THESIS

RAPID DETECTION OF VIABLE ESCHERICHIA coli O157:H7 BY ANTIMICROBIAL INCORPORATED MULTI-ANGLE LIGHT SCATTERING SPECTROSCOPY

Submitted by

Juan Carlos León

Department of Food Science and Human Nurtition

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2010

COLORADO STATE UNIVERSITY

December 18, 2009

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY JUAN CARLOS LEÓN ENTITLED "RAPID DETECTION OF VIABLE *ESCHERICHIA COLI* O157:H7 BY ANTIMICROBIAL INCORPORATED MULTI-ANGLE LIGHT SCATTERING SPECTROSCOPY" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

Committee on Graduate Work

Kendra K. Nightingale

Advisor: Lawrence D. Goodridge

Co-advisor: Marisa Bunning

Department Head: Chris Melby

ABSTRACT OF THESIS

RAPID DETECTION OF VIABLE *ESCHERICHIA COLI* O157:H7 BY ANTIMICROBIAL INCORPORATED MULTI-ANGLE LIGHT SCATTERING SPECTROSCOPY

Escherichia coli O157:H7 continues to cause outbreaks of produce-associated foodborne illness. Contaminated water has been shown to be a vehicle for transfer of *E. coli* O157:H7 to leafy greens during irrigation. Timely detection of this pathogen in irrigation water can prevent contamination of the final product. Current detection methods which are culture-based can be labor intensive and require several days to produce results. There is a need for development of rapid detection methods, which can detect *E. coli* O157:H7 in irrigation water. These methods have to be sensitive, robust and ideally should be able to differentiate between viable and non-viable microorganisms. Multi-angle light scattering spectroscopy (MALS) is a powerful technique that has been applied to qualitatively and quantitatively distinguish internal structural changes in cells upon perturbation by chemical/biological agents. We hypothesized that combining bacteriophage (phage) infection, which occurs only in viable bacterial cells, with MALS would allow for detection of the target bacteria and distinguish between viable and non-viable bacterial cells.

The objective of this study was to use *E. coli*-specific bacteriophages in conjunction with immunomagnetic separation and MALS to develop an assay for rapid detection of viable *E. coli* O157:H7 in irrigation water. We have termed this new method Antimicrobial

Incorporated Multi-angle Light Scattering (ANIMALS) to highlight the combination of a specific antimicrobial agent (phages) with the multi-angle light scattering technique to form a sensitive, rapid and specific assay for detection of *E. coli* O157:H7.

E. coli O157:H7 and *Salmonella* Typhimurium strains were diluted in lambda buffer to form concentrations of 10^0 to 10^4 CFU/ml and 10^8, respectively. The samples were subjected to immunomagnetic separation (IMS) using *E. coli* O157-specific IMS beads. Following IMS, the beads (and attached bacteria) were resuspended in 1 ml of tryptic soy broth (TSB) and one half (500 µl) of each sample was added to 10 mls of TSB that contained 1 ml of phage AR1 (10^{10} PFU/ml). The other half of the samples were added to TSB that did not contain phage AR1, but contained 1 ml of lambda buffer (to maintain constant volume), and these samples served as a reference. The samples were incubated at 37° C, with shaking, for 15 hours. Following incubation, 100 µl aliquots were removed from each sample, and separately assayed using a SpectraPoint light scattering spectrometer (Spectra Digital Corp., Toronto, Ontario, Canada).

E. coli O157:H7 was detected in 100% of pure culture samples in TSB containing various concentration (10^0 to 10^4) of the bacterium within 15 hours. An algorithm was developed to evaluate the area under the curve of each spectra. When compared to the light scattering spectra of the non-phage treated reference, the spectra of phage infected *E. coli* O157:H7 cells differed markedly. In contrast, the spectra of samples that contained *Salmonella* Typhimurium and the negative controls (containing no cells) were almost identical, because phage AR1 does not infect *Salmonella* Typhimurim and there was no

growth in the negative control samples, respectively. Using this method, *E. coli* O157:H7 could be detected following 15 hours of incubation in samples spiked at an initial concentration of 10^0 CFU/ml.

The sensitivity and specificity of the ANIMALS assay was determined using environmental water samples. River water samples were artificially contaminated with a 3-strain cocktail of *E. coli* O157:H7, followed by IMS and incubation in TSB supplemented with 20 mg/ml novobiocin at 42° C. MALS measurements were taken at 4, 6, 8 and 10 hours. The means and standard deviations of the ratios of the differences between the test and reference spectra for the four concentrations (10^0 to 10^3 CFU/ml) of *E. coli* O157:H7 were analyzed as well as for negative controls (samples of river water that were not inoculated with *E. coli* O157:H7). One concentration (10^3 CFU/ml) of *E. coli* O157:H7 was detected consistently after only 6 hours of enrichment. *E. coli* O157:H7 was consistently detected at concentrations from 10^1 to 10^3 CFU/ml in spiked river water within 8 hours. All concentrations (10^0 to 10^3 CFU/ml) were detected after 10 hours of enrichment. The presence of the pathogen in positive river water samples was confirmed by using lateral flow devices specific for *E. coli* O157:H7. The negative controls resulted in negative readings.

Additionally, reservoir water samples were prepared similarly to river water samples and MALS measurements were taken at 4, 6, 8 and 10 hours. The test and reference spectra for the four concentrations (10^0 to 10^3 CFU/ml) of *E. coli* O157:H7 were analyzed as well as for negative controls (samples of reservoir water that were not inoculated with *E. coli*

O157:H7). Similar results to experiments on river water were obtained with 10^3 concentrations of *E. coli* O157:H7 detected consistently at 6 hours. This demonstrated that the ANIMALS assay worked consistently across water samples with different physical and chemical characteristics. Regardless, *E. coli* O157:H7 was consistently detected in 100% of concentrations (10^0 to 10^3 CFU/ml) within 8 hours even against background levels of bacteria as high as 10^3 CFU/ml. Lateral flow devices specific for *E. coli* O157:H7 confirmed the presence of the pathogen in positive reservoir water samples. The negative controls resulted in negative readings.

These results demonstrate the ability of ANIMALS to rapidly and sensitively detect the presence and viability of *E. coli* O157:H7 following phage infection. This method has the potential to allow for rapid detection of viable *E. coli* O157:H7 in irrigation water against a high background of non-target microorganisms if coupled with selective enrichment.

Juan Carlos León
Department of Food Science and Human Nutrition
Colorado State University
Fort Collins, CO 80523
Spring 2010

ACKNOWLEDGEMENTS

I would like to thank Lawrence Goodridge for the help and guidance he provided for this project. I also would like to thank the United States Air Force for giving me the opportunity to pursue this advanced degree and for their financial support. I would like to also thank my fellow students Travis Steiner and Kellie Bauman for their help and friendly advice they offered me from day one. And last but not least, I would like to acknowledge Satish Deshpande and Bledar Bisha for their help and guidance during this project. To all of you, many thanks.

DEDICATION

This thesis is dedicated to my daughter whom endured many long days and nights of little companionship, and who behaved mature beyond her years. Thanks for always being there to make my day to day living less demanding and more enjoyable. And to my parents, who while monetarily poor, have always made me feel rich beyond measure thanks to their abundant love and unconditional support.

TABLE OF CONTENTS

List of Tables

List of Figures

OBJECTIVE OF THESIS

Collectively, studies show that detection methods based on light scattering may be used to detect bacteria in liquid suspension or on solid media. However, the limitations of these methods include the inability to reliably distinguish a particular bacterial species in liquid media, and the need for databases to identify target bacteria on solid media. Also, the methods as currently developed are time consuming (requiring up to several days in the case of conventional cultural methods for a result) making such methods impractical for rapidly detecting foodborne pathogens. Also, the need for extensive sample manipulation (plating the samples on selective solid media, followed by incubation) makes these methods unsuitable for field use. Finally, even after three decades of research on microbial morphology and identification using light scattering spectroscopy, there are no reports that the technique is able to definitively distinguish between bacterial strains of closely related bacteria (Rajwa et al., 2008).

The objectives of this study were to:

1. Develop a multi-angle light scattering (MALS) technique to rapidly detect *Escherichia coli* O157:H7 within 6-10 hours.

2. To demonstrate the rapid, repeatable, and sensitive detection of *E. coli* O157:H7 in pure culture using the newly developed MALS method.

3. To demonstrate the ability of the MALS method to detect *E. coli* O157:H7 in artificially contaminated environmental water samples, including river water and reservoir water.

CHAPTER ONE

Review of Literature

1.1. Introduction

The microbiological safety of food is a primary concern of the food industry. Traditional methods employed to detect foodborne pathogens include time consuming cultural methods, labor intensive immunoassays, complex molecular-based methods, or combinations of these methods (Goodridge and Griffiths 2002). The application of routine microbiological analysis to food products, and the increasing demand for greater sensitivity, speed and ease of use has driven the creation of new methods and procedures for the rapid and sensitive detection of foodborne pathogens that are no longer reliant on traditional microbiological culture on solid media (Goodridge and Griffiths 2004). This shift in detection technology is underscored by the number and variety of new detection systems that are constantly being developed (Acharya et al., 2006).

One of the more recent approaches to detect foodborne pathogens is based on the concept of the biosensor. Biosensors have been referred to as the offspring of the cross between biology and electronics (DeYoung, 1983). More recently, the International Union of Pure and Applied Chemistry (IUPAC) has defined a biosensor as a self-contained integrated device capable of specific quantitative or semi-quantitative analytical information using a biological recognition element which is retained in spatial contact with a transduction

element (Renneberg et al., 2008). Three main classes of biological recognition elements are most commonly used in biosensor applications including enzymes, antibodies and nucleic acids. However, enzymes are mostly used as labels rather than actual recognition elements (Lazcka et al., 2007). The development of one such enzyme-based biosensor dates back to the early 1960s, when Updike and Hicks (1967) described a glucose meter which employed a gel-entrapped enzyme (Renneberg et al., 2008). Since then, the development of biosensor technology has been dominated by the importance of measuring glucose for the management of diabetes (Renneberg et al., 2008), which has been the most successful application of biosensor technology thus far (Louie et al., 1998).

In addition to their biological-specificity conferring mechanism, biosensors may also be defined according to their mode of signal transduction, which could be physical or chemical in nature (Thevenot, et al., 2001). Generally, biosensors are divided into three broad categories, including electrochemical, optical and acoustic-based biosensors. They each have strengths and weaknesses which will be discussed in the following sections.

The potential of biosensors to meet the demands for speed, sensitivity, specificity, high throughput analysis and versatile real-time microbiological detection methods (Ruan et al., 2002; Louie et al., 1998), has led to their development and evaluation of their use to rapidly detect food and waterborne pathogens (Palchetti and Mascini, 2008; Banada et al., 2009; Acharya et al., 2006; Louie et al., 1998). In order to become more attractive, biosensors must be able to obtain detection levels comparable to more acceptable methods, with a high level of reproducibility. Additionally, in the food safety arena, there

is still a lack of portable, integrated biosensor systems (Palchetti and Mascini, 2008). These issues highlight the need for more research before biosensors can become a viable alternative to current detection methods.

1.2. Biosensors

Electrochemical Biosensors

Electrochemical biosensors are those that produce a signal through the use of an electrochemical transducer (Thevenot, et al., 2001). These biosensors could be further classified based on the type of signal measurement (potentiometric, amperometric, impedimetric and ion-charged) (Thevenot, et al., 2001). Regardless of differences in signal measurement, all electrochemical biosensors operate on the same principle: that the presence of the analyte will create a quantifiable change in the electrical conductivity of the media between the sensor and the sample matrix interface (Lazcka et al., 2007). Biological receptors (to capture the target analyte) are combined into electrochemical biosensors and may include antibodies, aptamers (oligonucleic acid or peptide molecules that bind to a specific target molecule), carbohydrate-based receptors and whole-cells, among others (Olsen et at., 2003; Ruan et al., 2002; Thevenot, et al., 2001).

An example of an electrochemical biosensor for the label-free detection of *Escherichia coli* using nonfaradaic electrochemical impedance spectroscopy (EIS) was described by Maalouf et al. (2007). Nonfaradaic refers to measuring the impedance without the use of a redox (oxidation-reduction) probe, which is normally used to amplify the net current flow through the biomolecular layers of a sensor (Kassanos et al., 2007). In this study,

Maalouf and colleagues linked a biotinylated polyclonal anti-*E. coli* antibody to a mixed, self-assembled monolayer (SAM) of biotin thiol and spacer alcohol thiol on a gold electrode via a biotin-neutravidin interaction. The authors exposed the gold electrode with self-assembled monolayers to various concentrations of *E. coli*, and determined the response by electrochemical impedance spectroscopy. The minimum detection limit of whole *E. coli* cells (in pure culture) was 10 CFU/ml. When whole-cell lysates were tested, the detection limit was 10^3 CFU/ml. The EIS method was then compared to *E. coli* detection by surface plasmon resonance (SPR), an optical biosensor. A surface plasmon is a spreading electromagnetic wave along the surface of a metal which can be optically excited with the use of a beam of light (Volpe and Palleschi, 2003). Surface plasmon resonance is obtained at a certain resonant angle of incidence of the light beam, which will change with the attachment of molecules to biological receptors, such as antibodies, immobilized on the metal surface (Volpe and Palleschi, 2003). The sensitivity of SPR detection of *E. coli* was determined by running an assay of different concentrations of bacteria ranging from 10 to 10^8 CFU/ml. The SPR response was determined by the difference between the signal at the injection of the sample and the signal at the end of washing. The detection limit of SPR was 10^7 CFU/ml of *E. coli*. Therefore, the EIS method was 6 orders of magnitude more sensitive. Several problems with the EIS method are noted. For example, a weakness of this system is that it tends to become saturated at concentrations above 10^4 CFU/ml. Also, because of the use of polyclonal antibodies, the assay could only detect *E. coli* among small numbers of Gram-positive bacteria, indicating that non-specific binding is a problem with the technique, and it is likely that more closely related bacteria (such as those belonging to the Enterobacteriaceae) would

produce a response. As such, the EIS method would seem to be impractical for use in most food matrix applications.

Ruan et al. (2002) described the development of an electrochemical biosensor which consisted of an amperometric biosensor combined with immunomagnetic separation (IMS) for the rapid and specific detection of *E. coli* O157:H7 in food samples. Samples of ground beef, fresh cut broccoli, and chicken carcass wash water were inoculated with *E. coli* O157:H7 and simultaneously mixed with IMS beads coated with anti–*E. coli* antibodies and alkaline phosphatase labeled anti–*E. coli* (APLAE) antibodies to form IMS beads–*E. coli* O157:H7–APLAE conjugates. The conjugates were separated by a magnetic field, followed by incubation with phenyl phosphate to produce phenol. An amperometric tyrosinase–horseradish peroxidase biosensor in a flow injection system was used to detect the phenol concentration, which was proportional to the *E. coli* O157:H7 concentration. The results indicated that the biosensor detected as few as 6×10^2 CFU/ml of heat-killed *E. coli* O157:H7 under optimized conditions in approximately two hours. While the test was also used to detect live bacterial cells, the signals between the control and the samples were not significantly different at concentrations below 6.0×10^3 CFU/ml. Additionally, the response of the system began to decline at concentrations greater than 6.0×10^5 CFU/ml. This was due to competition between the AP-labeled antibodies and the IMS beads in binding to the bacteria at higher concentrations. Finally, this technique also suffers from non-specific binding of other bacteria to the immunomagnetic beads which may result in false positive results (Ruan et al., 2002).

Chang and colleagues (2002) combined a conductance-based electrochemical biosensor with a bacteriophage for the identification of *E. coli* O157:H7. Conductance-based biosensors measure the conductivity of a medium to electricity, however, because the sensitivity of the sensor is hindered by the parallel conductance of the sample solution, typically measurements are performed in pairs between a sensor with an enzyme and an identical sensor without enzyme (Thevenot, et al., 2001). In this assay, the authors used bacteriophage AR1 to inhibit the growth of *E. coli* O157:H7 strains, stopping a change in the conductance of the sample media. They also utilized the inability of *E. coli* O157:H7 bacteria to ferment sorbitol as a probe for the bacteria. Hence, any *E. coli* strain that could not utilize sorbitol and caused no change in the conductance within an incubation period of 24 hours was considered a positive reaction (i.e. *E. coli* O157:H7).

The authors claimed a sensitivity of 100% in 41 out of 41 *E. coli* O157:H7 samples and of 99.4% in 154 of 155 non-O157:H7 samples. However, during testing, 14 of the 155 *E. coli* O157:H7 samples tested did not cause a change in conductance. The authors explain this by stating that 13 out of those 14 were sorbitol fermenters, and thus, only one non-fermenter caused a false positive reaction (Chang et al., 2002). Furthermore, the authors also stated that with the right inoculation levels (a bacterial inoculum of 10^6 CFU/ml and a cell/phage ratio of 1/10), negative results could be obtained in 4 hours. The main drawback of the technique appears to be that because phage AR1 exhibits cross-reactivity with *Shigella,* strains must be pre-determined to be *E. coli* before assessment by this method, which makes this method more of a serotyping method than a detection method.

The authors also obtained conflicting results between the plaque assays and the conductance measurements. For example, no changes in conductance were observed in certain strains of *E. coli* indicating that they should have been lysed by the phage, however, no lysis or very slight lysis was observed by plaque assay. Conversely, some strains showed very significant lysis, but the changes in conductance were not affected at all or were only delayed slightly compared to the controls. However, the authors did not try to investigate the reasons for the contradiction and only stated that the assays work by two very different mechanisms. Finally, no attempts were made to use a combination of phages which could theoretically enhance the lysis of *E. coli* O157:H7 strains not susceptible to AR1.

