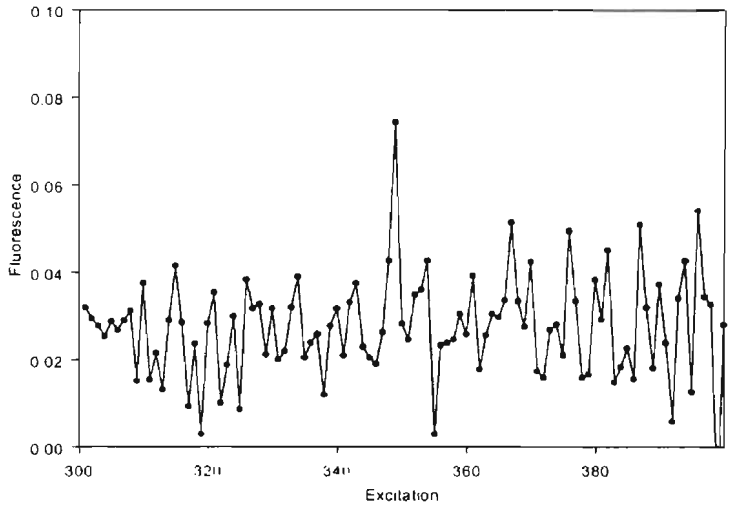

Figure 11: Fluorescence emission spectra of untreated *E. coli*. The x-axis represent the wavelength of the excitation monochromator scanned from 300 to 400nm by increments of 1nm while the y-axis represents the relative fluorescent intensity with the emission wavelength set to 420nm. Filled circles represent a cell concentration of 10^5 cells per ml.

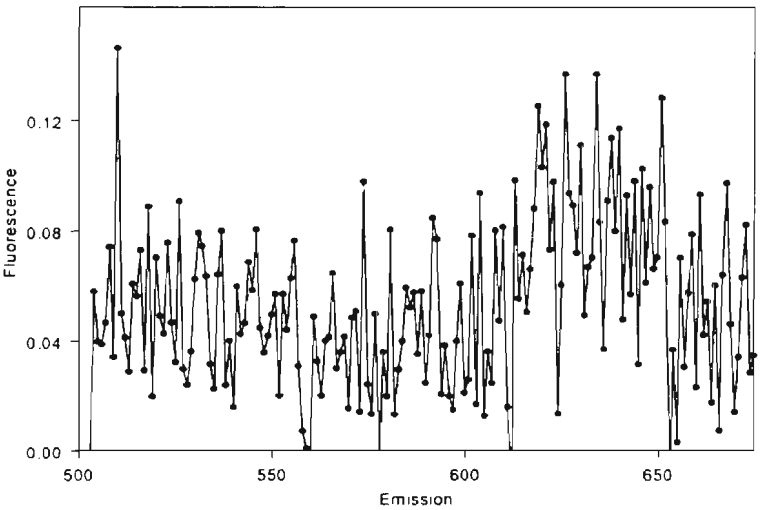

Figure 12: Fluorescence emission spectra of *E. coli* treated with the BacLight Viability Kit. The x-axis represent the wavelength of the emission monochromator while the y-axis represents the relative fluorescent intensity with the excitation wavelength set to 480nm. Filled circles represent a cell concentration of 10^5 cells per ml.

Chapter 4

Conclusion

The main objective of this study was to show that *E. coli*, *S. typhimurium*, and *S. aureus* autofluoresce, and that the bacterial autofluorescence is quantifiable, discriminating, and concentration dependent. We suspected that fluorescence spectra would discriminate between gram-negative and gram-positive bacteria. We did not believe it possible to discriminate between two closely related bacteria such as *E. coli* and *S. typhimurium*. The emission wavelengths used for Figs 3, 4, and 5 are not the only emission wavelengths that show differences among these three bacteria. When graphed three-dimensionally, the spectra of *E. coli*, *S. typhimurium*, and *S. aureus* have a definite topographical structure and is more complex than the emission wavelengths 425, 550 and 650nm can express.

Dilution experiments reflected that bacterial fluorescent intensity is dependent on concentration and reassures us that autofluorescence of bacteria is not an artifact because fluorescence is a linear function. Fluorescent emission scans performed on supernatants added greater reassurance to the autofluorescence coming from the bacteria and not another source such as the growth medium. Fluorescence did not change during cell growth phases or in cells grown in different media. This also assures us that the autofluorescence is not an artifact.

In experiments that compared autofluorescence to fluorescence of commercial dyes, we found that autofluorescence works as well as fluorescent dyes in detecting bacteria. Fluorescence of *E. coli* was not even detected when treated with Di-8-ANEPPS. The data collected from BacLight treated *E. coli* raises doubts to the reliability of the

BacLight Viabiltiy Kit as well. *E. coli* has fluorescent peaks at the same emission and excitation wavelengths used for detecting cells stained with the BacLight Viability Kit (see Figures 4 and 5). There is a strong possibility that bacterial autofluorescence could be contributing to the fluorescent intensity of Figure 12.

The verification of the autofluorescence of *E. coli*, *S. typhimurium*, and *S. aureus* has opened a Pandora's box of questions. These questions are leading our research into many different directions. We do not yet know the source of the autofluorescence or why these three bacteria have different autofluorescent spectra. Preliminary experiments suggest that the autofluorescence is due to a number of factors, such as cell wall components or cytochromes. Future research will elucidate the causal relationship between the bacteria and autofluorescence. We are also curious about identifying viable-but-nonculturable bacteria. Viable-but-nonculturable bacteria pose a threat particularly in the food industry because these bacteria can't be plated out, but can still cause serious illness (6, 15, 17, 27). We are very interested in seeing differences in autofluorescence spectra between viable-but-nonculturable and stationary phase bacteria and how this can be used as a tool in identifying bacteria.

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