Additional biosensor applications continue to be investigated using combinations of biological recognition elements and electrochemical means of transduction for the detection of food and waterborne pathogens. These include: nucleic acids in conjunction with electrochemical biochips (Pohlmann et al., 2009), and a portable impedance-based biosensor with disposable analyte-specific sensor modules for the detection of *E. coli* O157:H7 and *Salmonella*. The latter, could be used in both enzyme and affinity-based (i.e, receptor-based or antibody-based) biosensors (Louie et al., 1998).

In the latter, the authors used a proprietary immobilization and stabilization technology that allowed the assay to retain its bioactivity and preserve its stability for long periods (Louie et al., 1998). Additionally, the assay used an inter-digitated differential binding module (dubbed the ADL biosensor) that allowed for simultaneous measurements of the

test and reference samples. Finally, an electronic module quantitatively measured the analyte binding to the disposable module.

The ADL biosensor module was reusable after washing and was demonstrated to last up to 9 months at room temperature. As mentioned above, the sensor was designed to work with both an enzyme (e.g. ATP for the testing of cardiac glycosides) and with affinity-based biological recognition elements (examples: a nicotinic acetylcholine receptor for the detection of cholinergics, and antibodies against *E. coli* O157:H7 and *Salmonella*). Biosensor modules were prepared in pairs one with (test) and one without (reference) the specific biomolecule to be tested and were allowed to cure overnight. Then, the modules were prepared for used by hydration in aqueous buffer for 10-20 minutes and once hydrated they could be used directly. Between samples, the biosensor modules could be regenerated by washing with water or buffer solution. In the case of foodborne pathogens, the detection limit of *E. coli* O157:H7 for this assay was of 10^4 organisms and its detection limit for *Salmonella* is yet to be determined. Therefore, while the potential of the modules seems promising, it failed to deliver a practical detection method for food and waterborne pathogens; especially for *E. coli* O157:H7, whose infectious dose is 2 to 3 orders of magnitude below (Paton and Paton, 1998) the detectable level of the technique. However, the authors found an incremental dose response, indicating that the signal strength is directly proportional to the concentration of the analyte. As a result, the promise of portability, versatility of biological recognition element use and the disposable nature of the sensor modules make this idea attractive.

The collective scientific literature indicates that, although there are numerous electrochemical systems described for the detection of food and waterborne pathogens, only a few of them have been validated or applied to the determination of these microorganisms with sufficient sensitivity and/or specificity in real samples (Pedrero et al., 2009). For that reason, more research is required in the area of electrochemical biosensors before they can become a viable and reliable method for detection of foodborne pathogens. In general, electrochemical biosensors have the advantages of good sensitivity, speed, low cost and good suitability to micro-fabrication (Pedrero et al., 2009). However, electrochemical methods are slightly less selective and sensitive than optical biosensors (Lazcka et al., 2007).

Optical Biosensors

Optical biosensors utilize the properties of light (e.g. absorption, reflection and refraction) to facilitate detection of a target analyte. Due to their excellent selectivity and sensitivity, optical biosensors are currently attracting much interest in food analysis including the detection of foodborne pathogens (Lazcka et al., 2007; Bokken et al., 2003). Additionally, while other techniques require extensive sample preparation, samples for analysis by optical methods are easily prepared following incubation with low risk of contamination and the original samples are still preserved for subsequent analysis by other techniques (Bokken et al., 2003).

Optical biosensor classification is neither simple nor unanimous. In some cases, optical biosensors are classified by the types of optical measurement (i.e. luminescence,

absorption, surface plasmon resonance, etc.) (Vo-Dinh and Cullum, 2000), while piezoelectric and cantilever biosensors are placed within their own category of mass-sensitive measurements. Others define optical biosensors as including fluorescence detection, SPR and piezoelectric biosensors (Lazcka et al., 2007), ignoring the mass-sensitive category all together, and placing such methods (e.g. cantilever biosensors) within electronic, optical or other categories (Goodridge and Griffiths, 2002). Further complicating the issue is the fact that piezoelectric biosensors are also categorized as acoustic biosensors (Volpe and Palleschi, 2003; Olsen et al., 2003). In this review, optical biosensors are defined as including fluorescence detection, SPR, and fiber optic biosensors, while piezoelectric, quartz crystal microbalance and cantilevers biosensors are classified as acoustic sensors. Finally, light scattering biosensors, while optical in nature, will be discussed in their own section.

The application of optical biosensors for the detection of food and waterborne pathogens is well represented in the literature. For example, Ye et al. (2002) established the usefulness of a chemiluminescence fiber optic biosensor used in conjunction with immunomagnetic separation for the detection of *E. coli* O157:H7. Chemiluminescence is the emission of light as a result of a chemical reaction (Ye et al., 2002). For Ye and colleagues, the purpose of the fiber optic light guide was to transmit the light generated by a horseradish peroxidase-catalyzed chemiluminescence reaction from a reaction cell (a light-tight black box) to an ultra-sensitive photo detector. The sensitivity of the instrument was such that as few as 1.8×10^2 CFU/ml could be detected within 1½ hours, even in the presence of three other pathogenic bacteria (*Salmonella* Typhimurium,

Campylobacter jejuni and Listeria monocytogenes). However, since the infectious dose of *E. coli* O157:H7 is as few as 1-10 ingested cells (Paton and Paton, 1998), this method, while sensitive will still need to be coupled with an enrichment or concentration step. Nevertheless, the sensitivity of this method compares favorably with the electrochemical biosensor described above.

Another fiber optic biosensor utilizing the principle of fluorescence resonance energy transfer (FRET) was developed by Ko and Grant (2006). FRET is a phenomenon that involves the transfer of non-radiative energy from a donating fluorophore (a fluorescent donor molecule) to an accepting fluorophore, but only when the donor and acceptor fluorophore are in close proximity (e.g. when the analyte is present) (Lazcka et al., 2007). The transfer results in a change in fluorescence, which can be measured with a fluorometer (Ko and Grant, 2006). For the development of this assay, optical fiber probes containing antibodies and protein G were labeled with donor and acceptor fluorophores, respectively. In the absence of the analyte, the 3D structure of an antibody is such that the donor and acceptor fluorophores are not in close proximity (Ko and Grant, 2006). As a result, there is no exchange of energy and the fluorescence will be at the emission wavelength of the donor, with little or no fluorescence from the acceptor fluorophore. In contrast, as the antibody binds with the target analyte, a change in its 3D structure occurs. This leads to a decrease in distance between the two fluorophores and a transfer of energy from the excited donor fluorophores to the acceptor fluorophores and consequently the fluorescence will be at emission wavelength of the acceptor (Ko and Grant, 2006). To increase the sensitivity of the assay, the detectors were connected to signal amplifiers.

When the fiber optic probe was placed in a phosphate buffered solution containing *Salmonella* Typhimurium, the biosensor demonstrated a limit of detection of 10^3 cells/ml with an 8.2% change in fluorescence. Furthermore, when the probes were placed directly into homogenized pork samples inoculated with *Salmonella* Typhimurium, the limit of detection was 10^5 CFU/g within 5 minutes, with a 6.67% change in fluorescence. The latter result demonstrated a loss of sensitivity due to a complex food matrix, and highlights the challenges faced when using biosensors to detect low levels of bacterial pathogens in food samples.

Another optical phenomenon, which has also been successfully applied to the detection of pathogenic bacteria is surface plasmon resonance (SPR) (Lazcka et al., 2007). An SPR biosensor works by detecting changes in the total internal reflection of light (total refractive index) caused by structural alterations in the vicinity of a thin film metal surface (usually gold) coated with antibodies to a target analyte (Zordan et al., 2009). In a SPR biosensor, the evanescent wave, a component of the incident light momentum, interacts with surface plasmons (free oscillating electrons) in the thin metal film surface. As a result, energy from the incident light is lost to the metal film, resulting in a decrease in light intensity (Lan et al., 2008). The SPR phenomenon occurs only at a specific angle of incidence, which is dependent on the refractive index of the medium adjacent to the metal surface. In the presence of the target analyte the refractive index changes in direct proportion to the mass change (i.e. the amount of antibody-antigen complexes formed) and the composition of the medium present. By using antibodies specific to pathogens of interest, it is possible to measure this phenomenon to determine the presence and amount

of the analyte in a sample by simply measuring the change in refractive index (Lan et al., 2008). The main drawbacks of this type of biosensor are its complexity, cost, and the size of the equipment, which limits its portability (Lazcka et al., 2007).

SPR was used to develop an assay for detection of *Salmonella* spp. belonging to groups B, D and E (Bokken et al., 2003). Overnight cultures of *Salmonella* were prepared for testing by collecting 1 ml of the respective bacterial suspension in an Eppendorf vial followed by centrifugation and re-suspension of the pellet in 1 ml of HEPES-buffered saline, pH 7.4 (for complete description see: Bokken et al., 2003). The closed vial was heated to 95–100°C for 30 min, and was cooled down to ambient temperature before analysis on the SPR biosensor. To test each *Salmonella* isolate, 10 µl of each sample were injected into the biosensor, followed by a pulse with soluble anti-*Salmonella* immunoglobulins to intensify the signal. The authors obtained 100% specificity detecting *Salmonella* from complex mixtures such as those expected in cultures from feces, feed or food. The pure culture tests conducted using 10^7 CFU/ml of the bacteria showed strong positive responses for *Salmonella* groups B and D, however the response was less sensitive for group E. When experiments were conducted in the presence of non-target bacteria (10^8 CFU/ml each of *E. coli*, *Citrobacter freundii* and *Enterobacter*), the sensor detected as few as 6×10^5 CFU/ml of *Salmonella* in all samples tested. In mixed suspensions containing several background bacteria, the detection limit of *Salmonella* was 1.7×10^5 CFU/ml. Nevertheless, non-specific binding was observed in the assay when an anti-chloramphenicol antibody was employed as a negative control. Consequently, the authors concluded that the success of the technique will greatly depend

on the availability of antibodies with the necessary characteristics, such as high binding, low dissociation and high specificity for the complete *Salmonella* genus.

Surface plasmon resonance was also employed by Lan and colleagues (2008) for the detection of *Salmonella* Typhimurium in chicken carcasses. The assay employed an avidin-biotin anti-*Salmonella* antibody complex on a gold surface. The refractive index of test samples was measured approximately every 4.8 seconds for 3 minutes and compared to a baseline reference signal. The detection limit of the biosensor was 10^6 CFU/ml of *Salmonella* Typhmurium in pure culture, which is similar to results obtained by other researchers using SPR to detect bacterial pathogens (Seo et al., 1999; Meeusen et al., 2005).

Zordan et al. (2009) designed a hybrid microfluidic biochip to perform multiplexed detection of *E. coli* O157:H7 using a combination of SPR and fluorescence imaging. The biochip consisted of an array of gold spots, each functionalized with a capture biomolecule targeting a specific pathogen. The sample to be analyzed was imaged by SPR on the bottom of the biochip and epi-fluorescence on the top. Overnight cultures of *E. coli* O157:H7 were subjected to immunomagnetic separation (IMS) to concentrate the cells followed by fluorescent staining with the *Bac*Light Bacterial Viability Kit. Two strains of *E. coli*, *E. coli* O157:H7 and *E. coli* DH5-α (a negative control), were then selectively introduced to the array by pipetting 1 µl of each bacterial suspension onto a separate spot. The authors quantified the binding of *E. coli* O157:H7 to antibody coated gold spots by measuring the percentage of the gold spot area upon which the bacteria

bound, and used this value to determine that the detection limit for foodborne pathogens was as little as 10^2 cells. The results also showed that the bound bacteria retained their viability. The combination of SPR and fluorescent molecular imaging conferred several advantages to the detection of bacteria that neither method could achieve individually (Zordan et al., 2009). For example, the use of the fluorescent imaging allowed for a determination of cellular viability, and with the use of different fluorescent probes, the method could be expanded to derive information about specific metabolic activities of the target bacterial population. The technique required concentration of the sample prior to analysis, which could be facilitated through the use of magnetic separation via immobilized recognition elements that are specific for the target bacteria (Zordan et al., 2009).

While some issues such as sample concentration are being worked out, other more important issues continue to make optical biosensors unattractive to end-users. Issues such as cost and complexity need to be overcome prior to wide acceptance of the technology (Lazcka et al., 2007). However, since these biosensors offer better sensitivity and selectivity than electrochemical biosensors, it may be only a matter of time until these disadvantages are overcome (Lazcka et al., 2007). In contrast, acoustic wave biosensors offer a middle ground of reliability and cost, and as such, have been available for many years (Volpe and Palleschi, 2003).

Acoustic Wave Biosensors

Acoustic wave biosensors are mass sensitive sensors which operate by utilizing mechanical acoustic waves (of an oscillating piezoelectric quartz crystal that resonates at a fundamental frequency) as their transduction mechanism (Rocha-Gaso et al., 2009). Quartz crystal microbalance (QCM) and cantilevers are two types of biosensors described in the literature which use piezoelectricity to generate resonance changes during detection of foodborne pathogens (Maraldo and Mutharasan, 2007; Campbell and Mutharasan, 2005a,b; Pathirana et al., 2000; Vaughn et al., 2003; Vaughn et al., 2001). Coating the piezoelectric crystals with recognition elements and biological materials (antibodies, enzymes, etc.) that have a high specificity for a target molecule will cause binding of the target molecule to the crystal, causing a change in the mass, which is proportional to the amount of material bound. This, in turn, will cause a change in the frequency of the waves resonating from the crystal. This change can then be measured to determine the presence and amount of bound analyte (Babacan et al., 2000).

Piezoelectric instrumentation (including simplified piezoelectric sensors) has been available for many years. However, while not excessively expensive, the instrumentation is still considerably more costly than electrochemical transducers (Volpe and Palleschi, 2003).

Piezoelectric biosensors have great potential for the direct detection of foodborne pathogens, such as *Salmonella* Typhimurium (Babacan et al., 2000) as well as other food and waterborne pathogens. Nevertheless, one of the limitations that hinders the use of these systems is that the coating of the crystal by the biological material needs to be

uniform and as thin as possible, and its immobilization needs to be rigid and secure (Babacan et al., 2000). Problems with any of these parameters could result in a loss in the relation between the mass change and the frequency shift resulting in inaccurate readings (Volpe and Palleschi, 2003).

An example of a rapid, label free QCM sensor for the specific detection of *Bacillus cereus* was developed by Vaughn and colleagues (2003). The authors used a self-assembled monolayer of thiosalicylic acid to covalently immobilize antibodies to the quartz crystal surface. The analysis was performed in real time and assays were carried out directly in solution. Changes in vibrational frequency of the quartz crystal were measured and frequency changes proportional to mass of the bound material were observed and constituted a positive response. The authors were able to obtain a limit of detection of about 10^4 CFU/ml using this biosensor.

Another QCM biosensor was described by Su and Li (2005) for the detection of *Salmonella* Typhimurium. In this case, the biosensor was fabricated using protein A for the antibody immobilization; which is thought to produce a better orientation of the antibodies (Volpe and Palleschi, 2003). During direct detection of *Salmonella* Typhimurium in chicken meat samples, changes in resonance and motional resistance were proportional to the cell concentration. The immunomagnetic separation (IMS) lowered the detection limit to 10^2 cells/ml. No interference was observed from *E. coli* K12 or the sample matrix.

In an earlier study, Su and Li (2005) developed a self-assembled monolayer-based piezoelectric biosensor for rapid detection of *E. coli* O157:H7 (Su and Li, 2005). The assay involved the immobilization of antibodies specific to the target bacteria to a quartz crystal using protein A. The biosensor could detect *E. coli* O157:H7 in a range of 10^3–10^8 CFU/ml within 30–50 min.

Another type of biosensor based on the principles of piezoelectricity is the cantilever biosensor. Cantilever biosensors work on the principle that any physical, chemical or biological attachment at the micromechanical transducers can result in a measurable change which can be detected by electronic, optical or other methods (Sarid 1991). These sensors have been used extensively for the detection of food and waterborne pathogens (Campbell and Mutharasan, 2007; Campbell et al., 2007a, 2007b; Campbell and Mutharasan 2006; Campbell and Mutharasan 2005a, 2005b; Olsen et al., 2003). For example, Campbell and colleagues (2007a) used a piezoelectric-excited millimeter-sized cantilever (PEMC) biosensor for the detection of *E. coli* O157:H7 in meat samples. As in many other piezoelectric biosensors, this assay involved the use of affinity purified polyclonal antibodies immobilized to the surface of a glass layer. The authors were able to show that the PEMC sensor could detect the pathogen in beef samples under flow conditions in real time. The method did not require any sample preparation, and was able to sense about 50–100 CFU/ml of *E. coli* O157:H7 in meat particle containing broths. The authors also stated that the sensor was highly selective, and its response was reliable even when 10^6 non-*E. coli* CFU/ml were present in the broth.

Later, Campbell and Mutharasan (2007b), refined their cantilever-based instruments, for further use in the detection of food and waterborne pathogens. Here, a piezoelectric-excited cantilever with antibodies specific for *E. coli* O157:H7 immobilized to the cantilever's glass tip, was employed to detect as few as 1 CFU/ml in a1L water sample, suggesting that a very low concentration of the pathogen could be detected in large volumes of water in a short period of time without filtration or enrichment. However, these experiments were conducted using pure cultures, meaning that such an ambitious detection limit may not be achievable if conducted in a more complex food matrix. Earlier work by this group (Campbell and Mutharasan 2005a, 2006) demonstrated that the PEMC biosensor technology could detect as few as 700 CFU/ml of *E. coli* O157:H7 and 300 spores/ml of *Bacillus anthraxis* in pure cultures prepared in PBS solution (pH 7.4). However, the work showed that the sensors could not be used more than once, due to the requirement to regenerate the sensor prior to reuse, which resulted in higher resonance frequency changes following each regeneration, and could lead to false positive test results.

Other research groups have also focused their efforts on developing piezoelectric biosensors. Olsen et al. (2003) demonstrated the ability of a piezoelectric crystal biosensor to selectively detect *Salmonella* Typhimurium in liquid samples. Piezoelectric biosensor readings are based on changes in the resonance frequency of a quartz crystal microbalance (QCM) after mass changes on the transducer surface (Lazcka et al., 2007). When a monolayer of antibodies against *Salmonella* Typhimurium was deposited on a piezoelectric crystal, the sensor was able to selectively detect *Salmonella* Typhimurium

even when the number of *E. coli* cells exceeded the number of *Salmonella* by 3 orders of magnitude. Still, one disadvantage to immunoassay based biosensors is the inability to distinguish between viable and non-viable cells.

As a result of the great sensitivity to mass changes exhibited by acoustic biosensors, and the fact that the typical bacterial cell is 1 million times heavier than a typical (150kD) antibody molecule (Olsen et al., 2003), these biosensors are potentially more suited for the detection of microbial pathogens than for protein detection (i.e. detection of foodborne toxins) (Skottrup et al., 2008).

Cell-Based Biosensors

Cell-based biosensors (CBBs) represent what is likely the most elegant marriage of biology to electrical components. These biosensors use the naturally evolved sensitivity of living cells to their environment in order to detect a broad range of biochemical agents as a cellular response (DeBusschere, 2002). This cellular response is detected by a transducer which converts the response into an electronic signal which can be analyzed. As a result of this capability, CBBs allow for almost instantaneous and sensitive detection of bacterial pathogens. However, in certain applications, CBB detection of target analytes may be impractical due to the fragility and growth requirements of living cells (Zhao et al., 2006). Furthermore, applications of CBBs are limited by their cost, the need for specialized personnel, the short shelf-life of the test components, and size constraints due to media volume needs (i.e. the continuous need for "re-feeding" the cells to retain their viability may become cost-prohibitive) (DeBusschere, 2002).

The term cell-based biosensor has been used to describe detection systems based on prokaryotic cells, as well as systems that employ higher eukaryotic or mammalian cells (Banerjee and Bhunia, 2009). These biosensors employ single cells, cell layers, cellular networks, tissue, or even whole animals or plants (Ziegler et al., 2000). Three main approaches have been applied to whole-cell-based biosensors. The first approach involves the measurement of the mechanical contact between cells and between cells and substrates *via* alternate current (a.c.) conductivity measurements in which the cells act as resistors impeding the free flow of electricity. In the second approach, metabolic products delivered from cultured cells to the growth medium are measured using biochemical sensors. In the third approach, electrogenic cells such as neural cells, heart muscle cells or pancreas beta cells (or neural cell networks) are used to measure the direct electrical response to a target stimulus.

One example of a commercially available CBB is an electric cell-substrate-based impedance sensing (ECIS) system which measures the changes in impedance of a cell-culture. This particular system is recognized as one of the initial breakthroughs that brought the concept of mammalian or higher eukaryotic whole-cell-based biosensing to commercial and research diagnostics (Banerjee and Bhunia, 2009). In this approach, cells are grown such that they attach to a platform in a uniform monolayer. The system accepts numerous diverse cell cultures which can be tailored depending on the analyte to be detected. Endothelial cells, murine macrophages and epithelial cells are a few examples of the animal cell cultures that may be attached to the electrodes after these have been coated with an absorbed pure protein layer. The cells act as insulators and impede the free

flow of electricity (Giaever and Keese, 1993). Microelectrodes beneath the cells measure the conduction (Banerjee and Bhunia, 2009). When analytes either rupture the cell monolayer or cause disturbances upon it, a drop in resistance occurs, and a measurable increase in the flow of electricity occurs (Banerjee and Bhunia, 2009). This indicates the presence of potentially toxic substances.

Rider and colleagues (2003) developed a B-lymphocyte-based assay, termed CANARY (cellular analysis and notification of antigen risks and yields) and have demonstrated the sensitive detection of various microorganisms. The researchers chose the B lymphocyte as the basis for their biosensor for several reasons. First, B cells are a component of the immune system, and they display antibodies on their surface as pathogen receptors. An immense diversity of antibodies with various specificities can be selected and expressed in these cells with the use of recombinant methods. In addition, B lymphocytes link the surface immunoglobulin–receptor complex to a rapid-response system. The cross-linking of surface antibodies by cognate polyvalent antigen initiates a cascade of events that leads to the mobilization of intracellular calcium stores within seconds and, ultimately, to activation and proliferation of the cells. To develop the biosensor, Rider et al. (2003) engineered the B lymphocytes to express aequorin, a protein found in jellyfish that emits light in response to calcium flux, and they also modified these same cells so that they expressed surface antibodies with specificity for bacterial or viral agents of interest. When the target analyte is present, the immunoglobulins initiate a signal transduction cascade that results in the increase of intracellular calcium, which in turn, causes the

emission of light, measurable with a luminometer in less than five minutes, including the time needed to prepare the sample.

CANARY was shown to be capable of sensitive detection of foodborne pathogens in food matrices. B cells specific for *E. coli* O157:H7 detected as little as 500 CFU/g in lettuce in less than 5 min, which included the initial sample preparation time. Nevertheless, while the CANARY exhibits rapid and sensitive detection of microorganisms, the fact that it is based on affinity (antibody) recognition of the target agent means that this assay is subject to the same cross-reactivity problems observed with other antibody-based assays. Another issue to consider is the stability (i.e. storage and maintenance) of the assay, which is a problem observed in other cell-based sensor systems (Lim et al., 2005).

A third, commercially available, CBB is the bioelectric recognition assay (BERA) which utilizes mammalian cell membranes (Banerjee and Bhunia, 2009). These biosensors have been modified to carry antibodies or other molecules, which are inserted into the membrane by a method called electroinsertion which involves the attachment of enzymes or antibodies to the cell membrane by electroporation, improving the specificity of the sensor (Banerjee and Bhunia, 2009). When the antibodies bind to analytes such as virus particles, a change in the membrane potential of the cell is triggered, which can be measured by microelectrodes.

Cell-based biosensors have been employed to detect the presence of toxins produced by foodborne bacterial pathogens, as opposed to the actual bacterial cell. Such is the case in a study conducted by Zhao et al. (2006) where the authors used liposome-doped nanocomposites as artificial-cell-based biosensors for the detection of listeriolysin O. Listeriolysin O (LLO) is a pore-forming hemolysin produced by *Listeria monocytogenes.* The bacteriolysin is secreted by the bacterium and is needed for escape from the phagocytic vacuole (Coconnier et al., 2000). As such, the presence of this toxin is indicative of the presence of *L. monocytogenes*. The authors used fluorescent dye-filled liposomes either free or immobilized within porous silica, and these served as surrogates for membrane insertion and pore-formation by LLO. Pore-formation as a result of the presence of LLO caused dye leakage from the liposomes, which in turn, caused a fluorescent response of dose dependent intensity. The fluorescence intensities were measured with a fluorometer over time. Response times ranged from several minutes to 15 minutes for free liposomes and 30-100 minutes for the immobilized liposomes. The importance of this method is that liposomes can be used in situations where biological functionality is required but the use of living cells is problematic (Zhao et al., 2006). The free liposomes could be kept at room temperature for approximately one week, which is essential for producing a practical biosensor for the food industry. As an emerging technology, cell-based biosensors will continue to improve and likely reach superior analytical capabilities, which should revolutionize functional biosensing of pathogens and toxins in the future (Banerjee and Bhunia, 2009).

1.3. Multi-angle Light Scattering Spectroscopy

Similar to other optical biosensors, light scattering-based biosensors rely on the properties of light (in this case refraction and reflection) for their detection capabilities. Light scattering is a fundamental optical process by which electromagnetic waves deviate from a straight path as a result of non-uniformities in the medium which they traverse (Banada et al., 2009). When light strikes biological particles (eukaryotic cells, bacteria, viruses, among others) it scatters in all directions (Rajwa et al., 2008) depending on the wavelength of the light, the size of the object, the number of particles and the optical inhomogeneities (refractive index) of the particles (Bhunia et al., 2007; Jones et al., 1998). The measurement of this deviation as a function of angular intensity can provide information related to morphological changes in cells (Wyatt and Phillips, 1972). In addition to being extremely sensitive, due to its non-invasive nature, light scattering is also recognized as being non-destructive (Deshpande and Hall, 1994; Latimer, 1982).

Three types of light scattering measurements may be employed to detect the presence of microorganisms: forward-scattering, back-scattering and turbidimetric (McClatchey, 2002). Forward light scattering refers to measurements at angles $> 0°$ to angles $< 90°$, while back-scattering refers to measurements at angles $> 90°$ to angles $< 180°$. Turbidimetric light scattering is confined to measurements at $0°$ and $180°$ angles (McClatchey, 2002). Turbidimetric measurements are sensitive to the volume as well as the number of particles, and as a result, they are often used to detect the presence of particles in a media (McClatchey, 2002; Sung, 1976). Additionally, since there is a direct

relationship between microbial growth, dry weight and turbidity, turbidity is commonly used as a measurement of microbial growth (Flowers, 1977; Sung, 1976).

However, because factors such as cell size, aggregate size, the color of the culture as well as the amount of cell debris affect turbidity, it is necessary to generate a standard growth curve for a given organism and growth condition, relating turbidity to cell counts prior to using this method (Sung, 1976). Once this is established, a bacterial count of a specific organism can be calculated from its turbidity measurements and its growth curve. However, since most organisms have a specific growth rate, using a growth curve of a specific bacterial strain to calculate the number of cells of a different organism will undoubtedly result in incorrect growth calculations (Trinci, 1972). Consequently, caution should be employed if turbidity is used to quantitate growth; however, turbidity can adequately describe the presence or absence of particles.

Light scattering measurements can be obtained at a single angle (discrete angle light scattering) using a photodiode, or at multiple angles (multi-angle light scattering) using a complementary metal–oxide–semiconductor (CMOS) detector such as those found in digital cameras. CMOS detectors contain miniaturized arrays of discrete photodetectors and offer the ability to measure light scattering at multiple angles (Ma et al., 2002). Each individual photodetector in a CMOS array is referred to as a pixel (Ma et al., 2002). The intensity of light scattered by a particle is measured at each pixel as a function of the angle of scattering. The measurements at the pixel are independent and saturation at one pixel does not influence neighboring pixels (Ma et al., 2002). Based on this principle,

multi-angle light scattering (MALS) has been applied to qualitatively and quantitatively distinguish morphological changes of cells, which may include changes in their internal structure, changes in the cell membrane as well as reaction kinetics taking place in cells upon perturbation by chemical/biological agents (Deshpande and Hall, 1995). Since MALS provides a greater amount of information when compared to discrete angle light scattering, it appears to also provide a more accurate and more informative analysis of scattering spectra (Jones et al., 1998).

Light scattering has been used in microbiological applications since the 1960's when Wyatt (1969) used a laser and a photodetector to compare the scattering spectra of different bacteria in suspension. The author showed that the scattering spectra of *Staphylococcus lactis* (a Gram-positive cocci) and *Serratia marcescens* (a Gram-negative rod) differ and suggested that light scattering characteristics could serve as a method for microbial identification. However, he also recognized that normal variations in morphology and growth history of microorganisms may cause differences in the light scattering patterns which will render the method useless. Referring to this, Latimer (1982) explained that there are two major barriers for the use of predictive models for judging the morphology of microorganisms using light scattering. First, the simplicity of biological models used for theoretical purposes falls dramatically short of the complexity of biological cells as revealed by microscopy; and second (as Wyatt recognized), natural variations within ordinary cell populations would obscure differences in light scattering information. As a result, morphologically similar organisms (Gram-negative rods of similar sizes, for example) may appear identical under light scattering (Latimer, 1982).

This further implies that identification of closely related, but serologically distinct organisms (e.g. *E. coli* O157:H7 and *E. coli* K-12), would be almost impossible to achieve using light scattering.

Wyatt and Phillip (1972) continued studies on bioparticle morphology using light scattering by looking at the structure of a single bacteria. The authors reported their ability to deduce features of bacterial cell structure not seen with a microscope (Wyatt and Phillips, 1972). The radius, cell wall thickness, and approximate refractive index of the cell wall and the cytoplasm were inferred by analyzing the light scattering measurements. Later, Wyatt and Jackson (1989) were the first to use a three dimensional, underwater MALS spectrometer to look at the structure of a single phytoplankton. The setup allowed a single phytoplankton to enter a chamber and a scattering signal detected at a selected angle triggered repeated electronic scans of the particle. Data was collected at up to 16 distinct angles and was collected 100 times during a period of a few hundred milliseconds. The authors discovered twelve different spectral signatures among 1,265 individual measurements, hypothesizing that these represented 12 different species of phytoplankton, each with a distinct scattering pattern (Wyatt and Jackson, 1989). However, the differences in scattering measurements were almost exclusively based on the different sizes between phytoplankton species. This is supportive of the principle that forward light scattering at small angles depends primarily on particle size (Latimer, 1982). Thus, in the absence of size and shape differences the scattering patterns may become indiscernible.

In addition to evaluating the structure of microorganisms, light scattering has also been used to determine bacterial size. For example, Katz et al. (2003) studied three different bacteria of varying sizes and shapes including *Pseudomonas aureginosa* (a Gram-negative rod), *Staphylococcus aureus* (a Gram-positive cocci that can form pairs, chains or grape-like clusters), and *Bacillus subtilis* (a Gram-positive, spore forming rod that often grows in long chains) to determine their size differences based on their scattering patterns. Pure samples of each bacterium were diluted such that single scattering was occurring (i.e. a beam of light would only strike a single cell and was only scattered once before reaching the receptor). This prevented an increase in the angular spread of the scattered light (Katz et al., 2003). Transmission (forward-scattering) measurements at seven detector angles were used to generate spectra using the ratio of scattered light intensity to incidence intensity. The results were consistent with those of previous researchers, showing that the scattering spectra of bacteria of different genera were distinctive based on size (Wyatt, 1969). Light scattering was also proven an effective tool for measuring the size and shape of bacterial cells *in situ* (Katz et al., 2003). The authors concluded that the results demonstrated that light scattering could be used to identify microbial contamination in the environment (Katz et al., 2003).

Rajwa and colleagues (2008) observed similar results while trying to classify bacteria using MALS. Their method combined a flow cytometer and a prototypical enhanced scatter detection system to collect measurements at five pre-selected discrete angles followed by analysis by a vector machine classifier. Four different, unlabeled species of bacteria (of varying size and shape) were used for the experiments including:

E. coli K-12 (a Gram-negative rod), *Listeria innocua* (a Gram-postive rod), *Bacillus subtilis* (a Gram-positive spore forming rod) and *Enterococcus faecalis* (a Gram-positive cocci). Measuring at discrete angles meant that pre-selecting the scatter angles that allowed the ability to distinguish scatter angles for the target bioparticle was required. The authors presented the requirement of pre-selecting scattering angles based on the particle of interest as a strength as well as a weakness of the technique, since obtaining highly distinguishable scattering at the preselected angles demonstrated the predictive power of the technique, but as the system could only be optimized for a known bioparticle, purely exploratory cytometry for analysis of an unknown particle was not possible (Rajwa et a., 2008). Using this methodology, the bacteria were recognized with a 68-99% success rate. However, there was a noticeable misclassification of a large group of *E. faecalis* bacteria as *B. subtilis.* The authors attribute this to the fact that the intra-population variance in size and refractive indices had not been taken into account by the simple scatter model, a fact they found encouraging as it may be fixed in future work. Finally, the authors believed that overcoming the obstacles of the complexity of design and the lack of easy-to-use data analysis tools will ultimately allow for label-free detection of microbial particles (Rajwa et al., 2008).

Recognizing the limitations of light scattering for the identification of single bacteria or bacteria in suspension, other researchers have attempted to find specific spectral signatures of bacterial colonies in solid media, such as agar, with reasonable success (Banada et al., 2009). Like single particle light scattering, the scattering pattern of a

colony depends on its size, shape and refractive index, but additionally it also depends on the chemical composition of the colony and the composition of the growth media (Banada et al., 2007).

Banada and colleagues (2007) used this principle to create a scatterometer, and also generated algorithms to interpret the scattering spectra of colonies (non-invasively) after conventional incubation. The scatterometer consisted of a laser and a Petri-plate holder which held the plate at a perpendicular angle to the light path. The light path was directed to the center of a pure bacterial colony, which allowed the use of a forward light scattering configuration (Banada et al., 2007). Forward light scattering has been proven to be more effective in differentiating among different genera and species than back or turbidimetric scattering (Bae et al., 2007). Upon interacting with a colony, the light was scattered onto a detection plane (an 8 inch × 10 inch white board) and the resulting scattering patterns were captured using a digital camera. After overnight incubation in brain heart infusion agar, or other selective media, the authors subjected six *Listeria* species to the scatterometer and showed that distinct scattering patterns were generated allowing identification of even closely related *Listeria* species with an accuracy of 91-100% in 4 to 5 minutes. However, the authors did not include the overnight incubation as part of their assay which would add at least 18 to 24 hours to the identification. Additionally, because a complete library of scattering patterns will have to be developed to compare scattering patterns of tested bacteria to a reference (Banada et al., 2007), the detection of an unknown organism for which there has been no reference spectra gathered may prove difficult.

Later, Banada and colleagues (2009) expanded the scope of the whole-colony detection technique by testing bacteria of different genera including: *Escherichia, Vibrio, Salmonella, Staphylococcus* and *Listeria*. The bacteria were analyzed in pure culture, recovered from food matrices and from experimentally infected animals. The setup was very similar to the previous study, except that the detection plane was replaced by a camera that recorded the scattering pattern and sent it directly to a classifier for analysis. The authors claimed that the technique could detect the pathogens tested at concentrations of 1 CFU/25g of sample even in the presence of background bacteria with a specificity of 90-99% in less than 24 hours (Banada et al., 2009). The researchers also tested the robustness of the scattering patterns of the same bacteria when these had undergone various stresses including acid, osmotic and heat stress, and demonstrated that the scattering patterns did not differ from non-stressed colonies. However, the same limitations previously described have not been addressed, meaning that the technique still required the development of a scattering pattern library and required overnight, or sometimes longer, incubation of the samples prior to analysis.

Bae and colleagues (2007) used a similar setup to compare three different species of *Listeria* (*Listeria innocua, Listeria ivanovii* and *Listeria monocytogenes*) based on forward light scattering signatures. The results were analogous to those of Banada et al. (2007) showing that even closely related bacteria created distinctive scattering patterns. However, the technique suffered from the same issues previously described.

In addition to using light scattering for the study of single bioparticle morphology and identification, and the study of whole colonies in solid media, a third use of light scattering is its use for microbiological quantification. For example, Jones and colleagues (1998) studied the quantification of yeast cells using MALS. The authors investigated the relationship between cell counts and light scattering across a wide range of cell concentrations of yeast in suspension. Once standardized by the use of three serial dilution experiments, a supervised learning model was used to predict cell counts using MALS. While the results showed that the model provided an adequate method for predicting cells counts based on light scattering measurements, the authors described the prediction as cumbersome (Jones et al., 1998).

Regardless of the type, biosensor applications are gaining popularity and are regarded as the future of pathogen detection technologies. Traditional pathogen detection technologies, while sensitive enough, are presently too slow, costly or complicated to fulfill all the needs of the food industry (Lazcka et al., 2007). In recent years, biosensors (especially optical sensors, given their potential for excellent sensitivity and specificity) have become of great interest to the scientific community (Lazcka et al., 2007). While more research is needed to overcome issues such as the complexity of the systems, their cost and need for sample concentration and enrichment, the potential for label-free, rapid, simple and inexpensive detection of pathogens make them an exciting and important new technology which could become a true weapon in the near future for use by the food industry against foodborne disease (Acharya et al., 2006).

CHAPTER TWO

Antimicrobial Incorporated Multi-angle Light Scattering (ANIMALS) for the Detection of Escherichia coli O157:H7 *in Water Samples*

2.1. Introduction

Foodborne illnesses associated with the consumption of contaminated food and water are a public health and social concern. Outbreaks of foodborne illness account for 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States annually (Mead et al., 1999). *Escherichia coli* O157:H7 is a facultative anaerobic Gram-negative rod that causes an estimated 62,500 cases of foodborne illness and 1,800 hospitalizations each year (Mead et al., 1999). Symptoms related to an *E. coli* O157:H7 infection can range from mild, watery diarrhea to life-threatening conditions such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura. Furthermore, as few as 10-100 cells are required for *E. coli* O157:H7 to cause disease (Meeusen et al., 2005; Ochoa and Harrington, 2005).

Foods typically associated with *E. coli* O157:H7 illness include contaminated milk, beef and fresh produce (Ochoa and Harrington, 2005; Tu et al., 2003; Ruan et al., 2002; Wachtel et al., 2002). Irrigation water has been shown to be an important vehicle for the

transmission of *E. coli* O157:H7 to fresh produce (Wachtel et al., 2002). An example of a produce-borne *E. coli* O157:H7 outbreak occurred in 2006 and affected 26 states and Canada (Wendel et al., 2009). In this outbreak, 204 cases of *E. coli* O157:H7 infection were reported with a total of 31 cases of HUS resulting in 3 deaths. The source of the outbreak was traced to contaminated bagged spinach (Manning et al., 2008). The outbreak strain of *E. coli* O157:H7 was identified in samples of river water, cattle feces, and wild pig feces from a nearby ranch (Wendel et al., 2009). Additionally, ingestion of contaminated drinking water was the vehicle of a large *E. coli* O157:H7 outbreak in Walkerton, Ontario, Canada (Matsell and White, 2009). In this outbreak, 1,436 cases were reported with a total of 27 cases of HUS and 6 deaths attributed to contaminated ground water. The outbreak was attributed to an unchlorinated municipal water source.

Numerous techniques for detecting *E. coli* O157:H7 and other foodborne pathogens have been described, including cultural methods, immunoassays, molecular-based assays and biosensors (Volpe and Palleschi, 2003; Rider 2003; Ye et al., 2002; Chang et al., 2002; Goodridge et al., 1999a). The continuous demand for better detection methods is underscored by the number and variety of new detection systems that are constantly being developed (Acharya et al., 2006). Issues with sensitivity, specificity, speed, cross-reactivity, cost, and ease of use, among others, affect all of the current detection methods (Banada et al., 2007; Bae et al., 2007; Tu et al., 2003). For example, cultural methods are still considered the "gold standard" for microbiological analysis, but require long incubation times. Immunoassays and molecular-based methods are more sensitive and

specific, but these assays require costly specialized equipment, laboratory expertise conditions and extensive sample handling (Banada et al, 2007; Volpe and Palleschi, 2003).

There is an acute need to develop faster, more robust and sensitive methods to detect E. coli O157:H7 in water which should be cost-effective and able to detect viable organisms. Optical detection based on light scattering techniques may be able to provide an effective alternative. Light scattering is a fundamental optical process by which electromagnetic waves deviate from a straight path as a result of non-uniformities in the medium which they traverse (Banada et al., 2009). When light strikes biological particles (eukaryotic cells, bacteria, viruses, among others) it scatters in all directions (Rajwa et al., 2008) depending on the wavelength of the light, the size of the object, the number of particles and the optical inhomogeneities (refractive index) of the particles (Bhunia et al., 2007; Jones et al., 1998). The measurement of this deviation as a function of angular intensity can provide information related to the morphological changes in cells. Multi-angle light scattering (MALS), the measurement of light scattered at multiple angles, is a powerful technique that has been applied, based on this principle, to qualitatively and quantitatively distinguish morphological changes that may include changes in the internal structure of cells, changes in the cell membrane as well as reaction kinetics taking place in cells upon perturbation by chemical/biological agents (Rajwa et al., 2008; Jones et al., 1998; Deshpande and Hall, 1995).

Light scattering measurements can be obtained at a single (discrete) angle using a photodiode, or at multiple angles using a complementary metal–oxide–semiconductor (CMOS) detector such as those found in digital cameras. CMOS detectors contain miniaturized arrays of discrete photodetectors and offer the ability to measure light scattering at multiple angles (Ma et al., 2002). Each individual photodetector in a CMOS array is referred to as a pixel (Ma et al., 2002). The intensity of light scattered by a particle is measured at each pixel as a function of the angle of scattering. The measurements at the pixel are independent and saturation at one pixel does not influence neighboring pixels (Ma et al., 2002).

The use of MALS for detection of bacteria *in situ* has been described based on size (the difference between prokaryotic and eukaryotic cells) and shape (the difference between rods, cocci, spirochetes) and has been suggested as a possible method to monitor environmental microbial contamination (Katz et al., 2003). However, to date, no claims have been made on the ability of the technique to differentiate between morphologically similar bacteria (e.g. distinguishing between non-pathogenic and pathogenic isolates of the same species or closely related species).

The objective of this study was to develop a MALS assay that specifically detects *E. coli* O157:H7. The developed method utilizes bacteriophages (phages) to specifically lyse *E. coli* O157:H7 cells in a test sample. When the test sample is compared to another sample that does not contain phages (the reference sample), the resulting light scattering spectra can be used to determine the presence or absence of the *E. coli* O157:H7 cells. We have

termed this new method Antimicrobial Incorporated Multi-angle Light Scattering (ANIMALS) to highlight the combination of a specific antimicrobial agent (phages) with the multi-angle light scattering technique to form a sensitive, rapid and specific assay for detection of *E. coli* O157:H7.

2.2. Materials and Methods

Bacterial Strains

A total of 21 strains of bacteria (Table 2.1) were used for host range, sensitivity and specificity studies. Two additional bacterial strains (*E. coli* B and *E. coli* C) were used for phage propagation. The bacteria studied comprised 10 strains of *E. coli* O157:H7, and 11 strains of non-O157:H7 bacteria. All strains were from our culture collection, housed in the Department of Animal Sciences at Colorado State University. Stock cultures were maintained in 20% glycerol and were frozen at -80°C. Fresh bacterial host cultures for use in experiments were produced by inoculating frozen stock cultures onto Tryptic Soy Agar (TSA) plates (Teknova, Hollister, CA) and incubating the plates overnight at 37°C. For ANIMALS experiments, the inocula consisted of stationary-phase cells that were obtained by inoculating Tryptic Soy Broth (TSB) (DIFCO, Sparks, MD) with cells from an overnight TSA plate and incubating the preparations overnight with shaking at 37°C.

Bacteriophage Propagation

Thirty seven phages (Table 2.2) from our phage collection were used in this study. Each phage was individually amplified on its bacterial host (*E. coli* O157:H7 920333, *E. coli* B, or *E. coli* C). To amplify the phages, a modified procedure of the traditional double-layer plaque technique (Adams, 1959) was employed. The top agar layer consisted of 1%

(wt/vol) tryptone (DIFCO, Sparks, MD), 0.8% (wt/vol) sodium chloride (Fisher Scientific, Pittsburgh, PA), and 0.5% Bacto-agar (DIFCO, Sparks, MD).

For each phage to be amplified, 5 mls of top agar were steamed for approximately 10 min and allowed to cool to 47°C. One hundred microliters of an overnight culture of the host bacterium were added to the top layer, and the mixture was vortexed and poured onto the bottom layer (TSA). The top agar was allowed to solidify at room temperature, and 50 µl of a bacteriophage suspension (from frozen stock) was pipetted onto the top agar layer. The plates were incubated at 37°C overnight. Following incubation, the top agar was removed from the plate and placed in 10 mls of lambda buffer (LB) (5.8 grams sodium chloride, 2.0 grams magnesium sulfate heptahydrate, 50 milliliters of 1 molar tris hydrochloride buffered to pH 7.5 and 0.1 grams of gelatin). Two milliliters of chloroform were added to the tube to release any progeny phage which may still have been in the host cells, and the suspension was incubated with shaking at 37°C for an additional 15 min. The suspension was centrifuged at 5,250 × g for 25 minutes, and the supernatant was withdrawn and filtered through 0.2-µm-membrane syringe filters (AcroCap Filter Unit, Pall Corp., Ann Arbor, MI) to remove the bacterial debris. The phage were titered and stored in the dark at 4°C. A single phage, AR1, was used in the pure culture experiments, while a cocktail containing all 37 phages was used in the environmental water experiments. The concentrations of each phage were individually adjusted to 10^8 PFU/ml and equal volumes of each phage were combined to produce the cocktail.

Host Range Determination

The host range of the cocktail was determined by testing against 21 bacterial strains (Table 2.1). Phage lysis assays were conducted in 10-cm-diameter petri plates (Fisher Scientific, Pittsburg, PA). The top agar layer consisted of 1% (wt/vol) tryptone (DIFCO, Sparks, MD), 0.8% (wt/vol) sodium chloride (Fisher Scientific, Pittsburgh, PA), and 0.5% Bacto-agar (DIFCO, Sparks, MD). For each strain tested, 3 ml of top agar was steamed for approximately 10 min and allowed to cool to 47°C. One hundred microliters of an overnight culture of the bacterium to be tested was added to the top layer, and the mixture was vortexed and poured onto the bottom layer (TSA). The top agar was allowed to solidify at room temperature, and 20 µl of the phage cocktail (10^8 PFU/ml) was pipetted onto the top agar layer. The plates were incubated upright, at 37°C overnight, and then examined for the presence of clear zones of lysis. The host range of phage AR1 was determined in a similar manner.

Development of the ANIMALS Assay

The Antimicrobial Incorporated Multi-angle Light Scattering Assay is an integrated method that combines immunomagnetic separation with phage-based MALS. The ANIMALS assay was developed using pure cultures of bacteria, and the method was also evaluated using environmental water samples.

Sample Preparation

Pure Culture Studies

The ability of the ANIMALS assay to detect *E. coli* O157:H7 in broth culture was evaluated. An overnight culture of *E. coli* O157:H7 (strain EC920333) was diluted by preparing a series of 1:10 dilutions in lambda buffer until the final dilution was 10^{-9}. The concentration of the *E. coli* O157:H7 was determined by plate count on TSA. Ten ml dilutions that contained 10^0, 10^1, 10^2,10^3 and 10^4 CFU/ml of *E. coli* O157:H7 were evaluated by ANIMALS as described below. Samples inoculated with *Salmonella* Typhimurium (strain SPM0000437) and samples that did not contain cells were also included as negative controls. An overnight culture of *Salmonella* Typhimurium was diluted 1/10 and subjected to ANIMALS. A total of three technical replicates were conducted across 10 independent replications.

Environmental Water Studies

Environmental water samples were obtained from two different sources to approximate the types of water used in vegetable irrigation. The water samples included reservoir water obtained from a farm in Larimer County, Colorado, and river water from the Poudre River, obtained in Greely, Colorado. Water samples were collected from each site on the same day (once per experiment, 3 experiments in total). The temperature of the water was measured immediately prior to collection. Water was collected in sterile 1 liter glass bottles (Fisher Scientific, Pittsburgh, PA), and transported back to the Laboratory on ice. Water samples were stored at 4°C until use. No more than 3 days passed between water collection and use in experiments. Upon arrival at the laboratory, each water

sample was assayed for microbial counts by filtering 100 ml of the water through a MicroCheck II Beverage Monitor (Pall Corporation, Ann Arbor, MI). The filters from the monitor device were removed and placed onto a TSA plate, followed by incubation for up to 48 hours at 30°C. Where necessary, 10-fold dilutions were performed on the water samples to obtain microbial colonies in the countable range. Additionally, aliquots of each water sample were sent to the Soil, Water and Plant Testing Laboratory at Colorado State University for physical and chemical analysis (Table 2.3) using the routine package of tests as described at http://www.extsoilcrop.colostate.edu/SoilLab/documents/waterpricelist2009.pdf.

For experiments, 50 mls of each water sample were spiked with a 3-strain cocktail of *E. coli* O157:H7 to obtain final concentrations of 10^0, 10^1, 10^2 and 10^3 CFU/ml. The cocktail was prepared by growing overnight cultures and adjusting their individual concentrations to 10^8 CFU/ml as determined by plate counts. Additionally, un-spiked water samples were also included as negative controls. The water samples were then subjected to IMS followed by ANIMALS at 4, 6, 8 and 10 hours of incubation. All experiments were conducted in triplicate.

Immunomagnetic Separation

Pure cultures and spiked environmental water samples were subjected to immunomagnetic separation (IMS) in order to concentrate *E. coli* O157:H7 cells. For pure culture studies, 10 ml portions of stationary-phase cultures (diluted as described above) were added to 15 ml Falcon tubes containing 40 µl of *E. coli* O157-specific

immunomagnetic beads (Invitrogen, Carlsbad, CA), and allowed to incubate at 37° C with shaking at 250 rpm for 15 minutes. After incubation, the samples were placed in a magnetic rack so that the magnetic beads could be separated from the sample. The beads were washed three times with 1 ml of wash buffer (1× Omni*Pur* phosphate buffered saline (Gibbstown, NJ) with 0.1% Tween 20 (Cayman Chemical Co., Ann Arbor, MI)), applying a magnet in between washes to retain the beads. Once separated and washed, the beads and attached cells were re-suspended in 1 ml of TSB for pure culture experiments, 500 µl of the resuspended samples were added to a test tube containing 10 mls of TSB and 1 ml of phage ARI (10^{10} PFU/ml); this constituted the test sample. The remaining 500 µl of sample were added to another test tube (reference sample) that contained 10 mls of TSB and 1 ml of lambda buffer. The samples were allowed to incubate for 15 hours at 37°C with shaking. A complete schematic of the sample preparation process is shown in Figure 2.1.

For environmental water studies, 50 ml portions of river or reservoir water (spiked with different concentrations of a 3-strain *E. coli* O157:H7 cocktail as described above) were added to 50 ml Falcon tubes containing 40 µl of *E. coli* O157-specific immunomagnetic beads (Invitrogen, Carlsbad, CA), and allowed to incubate for 2 hours at room temperature while rotating at 20 rpm in a mixing device (Invitrogen, Carlsbad, CA). After incubation, the samples were transferred to sterile wash tubes (Matrix Microscience, Golden, CO) and placed in a magnetic rack so that the magnetic beads could be separated from the water sample. The beads were washed three times as described above. Once separated and washed, the beads and attached cells were re-suspended using 1 ml of TSB.

Five hundred microliters of the resuspended sample were added to a test tube (test) containing 10 mls of TSB supplemented with 20mg/ml of novobiocin (Sigma-Aldrich, St. Louis MO) and 1 ml of a 37 phage cocktail (10^8 PFU/ml). The remaining 500 µl of sample were added to another test tube (reference) that contained 10 mls of TSB supplemented with 20 mg/ml of novobiocin and 1 ml of lambda buffer. The samples were allowed to incubate for 4, 6, 8 and 10 hours at 42°C with shaking.

2.3. Multi-angle Light Scattering Measurements

For MALS measurements, 100 µl of each sample to be tested were removed at each time point and placed into a disposable cuvette (UVette®, Eppendorf North America, Inc., Westbury, NY) with a 2mm path length. A SpectraPoint light scattering spectrometer (SpectraDigital Corp., Guelph, Ontario, Canada) (Figure 2.2) was used to collect 100 measurements per pixel of scattered light at low forward light scattering angles < 4°. An algorithm was used to compare the scattering intensity of the test (phage-treated) sample to that of the reference sample and a ratio of the scattering intensity difference between the two samples was generated. Triplicate measurements of the scattering intensity ratio of the difference between the test and reference samples were averaged to determine the mean intensity ratio for each beginning bacterial concentration (10^0 to 10^4) per experiment. The means for each experiment were then used to calculate means and standard deviations for each concentration. The same procedure was used to determine means and standard deviations for the negative controls. A positive test result was defined as a ratio between the test and reference sample that was greater than 3× the standard deviation of the ratio of the control (test and reference) samples.

2.4. Statistical Analysis

Statistical analysis of the means and standard deviations of the ratio of the scattering intensity differences was conducted using a general linear model (GLM) in one-way ANOVA followed by least significant difference (LSD) to compare the means with an $\alpha = 0.05$ using the SAS software (SAS Institute Inc., Cary, NC).

2.5. Results

Host Range Determination

Phage AR1, individually, and the 37-phage cocktail were characterized according to host range. The phages were tested against 21 bacterial isolates which included 10 *E. coli* O157 strains and 11 non-O157 strains (Table 2.1). Phage AR1 lysed 9 out of 10 *E. coli* O157 strains and 0 out of 10 non-O157 strains. The 37-phage cocktail resulted in lysis of 10 out of 10 O157 strains and 4 out of 11 non-O157 strains.

Development of ANIMALS Using Pure Cultures

The sensitivity and specificity of the ANIMALS assay was determined in pure culture. *E. coli* O157:H7 (strain EC920333) cultures were diluted to give final concentrations of 10^0 to 10^4 CFU/ml in 10 milliliters of lambda buffer. Each individual sample was subjected to IMS followed by addition of phage AR1 to the test samples and measurement using ANIMALS after a 15-hour incubation (Figure 2.3). A total of three technical replicates were conducted across 10 independent replications. Differences in scattering intensity that were 3× the standard deviations of the ratio of the no-cells controls were considered

positive. *E. coli* O157:H7 was consistently detected in all samples after 15-hour enrichment. Negative controls resulted in negative readings, even when concentrations as high as 10^7 CFU/ml of *Salmonella* Typhimurium were used. Statistical analysis revealed significant differences between samples containing *E. coli* O157:H7 and those containing *E. coli* O157:H7 and AR1 ($p < 0.0001$), but not between the *Salmonella* Typhimurium or no-cell controls ($p = 0.699$). Figure 2.4 shows a boxplot graph of means and variance for the difference in scattering intensity ratios between test and reference samples of pure culture studies.

Environmental Water Studies

The sensitivity and specificity of the ANIMALS assay was tested using environmental water samples. River water samples were artificially contaminated with a 3-strain cocktail of *E. coli* O157:H7 and MALS measurements were taken at 4, 6, 8 and 10 hours. Figure 2.5 shows the means and standard deviations of the ratios of the differences between the test and reference spectra for the four concentrations (10^0 to 10^3 CFU/ml) of *E. coli* O157:H7 analyzed as well as for negative controls (samples of river water that were not inoculated with *E. coli* O157:H7). Two technical replicates across the three independent experiments were conducted and measured using ANIMALS. Differences in the scattering intensity ratios in excess of 3× the standard deviations of the ratios of the controls were considered positive. One concentration of *E. coli* O157:H7 (10^3 CFU/ml) was detected at 6 hours, helped by the good congruency between the negative control samples which resulted in very small standard deviations for the controls. Average aerobic plate counts for river water samples were $3.4 \times 10^3 \pm 1.6 \times 10^3$. *E. coli* O157:H7

was consistently detected at all concentrations (10^0 to 10^3 CFU/ml) within 8 hours. Lateral flow devices (Neogen Corporation, Lansing, MI) specific for *E. coli* O157:H7 confirmed the presence of the pathogen in positive river water samples. The negative controls resulted in negative readings. Figure 2.6 shows a boxplot graph of means and variance for the difference in scattering intensity ratios between test and reference samples of environmental sample studies.

Reservoir water samples were prepared similarly to river water samples and MALS measurements were taken at 4, 6, 8 and 10 hours. Figure 2.7 shows the means and standard deviations of the ratios of the differences between the test and reference spectra for the four concentrations (10^0 to 10^3 CFU/ml) of *E. coli* O157:H7 analyzed as well as for negative controls (samples of reservoir water that were not inoculated with *E. coli* O157:H7). Similar results to experiments on river water were obtained with 10^3 concentrations of *E. coli* O157:H7 detected consistently at 6 hours. This demonstrated that the ANIMALS assay worked consistently across water samples with different physical and chemical characteristics. Average aerobic plate counts for river water samples were $1.0 \times 10^4 \pm 5.7 \times 10^3$. Regardless, *E. coli* O157:H7 was consistently detected in 100% of concentrations (10^0 to 10^3 CFU/ml) within 8 hours even against background levels of bacteria as high as 10^3 CFU/ml. Lateral flow devices (Neogen Corporation, Lansing, MI) confirmed the presence of the pathogen in positive reservoir water samples. The negative controls resulted in negative readings. Figure 2.8 shows a boxplot graph of means and variance for the difference in scattering intensity ratios between test and reference samples of environmental sample studies.

2.6. Discussion

The objective of this work was the development of a bacterial detection method, based on multi-angle light scattering for rapid detection of the foodborne bacterial pathogen *E. coli* O157:H7. The method combines immunomagnetic separation, phage-based inhibition of the target cells, and MALS to form an integrated method capable of detecting viable *E. coli* O157:H7 cells within 8-10 hours. IMS was used to specifically isolate and concentrate the target *E. coli* O157:H7 cells from the sample. The addition of phages (either individually or in a cocktail) specifically inhibited the growth of the *E. coli* O157:H7 cells in the test sample, allowing for a comparison between the test and reference spectra to determine the presence or absence of the bacterial cells. During MALS, low forward scattering angles were collected because they provided data about large structures (such as the bacterial cell as a whole), and thus, are most consistent with the mechanism of actions of phages which lyse bacterial cells causing scattering intensity changes at the low scattering angles which indicate a loss of structure. The measurements were averaged to produce an average scattering intensity per pixel. These averages could be plotted to produce the characteristic spectra shown in Figure 2.9. When plotted together, a visual comparison of the spectra of the test and reference samples provided a quick answer as to whether a sample was positive or negative. Spectra which approximated each other (Figure 2.9. Panels B and C) were consistent with negative samples, (i.e. no difference in scattering was noticeable), while spectra that differed were indicative of a positive sample (Figure 2.9. Panel A).

In the pure culture experiments, the addition of a single phage (AR1) was sufficient to inhibit the growth of the *E. coli* O157:H7 cells. Phage AR1 is a lytic myophage belonging to the T4-like phage family which has been shown to specifically infect *E. coli* (Goodridge et al., 2003; Yu et al., 2000). However, a single *E. coli* O157:H7 strain was used in these experiments, and subsequent experiments indicated that during long periods of incubation, large initial concentrations of *E. coli* O157:H7 could produce resistant mutants to phage AR1, which could proliferate and produce a false negative result in the assay. Other studies have shown that phage resistance in *E coli* O157:H7 can readily develop when incubated with a single phage (Tanji et al., 2004; Mizoguchi et al., 2003)). These studies also showed that when the *E. coli* O157:H7 cells were incubated with two phages which used different bacterial receptors for infection, resistance was delayed for many hours (Tanji et al., 2004). Therefore, a cocktail consisting of 37 phages was developed and used in subsequent development of the ANIMALS assay. The rationale for using such a large number of phages in the cocktail was twofold. First, the chances of resistance developing to the cocktail was minute (due to the presence of such a large number of phages, many of which employed different cellular receptors for adsorption (data not shown)), and the use of so many phages alleviated concerns about the ability of the cocktail to inhibit the growth of many different isolates of *E. coli* O157:H7. The cocktail was previously screened on 58 *E. coli* O157:H7 isolates and results showed that all isolates were effectively lysed (data not shown). In this study, when the cocktail was employed against a 3-strain mixture of *E. coli* O157:H7 that were spiked into environmental water samples, the results showed that the phage cocktail effectively limited the growth of the *E. coli* O157:H7 cells in the test samples, at all concentrations

tested. Host range analysis showed that the cocktail lyses several non O157:H7 *E. coli* isolates, and this could cause false positive results. Future work will center around modifying the cocktail to make it more specific for *E. coli* O157:H7.

The ANIMALS assay was capable of detecting as few as 10^0 CFU/ml of *E. coli* O157:H7 in water within 8-10 hours, in the presence of a high concentration of background microflora (10^3 CFU/ml), and in water samples with high concentrations of minerals and total suspended solids (Table 2.3). These results compare favorably with other phage-based detection methods. Goodridge et al. (1999a) developed an assay that combined IMS and a fluorescently stained phage for detection of *E. coli* O157:H7 in broth. When it was combined with flow cytometry, the fluorescent-bacteriophage assay (FBA) was capable of detecting 10^4 cells/ml. A modified direct epifluorescent-filter technique (DEFT) was employed in an attempt to estimate bacterial concentrations. Using regression analysis, it was calculated that the lower detection limit was between 10^2 and 10^3 cells/ml. Further studies indicated that the FBA was able to detect 2.2 CFU/g of artificially contaminated ground beef following a 6-hour enrichment, and between 10^1 and 10^2 CFU/ml of artificially contaminated raw milk after a 10-hour enrichment step. Reporter phage assays have been developed to detect several species of foodborne bacteria including *E. coli*, *Salmonella* spp., and *Listeria monocytogenes* (Willford and Goodridge, 2008; Chen and Griffiths, 1996; Loessner et al., 1996; Pearson et al., 1996; Turpin et al., 1993; Kodikara et al., 1991; Ulitzur and Kuhn, 1987). In general, these assays allowed detection of between 1 and 10 cells of the target bacteria after an enrichment period of between 4 and 16 hours. Chang and colleagues (2002) combined a

conductance-based electrochemical biosensor with phage AR1 to develop a serotyping method for *E. coli* O157:H7. Any *E. coli* strain that could not utilize sorbitol and caused no change in the conductance of the media within an incubation period of 24 hours was considered to be *E. coli* O157:H7.

Shabani et al. (2008) used phages as recognition receptors, and covalently immobilized them onto functionalized screen-printed carbon electrode (SPE) microarrays. The SPE networks were functionalized through electrochemical oxidation in acidic media of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) by applying a potential of +2.2 V to the working electrode. Immobilization of phage T4 onto the SPEs was achieved via EDC by formation of amide bonds between the protein coating of the phage and the electrochemically generated carboxylic groups at the carbon surface. The immobilized T4 phages were then used to specifically detect *E. coli*. The presence of surface-bound bacteria was verified by scanning electron and fluorescence microscopy. Impedance measurements showed shifts on the order of 10^4 Ω due to the binding of *E. coli* bacteria to the T4 phages. No significant change in impedance was observed for control experiments using immobilized T4 phage in the presence of *Salmonella*. Impedance variations as a function of incubation time showed a maximum shift after 20 min, indicating the onset of bacterial lysis, as also confirmed by fluorescence microscopy. Concentration-response curves were used to determine the detection limit of this method, which was observed to be 10^4 CFU/ml.

This study describes the creation and evaluation of a method that combines the principles of IMS, phage-based bacterial lysis and MALS into an easy to perform assay that can detect low levels of *E. coli* O157:H7 in environmental water within as few as 6 hours. When coupled with a pre-concentration step that can concentrate large volumes of water, the ANIMALS assay should be able to detect extremely low concentrations of *E. coli* O157:H7 in shorter time periods. The use of unmodified phages in the assay allows for the creation of ANIMALS assays for other bacterial pathogens without the need to conduct time consuming genetic modification or labeling steps. This should dramatically decrease the time needed to develop additional assays. Future work will be focused on further refining the assay to improve sensitivity and specificity, especially through tailoring the phage cocktail to a more narrow range of *E. coli O157:H7* bacteria. Additionally, work on developing ANIMALS methods for other foodborne pathogens (e.g. *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter* spp.) will be conducted. Finally, future work will include coupling the ANIMALS method with sample concentration methods (such as continuous centrifugation, filtration) to allow for detection of foodborne pathogens in larger volumes (10 L and up) of water.

TABLES AND FIGURES

Table 2.1. Bacterial strains used in bacteriophage host range and specificity studies.

Bacterial Strain	Sensitivity to Bacteriophage AR1	Sensitivity to Bacteriophage Cocktail[a]
Escherichia coli O157:H7 (EC920333)[b]	+[c]	+
Escherichia coli O157 (LG 36)	+	+
Escherichia coli O157 (LG 37)	+	+
Escherichia coli O157 (LG 38)	+	+
Escherichia coli O157 (LG 39)	+	+
Escherichia coli O157 (LG 124)	-	+
Escherichia coli O157 (E.C. 1)[d]	-	+
Escherichia coli O157 (E.C. 2)[d]	+	+
Escherichia coli O157 (E.C. 3)[d]	+	+
Escherichia coli O157 (E.C. 4)	+	+
Escherichia coli O157 (E.C. 5)	+	+
Enterococcus faecalis (29212)	-	-
Enterococcus faecalis (33186)	-	-
Listeria monocytogenes (FSL-C1 115)	-	-
Listeria monocytogenes (FSL-C1 122)	-	-
Salmonella Typhimurium (14028)	-	-
Salmonella Anatum (24594)	-	-
Salmonella Enteritidis (PT-30)	-	-
Escherichia coli (ECOR[e] 39)	-	+
Escherichia coli (ECOR 42)	-	+
Escherichia coli (ECOR 43)	-	+

[a] Cocktail of 37 bacteriophages as described in Table 2.2.
[b] Bacterial strain used for pure culture studies.
[c] (–) indicates no lysis, (+) indicates lysis of the corresponding target bacteria.
[d] Bacterial strains used to prepare 3-strain cocktail for environmental water studies.
[e] *Escherichia coli* Reference Collection.

Table 2.2. Bacteriophage strains used to prepare the 37-phage cocktail used in Antimicrobial Incorporated Multi-angle Light Scattering (ANIMALS) during detection of *Escherichia coli* O157:H7 in environmental water samples.

Bacteriophage	Host[a]	Family	Ave. Head Size (nm)	Tail Length (nm)
1	A	Myoviridae	79.9/±/7.3 x 95.2/±/10.3	110.1/±/12.5
2	A	Myoviridae	73.4/±/4.5 x 85.6/±/6.6	105.2/±/7.9
3	A	Myoviridae	78.7/±/5.4 x 97.5/±/6.3	103.7/±/10.8
5	A	Myoviridae	78.4/±/5.9 x 95.9/±/3.1	109.2/±/2.7
6	A	Myoviridae	82.5/±/2.6 x 108.3/±/2.3	108.0/±/2.2
7	A	Siphoviridae	53.9/±/2.7	
8	A	Myoviridae	62.4/±/2.2 x 83.6/±/1.4	118.1/±/2.3
8a	B	Myoviridae	74.2/±/3.2 x 98.0/±/5.3	112.2/±/3.5
9	B	Siphoviridae	51.58/±/0.70	
10	A	Myoviridae	73.2/±/4.2 x 88.0/±/1.7	106.7/±/6.0
10a	A	Myoviridae	71.2/±/2.6 x 95.6/±/7.4	111.8/±/5.9
11	A	Siphoviridae	49.2/±/1.6	
11a	A	Myoviridae	73.8/±/5.8 x 102.6/±/2.4	108.7/±/2.2
12	A	Siphoviridae	49.3/±/5.0	
13	A	Myoviridae	72.1/±/9.2 x 92.5/±/4.3	120.4/±/7.5
15	A	Myoviridae	77.5/±/7.8 x 95.8/±/1.6	117.6/±/3.2
16	A	Myoviridae	76.6/±/4.6 x 91.9/±/4.6	118.9/±/5.8
17	A	Siphoviridae	53.3/±/3.9	
20	A	Myoviridae	79.0/±/3.31 x 98.5/±/2.6	108.7/±/5.3
21	B	Myoviridae	79.1/±/7.9 x 90.2/±/2.7	115.2/±/8.7
22	B	Myoviridae	74.2/±/7.4 x 104.5/±/4.9	109.2/±/3.4
23	A	Myoviridae	70.6/±/5.1 x 99.6/±/7.4	110.4/±/5.4
24	B	Siphoviridae	52.4/±/3.1	
25	B	Siphoviridae	51.7/±/0.8	
27	A	Myoviridae	64.4/±/2.9 x 85.2/±/11.2	124.6/±/5.0
38	A	Myoviridae	69.4/±/2.2 x 92.4/±/5.0	119.3/±/3.6
41	A	Myoviridae	63.1/±/0.60 x 74.4/±/1.2	115.7/±/3.2
50	A	Myoviridae	70.1/±/3.2 x 94.1/±/3.0	120.5/±/1.7
52	A	Myoviridae	61.2/±/2.2 x 79.1/±/5.1	120.7/±/4.7
53	B	Myoviridae	65.9/±/2.5 x 83.4/±/4.4	119.2/±/3.1
54	A	Myoviridae	69.1/±/5.4 x 99.3/±/6.2	117.1/±/5.1
56	A	Myoviridae	68.9/±/4.9 x 92.5/±/4.1	115.5/±/4.6
57	A	Myoviridae	60.9/±/3.6 x 80.7/±/3.5	119.1/±/5.1
AR1[b]	C	Myoviridae	76.0/±/2.8 x 93.7/±/2.4	112.3/±/5.7
RB33	A	Myoviridae	73.7/±/2.5 x 95.6/±/4.9	107.8/±/3.6
RB34	C	Myoviridae	71.2/±/5.7 x 93.4/±/6.2	112.4/±/6.6
T4	A	Myoviridae	68.0/±/7.9 x 90.7/±/4.1	112.9/±/6.8

[a] (A) = *Escherichia coli* B, (B) = *E. coli* C and (C) = *E. coli* 920333

[b] Bacteriophage strain used in pure culture studies.

Table 2.3. Physical and chemical characteristics of environmental water samples subjected to Antimicrobial Incorporated Multi-angle Light Scattering (ANIMALS).

Parameter	River Water Results	Reservoir Water Results
Conductivity	1493 µmhos/cm	1561 µmhos/cm
pH	7.9	7.5
	mg/L	**mg/L**
Calcium	60.6	108.3
Magnesium	63.05	57.74
Sodium	68.8	224.9
Potassium	2.18	2.31
Carbonate	0	0
Chloride	21.7	12.8
Sulfate	3,720	4,311
Nitrate	15.2	32.9
Nitrate-Nitrogen	3.42	7.42
Total Alkalinity as $CaCO_3$	161	281
Total Hardness as $CaCO_3$	410	508
Total Dissolved Solids	3,955	4,757

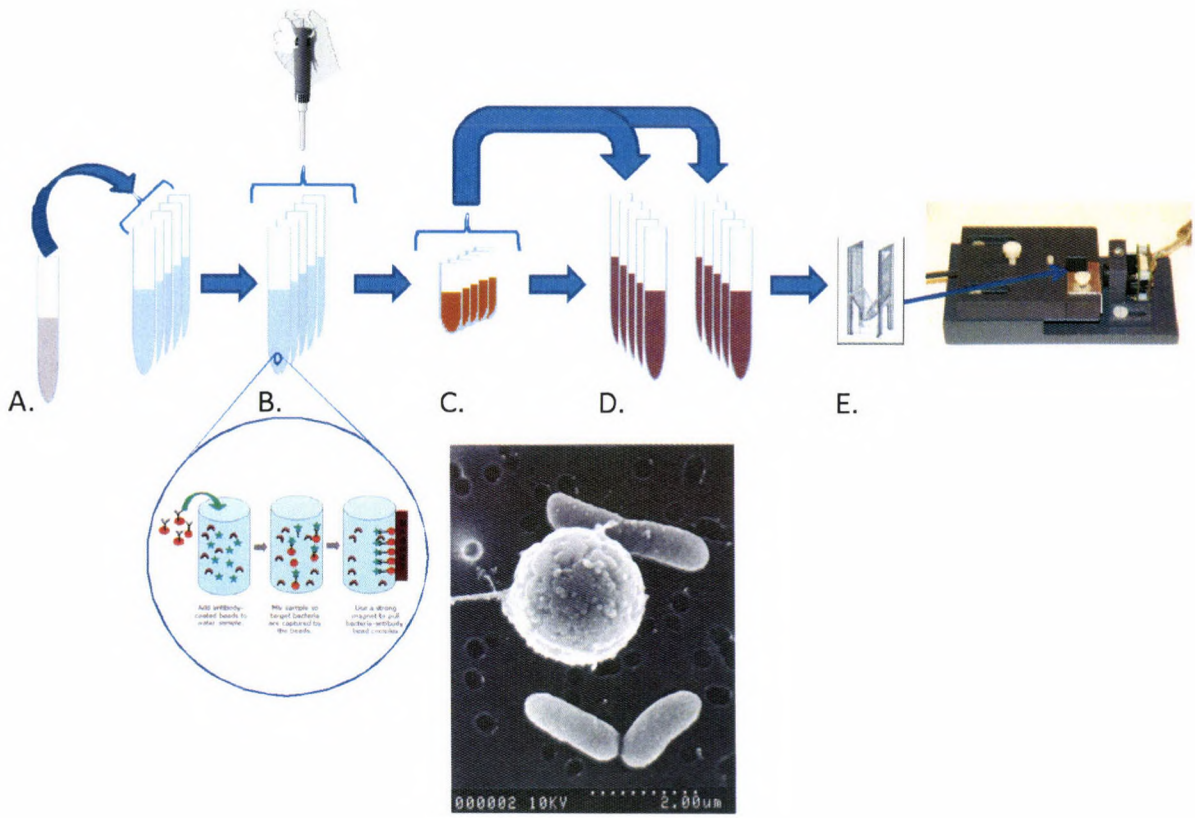


Figure 2.1. The Antimicrobial Incorporated Multi-angle Light Scattering (ANIMALS) Assay- A) Beginning with a pure culture of *Escherichia coli* O157:H7 (EC920333), 1/10 serial dilutions were prepared in lambda buffer and selected to contain a final concentration of 10^0, 10^1, 10^2, 10^3 and 10^4 CFU/ml. B) 40µl of *E. coli* O157:H7 specific immunomagnetic separation (IMS) beads were added to each tube and allowed to incubate for 15 minutes at 37°C with shaking. The magnified image shows capture of *Escherichia coli* O157:H7 by the antibody coated IMS beads. C) Once separated and washed, the beads and any attached cells were re-suspended using 1 ml of Tryptic Soy Broth (TSB). D) The samples were split into test and reference tubes and 500µl were added to each pair of tubes containing 10 mls of TSB. Test samples received 1 ml of bacteriophage AR1 (10^{10} PFU/ml), while reference samples received 1 ml of lambda buffer. All samples were then allowed to incubate for 4, 6, 8, 10 or 15 hours at 37° C (pure culture) or 42° C (environmental water) with shaking. Following incubation, each sample was analyzed using a SpectraPoint Light Scattering Spectrometer.

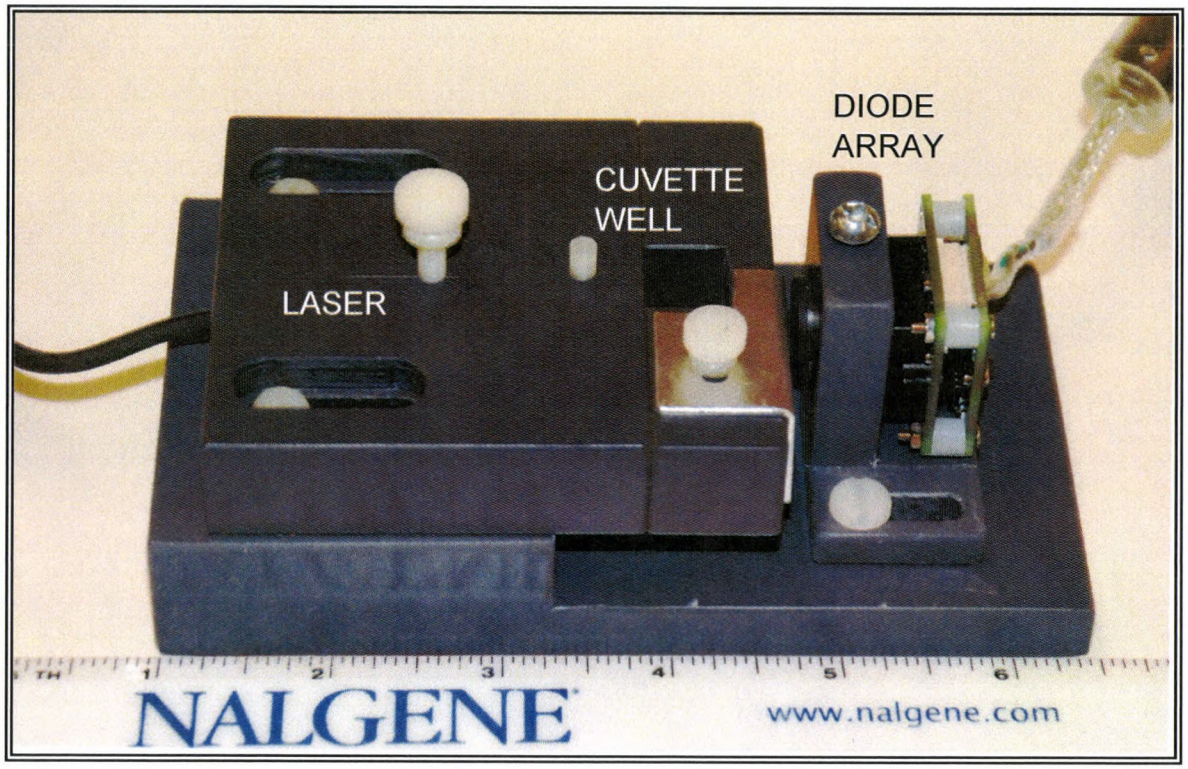


Figure 2.2. SpectraPoint light scattering spectrometer (SpectraDigital Corp., Guelph, Ontario, Canada). The prototype device included a 780nm wavelength infrared laser light source, a cuvette well for use with a 2mm path length disposable cuvette (UVette®, Eppendorf North America, Inc., Westbury, NY), and a receptor diode array. One hundred (100) forward light scattering measurements per pixel were collected at low light scattering angles (< 4°).

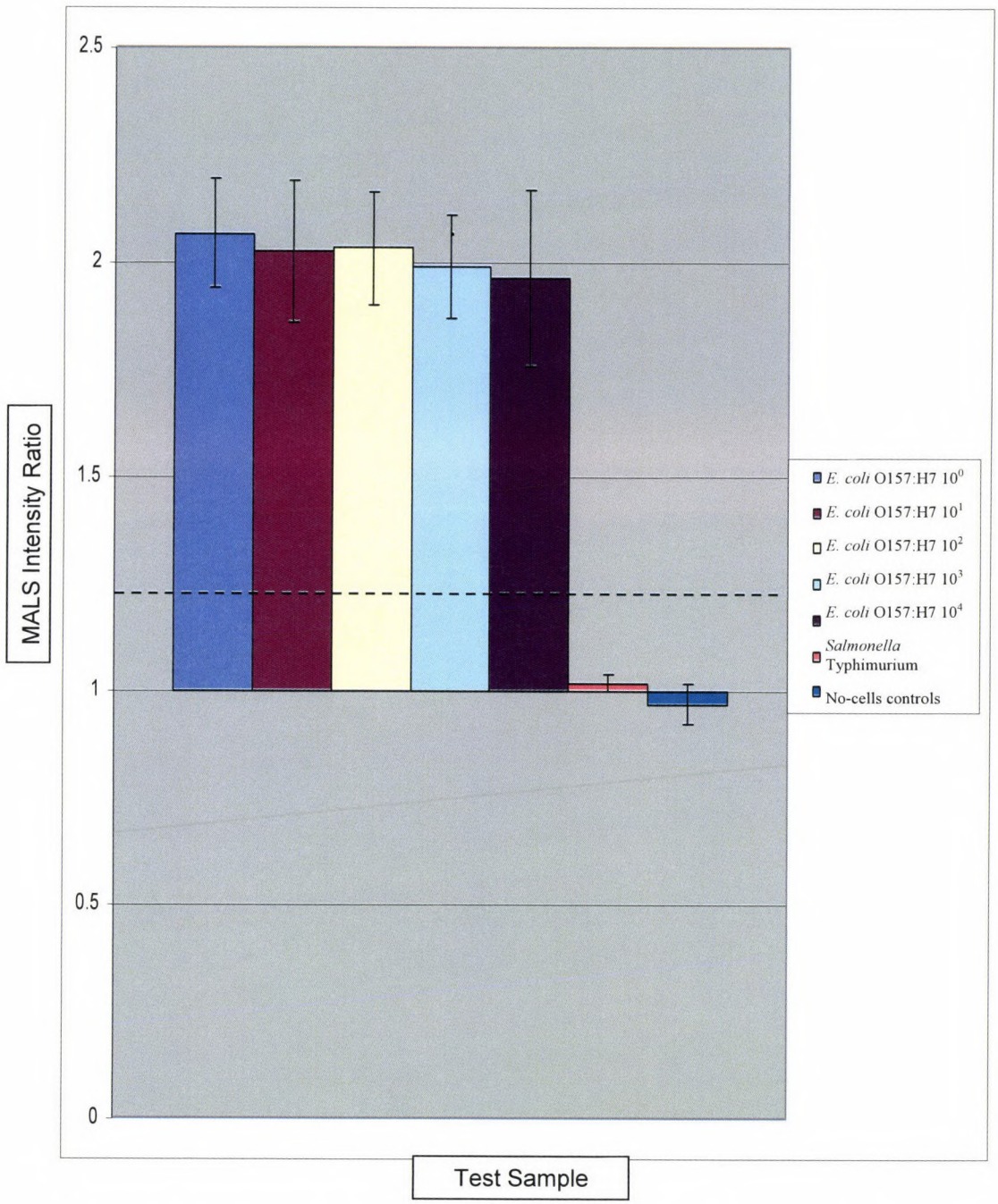


Figure 2.3. Means and standard deviations for the difference in scattering intensity ratios between test and reference samples of pure cultures subjected to ANIMALS. Means higher than 3× the standard deviations of the control (represented by the dashed line) were considered positive. There were significant differences ($p < 0.0001$) between the means of all *Escherichia coli* O157:H7 concentrations and the negative controls. However, no statistically significant differences ($p = 0.6909$) were found between the negative controls.

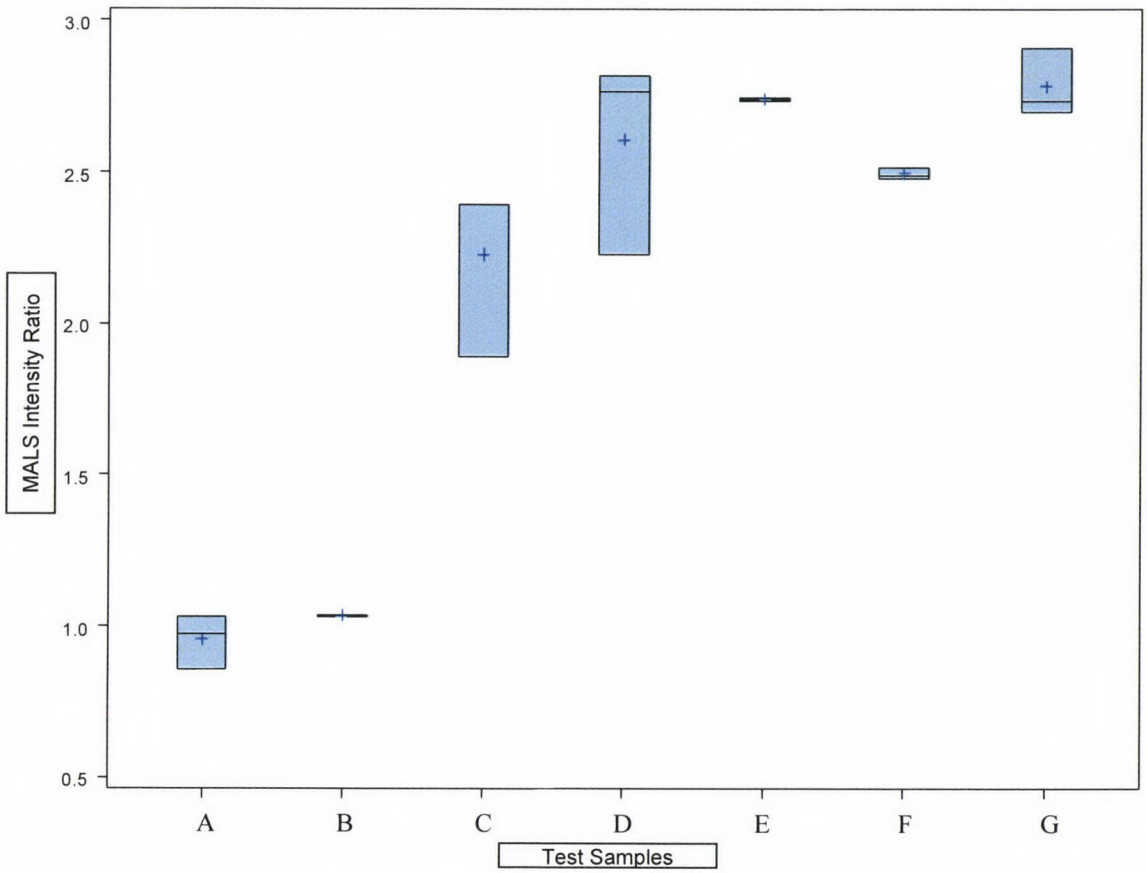


Figure 2.4. Boxplot of means and variance for the difference in scattering intensity ratios between test and reference samples of pure culture studies at 15 hours. Data was generated using the GLM procedure of the one-way ANOVA in SAS. The graph shows statistically significant differences ($p < 0.0001$) for all concentrations of *Escherichia coli* O157:H7. No statistically significant differences ($p = 0.6909$) were present among the negative controls of *Salmonella* Typhimurium and uninoculated samples. (A) = Uninoculated controls, (B) = *Salmonella* Typhimurium, (C) = *Escherichia coli* O157:H7 10^0, (D) = *E. coli* O157:H7 10^1, (E) = *E. coli* O157:H7 10^2, (F) = *E. coli* O157:H7 10^3, (G) = *E. coli* O157:H7 10^4.

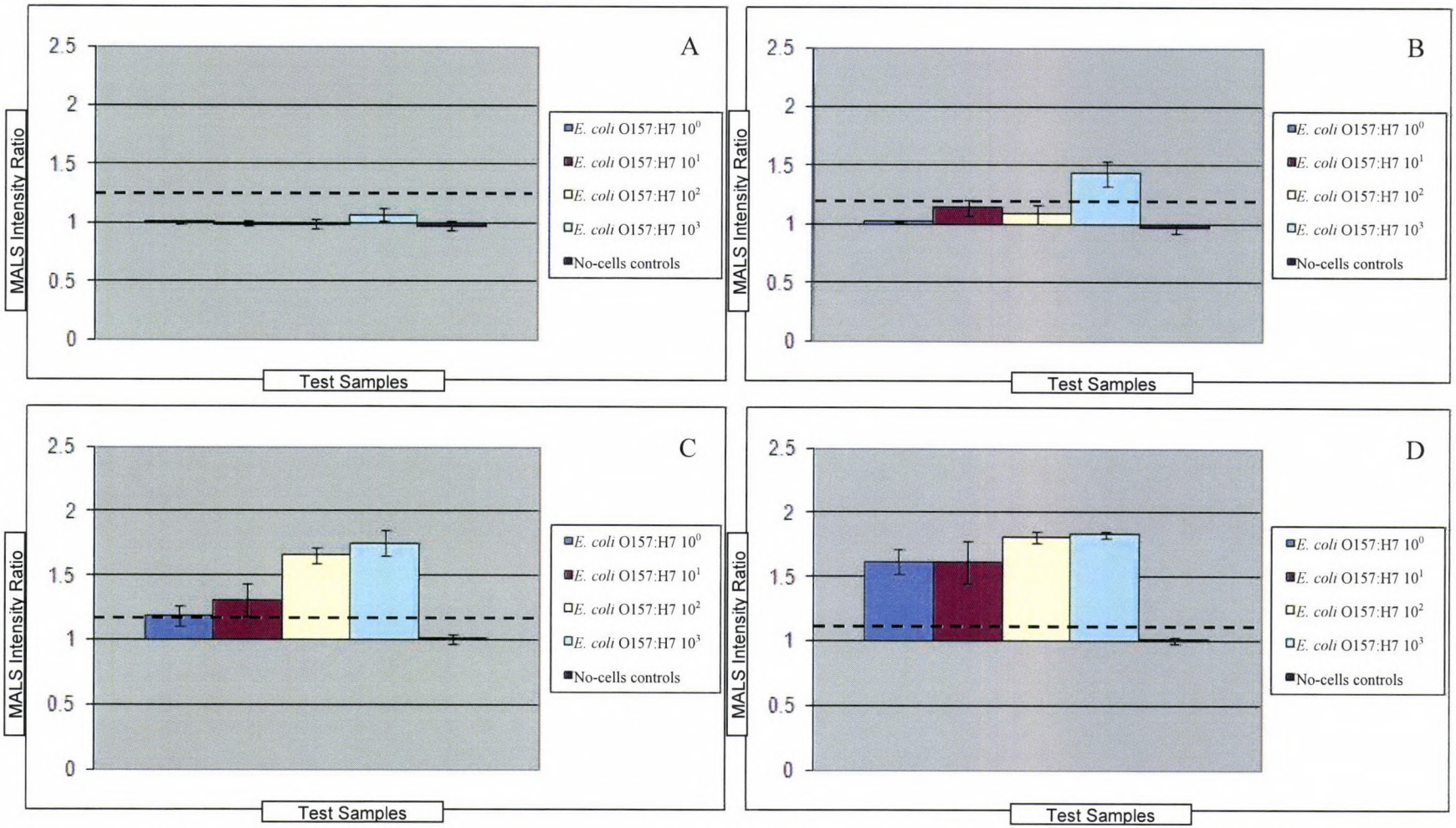


Figure 2.5. Detection of a 3-strain cocktail of *Escherichia coli* O157:H7 in spiked river water samples using ANIMALS. The means and standard deviations for the difference in scattering intensity ratios between test and reference samples at 4, 6, 8 and 10 hours are shown. Means higher than 3× the standard deviations of the control (represented by the dashed lines) were considered positive. Detection of all concentrations of *E. coli* O157:H7 was possible after only 10 hours of enrichment. (A) = 4hrs, (B) = 6hrs, (C) = 8hrs, (D) = 10hrs.

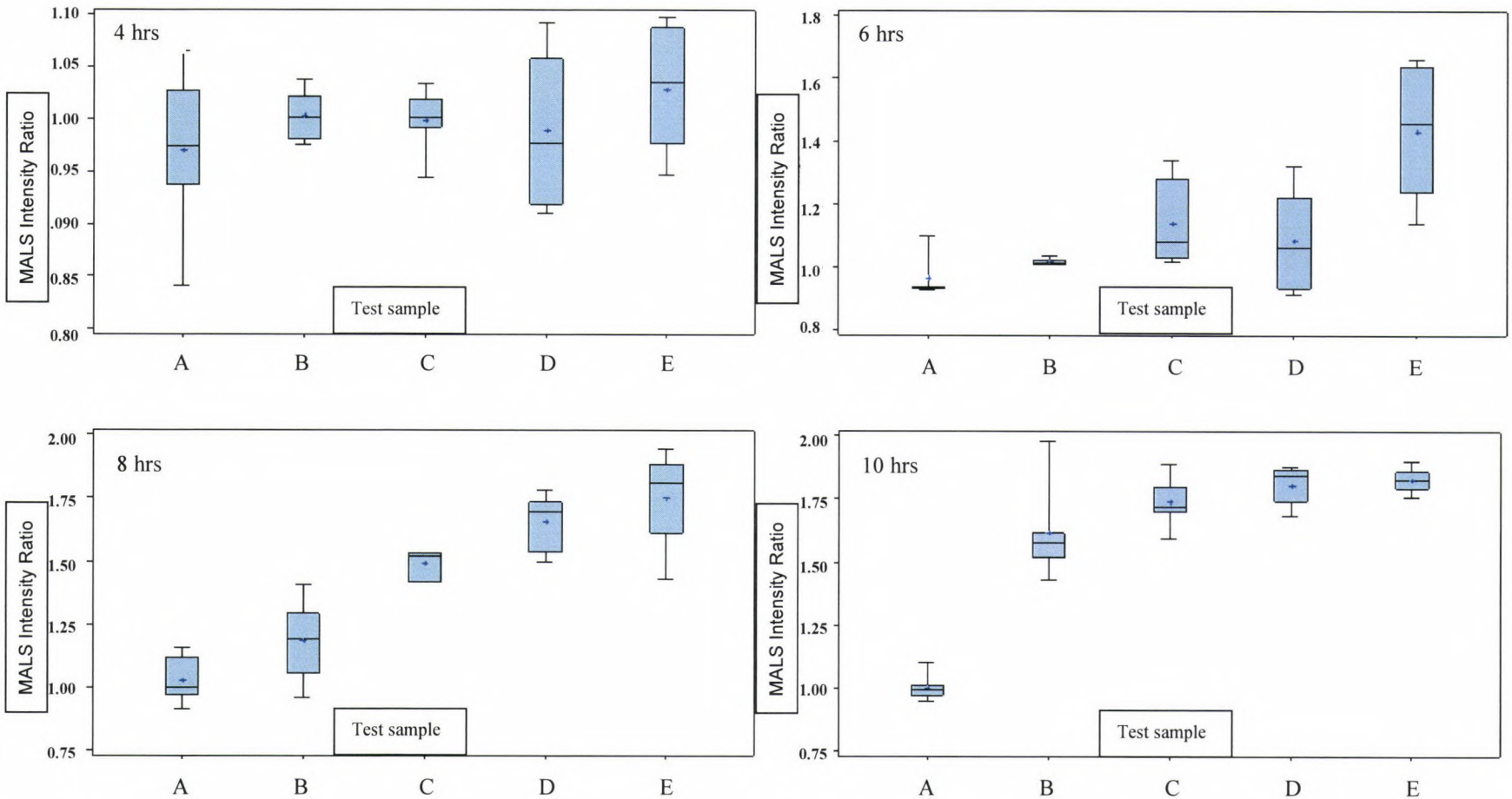


Figure 2.6. Boxplot of means and variance for the difference in scattering intensity ratios between test and reference samples of spiked environmental water studies (river water). Data was generated using the GLM procedure of the one-way ANOVA in SAS. The graph shows statistically significant differences ($p < 0.0001$) for the ten-hour readings for all concentrations of *Escherichia coli* O157:H7. No statistically significant differences were present in readings taken at 4, 6 or 8 hours. (A) = Uninoculated controls, (B) = *E. coli* O157:H7 10^0, (C) = *E. coli* O157:H7 10^1, (D) = *E. coli* O157:H7 10^2, (E) = *E. coli* O157:H7 10^3.

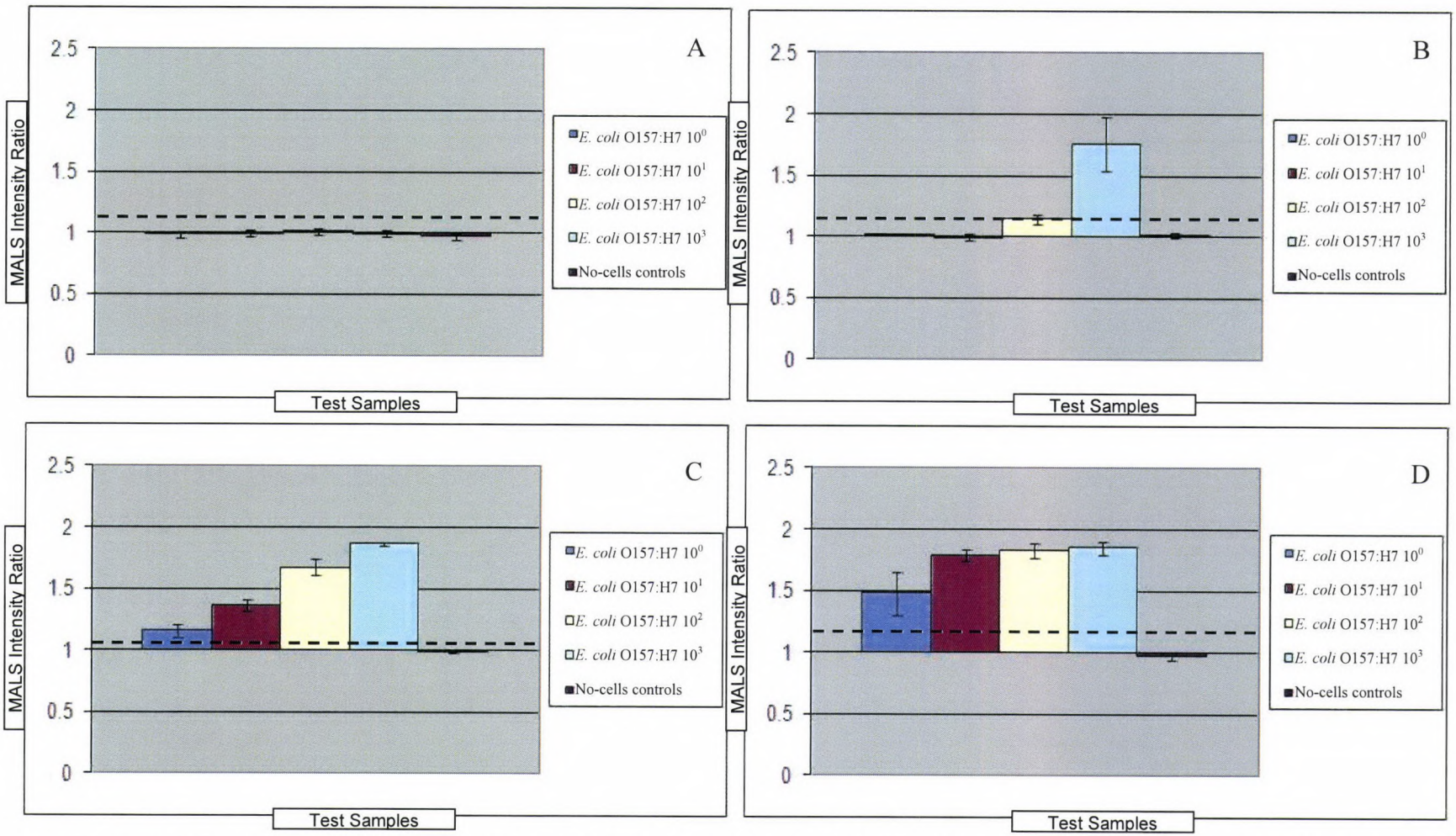


Figure 2.7. Detection of a 3-strain cocktail of *Escherichia coli* O157:H7 in spiked reservoir water samples using ANIMALS. The means and standard deviations for the difference in scattering intensity ratios between test and reference samples at 4, 6, 8 and 10 hours are shown. Means higher than 3× the standard deviations of the control (represented by the dashed lines) were considered positive. Detection of all concentrations of *E. coli* O157:H7 was possible after only 8 hours of enrichment. (A) = 4hrs, (B) = 6hrs, (C) = 8hrs, (D) = 10hrs.

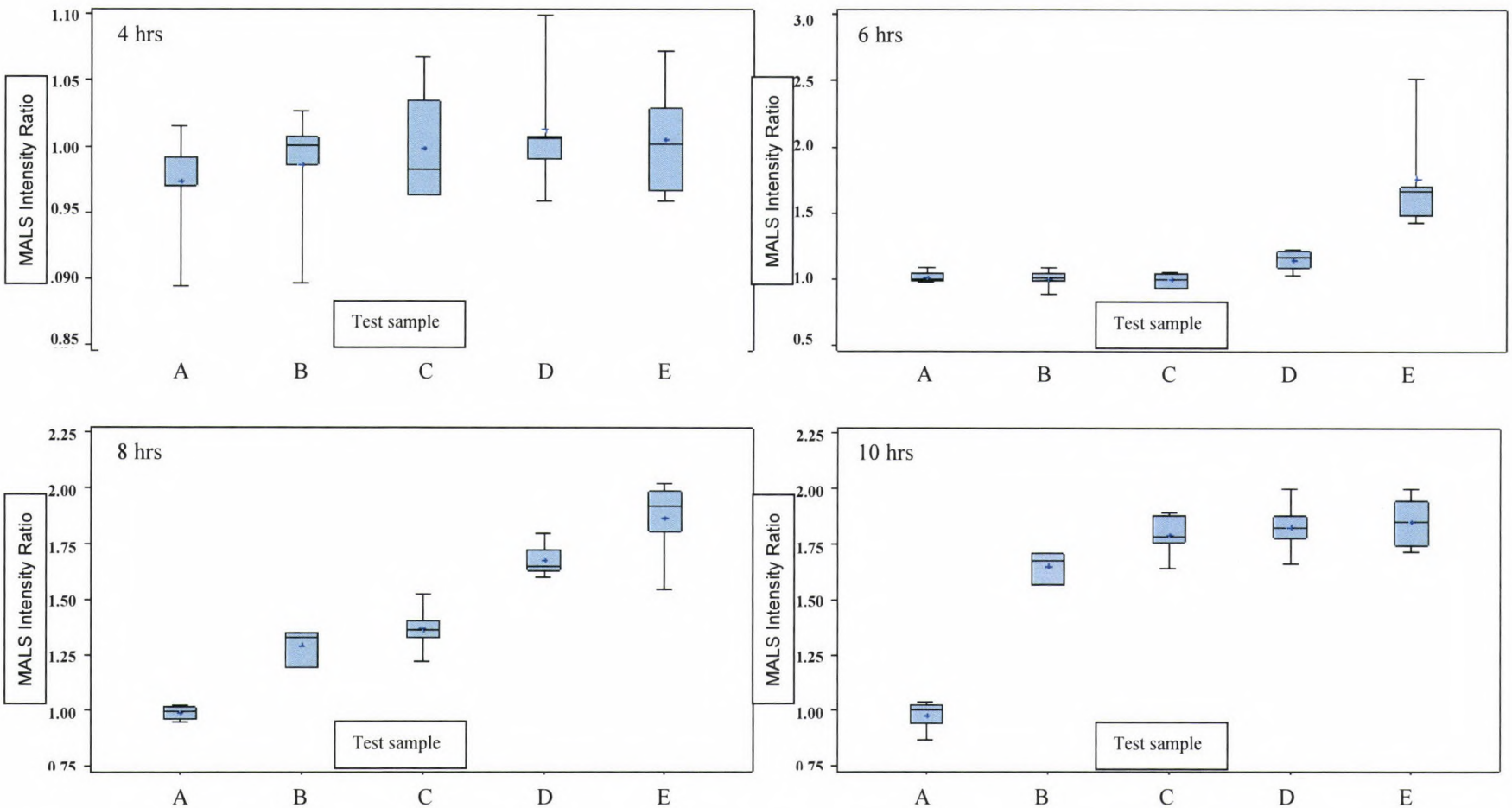
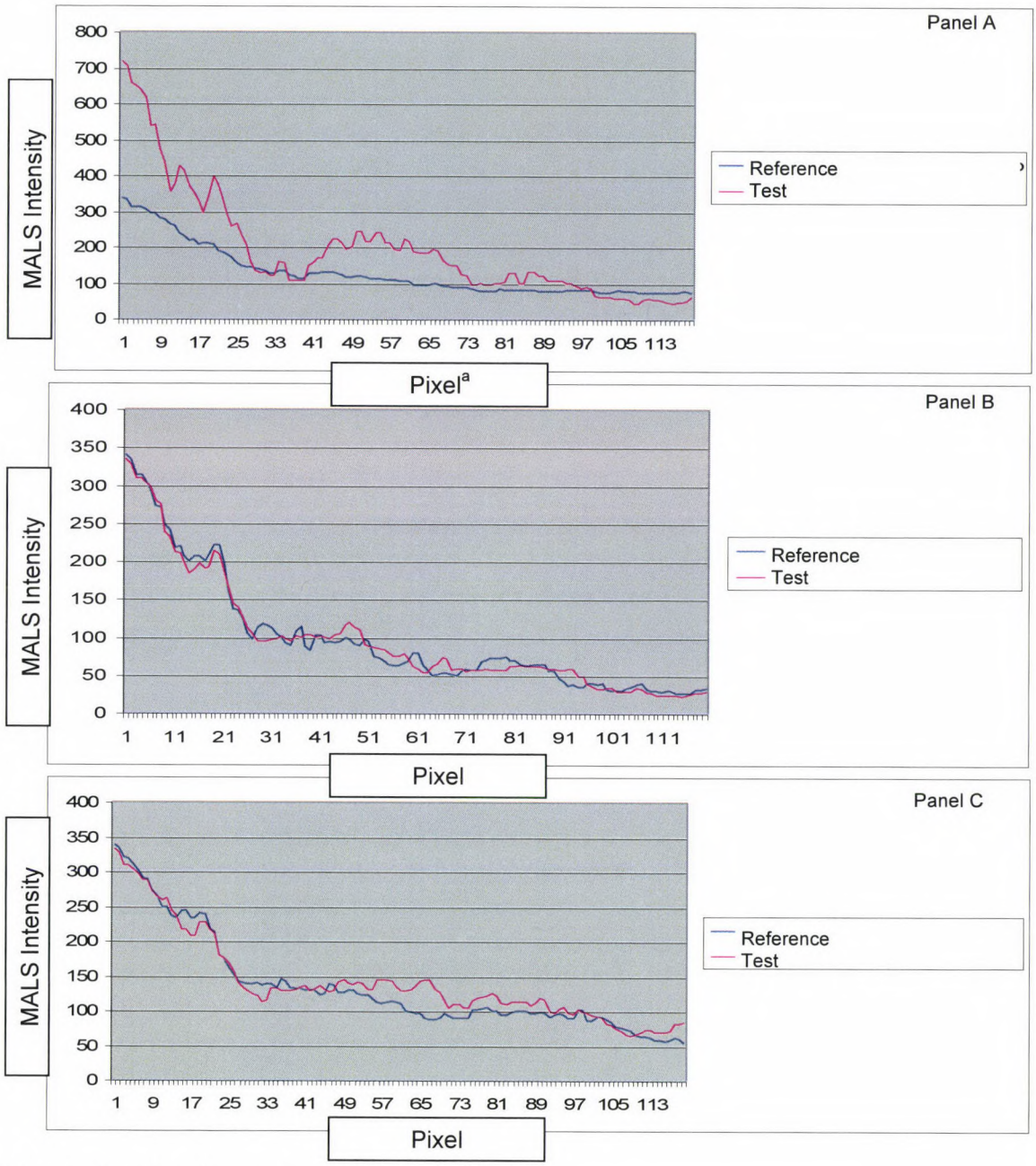


Figure 2.8. Boxplot of means and variance for the difference in scattering intensity ratios between test and reference samples of spiked environmental water studies (reservoir water). Data was generated using the GLM procedure of the one-way ANOVA in SAS. The graph shows statistically significant differences ($p < 0.0001$) for the ten-hour readings for all concentrations of *Escherichia coli* O157:H7. No statistically significant differences were present in readings taken at 4, 6 or 8 hours. (A) = Uninoculated controls, (B) = *E. coli* O157:H7 10^0, (C) = *E. coli* O157:H7 10^1, (D) = *E. coli* O157:H7 10^2, (E) = *E. coli* O157:H7 10^3.



[a] 36 pixels = 1° of scattering

Figure 2.9. (Panel A) Typical spectra of a positive test sample containing *Escherichia coli* O157:H7 subjected to ANIMALS. Lysis of *E. coli* O157:H7 in the test sample to which phage AR1 has been added, causes a difference in scattering intensity of the test sample when compared to a reference in which *E. coli* O157:H7 cells were allowed to grow unobstructed. The presence of intact cells in the reference samples increases light scattering at low angles and produces lower intensity values when compared to the less refractive test sample. In panels B and C, there is no difference in the scattering intensities of the test and reference samples because there is either no cell growth (Panel B) or there is equal cell growth of *Salmonella* Typhimurium in test and reference samples (Panel C) as these bacteria are non-susceptible to lysis by the phage.

CHAPTER THREE
REFERENCES

Acharya, G., Chun-Li, C., Savran, C. 2006. An optical biosensor for rapid and label-free detection of [bacterial] cells. Journal of the American Chemical Society 128, 3862-3863.

Adams, M.H. 1959. Bacteriophages. Interscience Publishers, New York.

Babacan, S., Pivarnik, P., Letcher, S., Rand, A.G. 2000. Evaluation of antibody immobilization methods for piezoelectric biosensor application. Biosensors & Bioelectronics 15, 615-621.

Bae, E., Banada, P.P., Huff, K., Bhunia, A.K., Robinson, J.P., Hirleman, E.D. 2007. Biophysical modeling of forward scattering from bacterial colonies using scalar diffraction theory. Applied Optics 46, 3639-3648.

Banada, P.P., Guo, S.L., Bayraktar, B., Bae, E., Rajwa, B., Robinson, J.P., Hirleman, E.D., Bhunia, A.K. 2007. Optical forward-scattering for detection of *Listeria monocytogenes* and other *Listeria* species. Biosensors & Bioelectronics 22, 1664-1671.

Banada, P.P., Huff, K., Bae, E., Rajwa, B., Aroonnual, A., Bayraktar, B., Adil, A., Robinson, J.P., Hirleman, E.D., Bhunia, A.K. 2009. Label-free detection of multiple bacterial pathogens using light-scattering sensor. Biosensors & Bioelectronics 24, 1685-1692.

Banerjee, P., Bhunia, A.K. 2009. Mammalian cell-based biosensors for pathogens and toxins. Trends in Biotechnology 27, 179-188.

Bhunia, A.K., Banada, P., Banerjee, P., Valadez, A., Hirleman, E.D. 2007. Light scattering, fiber optic- and cell-based sensors for sensitive detection of foodborne pathogens. Journal of Rapid Methods and Automation in Microbiology 15, 121-145.

Bokken, G.C.A.M., Corbee, R.J., Knapen, F.v., Bergwerff, A.A. 2003. Immunochemical detection of *Salmonella* group B, D and E using an optical surface plasmon resonance biosensor. FEMS Microbiology Letters 222, 75-82.

Campbell, G.A., Mutharasan, R. 2005a. Detection of pathogen *Escherichia coli* O157:H7 using self-excited PZT-glass microcantilevers. Biosensors & Bioelectronics 21, 462-473.

Campbell, G.A., Mutharasan, R. 2005b. *Escherichia coli* O157:H7 detection limit of millimeter-sized PZT cantilever sensors is 700 cells/mL. Analytical Sciences 21, 355-357.

Campbell, G.A., Mutharasan, R. 2006. Piezoelectric-excited millimeter-sized cantilever (PEMC) sensors detect *Bacillus anthracis* at 300 spores/mL. Biosensors & Bioelectronics 21, 1684-1692.

Campbell, G.A., Mutharasan, R. 2007. A method of measuring *Escherichia coli* O157:H7 at 1 cell/ml in 1 liter sample using antibody functionalized piezoelectric-excited millimeter-sized cantilever sensor. Environmental Science & Technology 41, 1668-1674.

Campbell, G.A., Delesdernier, D., Mutharasan, R. 2007a. Detection of airborne *Bacillus anthracis* spores by an integrated system of an air sampler and a cantilever immunosensor. Sensors and Actuators B-Chemical 127, 376-382.

Campbell, G.A., Uknalis, J., Tu, S.I., Mutharasan, R. 2007b. Detect of *Escherichia coli* O157:H7 in ground beef samples using piezoelectric excited millimeter-sized cantilever (PEMC) sensors. Biosensors & Bioelectronics 22, 1296-1302.

Chang, T.C., Ding, H.C., Chen, S.W. 2002. A conductance method for the identification of *Escherichia coli* O157:H7 using bacteriophage AR1. Journal of Food Protection 65, 12-17.

Chen, J., Griffiths, M.W. 1996. *Salmonella* detection in eggs using *Lux*(+) bacteriophages. Journal of Food Protection 59, 908-914.

Coconnier, M.H., Lorrot, M., Barbat, A., Laboisse, C., Servin, A.L. 2000. Listeriolysin O-induced stimulation of mucin exocytosis in polarized intestinal mucin-secreting cells: evidence for toxin recognition of membrane-associated lipids and subsequent toxin internalization through caveolae. Cellular Microbiology 2, 487-504.

DeBuschere, B.D. 2002. Portable cell-based biosensors. Ph.D. Thesis, Stanford University, CA.

Deshpande, S., Hall, J.C. 1995. Comparison of flash-induced light-scattering transients and protons efflux from auxinic-herbicide resistant and susceptible wild mustard protoplasts – a possible role for calcium in mediating auxinic herbicide resistance. Biochimica Et Biophysica Acta-General Subjects 1244, 69-78.

DeYoung, H.G. 1983. Biosensors: The mating of biology and electronics. High Technology 3, 41-49.

Flowers, T.H. 1977. Measurement of growth rates of *Streptomycetes*: comparison of turbidimetric and gravimetric techniques. Journal of General Microbiology 98, 285-289.

Giaever, I. 1993. A morphological biosensor for mammalian cells. Nature 366, 591-592.

Goodridge, L. 2004. Isoelectric point determination of norovirus virus-like particles by capillary isoelectric focusing with whole column imaging detection. Analytical Chemistry 1, 48-52.

Goodridge, L., Chen, J.R., Griffiths, M. 1999a. Development and characterization of a fluorescent-bacteriophage assay for detection of *Escherichia coli* O157:H7. Applied and Environmental Microbiology 65, 1397-1404.

Goodridge, L., Chen, J.R., Griffiths, M. 1999b. The use of a fluorescent bacteriophage assay for detection of *Escherichia coli* O157:H7 in inoculated ground beef and raw milk. International Journal of Food Microbiology 47, 43-50.

Goodridge, L., Gallaccio, A., Griffiths, M.W. 2003. Morphological, host range, and genetic characterization of two coliphages. Applied and Environmental Microbiology 69, 5364-5371.

Goodridge, L., Griffiths, M. 2002. Reporter bacteriophage assays as a means to detect foodborne pathogenic bacteria. Food Research International 35, 863-870.

Jones, A., Young, D., Taylor, J., Kell, D.B., Rowland, J.J. 1998. Quantification of microbial productivity via multi-angle light scattering and supervised learning. Biotechnology and Bioengineering 59, 131-143.

Kassanos, P., Iles, R.K., Bayford, R.H., Demosthenous, A. 2007. Development of a Biosensor for hCGβ Detection. 13th International Conference on Electrical Bioimpedance and the 8th Conference on Electrical Impedance Tomography, 620-623.

Katz, A., Alimova, A., Xu, M., Rudolph, E., Shah, M.K., Savage, H.E., Rosen, R.B., McCormick, S.A., Alfano, R.R. 2003. Bacteria size determination by elastic light scattering. Journal of Selected Topics in Quantum Electronics 9, 277-287.

Ko, S.H., Grant, S.A. 2006. A novel FRET-based optical fiber biosensor for rapid detection of *Salmonella* Typhimurium. Biosensors & Bioelectronics 21, 1283-1290.

Kodikara, C.P., Crew, H.H., Stewart, G. 1991. Near online detection of enteric bacteria using *lux* recombinant bacteriophage. Fems Microbiology Letters 83, 261-266.

Lan, Y.B., Wang, S.Z., Yin, Y.G., Hoffmann, W.C., Zheng, X.Z. 2008. Using a Surface Plasmon Resonance Biosensor for Rapid Detection of *Salmonella* Typhimurium in Chicken Carcass. Journal of Bionic Engineering 5, 239-246.

Latimer, P. 1982. Light-scattering and absorption as methods of studying cell-population parameters. Annual Review of Biophysics and Bioengineering 11, 129-150.

Lazcka, O., Campo, F.J.D., Muñoz, F.X. 2007. Pathogen detection: A perspective of traditional methods and biosensors. Biosensors and Bioelectronics 22, 1205-1217.

Lim, D.V., Simpson, J.M., Kearns, E.A., Kramer, M.F. 2005. Current and developing technologies for monitoring agents of bioterrorism and biowarfare. Clinical Microbiology Reviews 18, 583.

Loessner, M.J., Rees, C.E.D., Stewart, G., Scherer, S. 1996. Construction of luciferase reporter bacteriophage A511::*lux*AB for rapid and sensitive detection of viable *Listeria* cells. Applied and Environmental Microbiology 62, 1133-1140.

Louie, A.S., Marenchic, I.G., Whelan, R.H. 1998. A fieldable modular biosensor for use in detection of foodborne pathogens. Field Analytical Chemistry and Technology 2, 371-377.

Ma, Z.H., Merkus, H.G., van der Veen, H.G., Wong, M., Searlett, B. 2002. On-line measurement of particle size and shape using laser diffraction. Optic Letters, 18. 243-247.

Maalouf, R., Fournier-Wirth, C., Coste, J., Chebib, H., Saikali, Y., Vittori, O., Errachid, A., Cloarec, J.P., Martelet, C., Jaffrezic-Renault, N. 2007. Label-free detection of bacteria by electrochemical impedance spectroscopy: Comparison to surface plasmon resonance. Analytical Chemistry 79, 4879-4886.

Manning, S.D., Motiwala, A.S., Springman, A.C., Qi, W., Lacher, D.W., Ouellette, L.M., Mlaclonicky, J.M., Somsel, P., Rudrik, J.T., Dietrich, S.E., Zhang, W., Swaminathan, B., Alland, D., Whittam, T.S. 2008. Variation in virulence among clades of *Escherichia coli* O3157:H7 associated with disease outbreaks. Proceedings of the National Academy of Sciences of the United States of America 105, 4868-4873.

Maraldo, D., Mutharasan, R. 2007. Preparation-free method for detecting *Escherichia coli* O157:H7 in the presence of spinach, spring lettuce mix, and ground beef particulates. Journal of Food Protection 70, 2651-2655.

Matsell, D.G., White, C.T. 2009. An outbreak of diarrhea-associated childhood hemolytic uremic syndrome: the Walkerton epidemic. Kidney International 75, 35-37.

McClatchey, K.D. 2002. Clinical Laboratory Medicine. Lippincott Williams & Wilkins, Hagerstown, Maryland.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R.V. 1999. Food-related illness and death in the United States. Emerging Infectious Diseases 5, 607-625.

Meeusen, C.A., Alocilja, E.C., Osburn, W.N. 2005. Detection of *E. coli* O157:H7 using a miniaturized surface plasmon resonance biosensor. Transactions of the ASAE 48, 2409-2416.

Mizoguchi, K., Morita, M., Fischer, C.R., Yoichi, M., Tanji, Y., Unno, H. 2003. Coevolution of bacteriophage PP01 and *Escherichia coli* O157 : H7 in continuous culture. Applied and Environmental Microbiology 69, 170-176.

Ochoa, M.L., Harrington, P.B. 2005. Immunomagnetic isolation of enterohemorrhagic *Escherichia coli* O157:H7 from ground beef and identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and database searches. Analytical Chemistry 77, 5258-5267.

Olsen, E.V., Pathirana, S.T., Samoylov, A.M., Barbaree, J.M., Chin, B.A., Neely, W.C., Vodyanoy, V. 2003. Specific and selective biosensor for *Salmonella* and its detection in the environment. Journal of Microbiological Methods 53, 273-285.

Palchetti, I., Mascini, M. 2008. Electroanalytical biosensors and their potential for food pathogen and toxin detection. Analytical and Bioanalytical Chemistry 391, 455-471.

Pathirana, S.T., Barbaree, J., Chin, B.A., Hartell, M.G., Neely, W.C., Vodyanoy, V. 2000. Rapid and sensitive biosensor for *Salmonella*. Biosensors & Bioelectronics 15, 135-141.

Paton, A.W., Paton, J.C. 1998. Detection and characterization of shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx(1), stx(2), eaeA, enterohemorrhagic *E. coli* hlyA, rfb(O111), and rfb(O157). Journal of Clinical Microbiology 36, 598-602.

Pearson, R.E., Jurgensen, S., Sarkis, G.J., Hatfull, G.F., Jacobs, W.R. 1996. Construction of D29 shuttle phasmids and luciferase reporter phages for detection of mycobacteria. Gene 183, 129-136.

Pedrero, M., Campuzano, S., Pingarron, J. 2009. Electroanalytical Sensors and Devices for Multiplexed Detection of Foodborne Pathogen Microorganisms. Sensors 9, 5503-5520.

Pohlmann, C., Wang, Y., Humenik, M., Heidenreich, B., Gareis, M., Sprinzl, M. 2009. Rapid, specific and sensitive electrochemical detection of foodborne bacteria. Biosensors and Bioelectronics 24, 2766-2771.

Rajwa, B., Venkatapathi, M., Ragheb, K., Banada, P.P., Hirleman, E.D., Lary, T., Robinson, J.P. 2008. Automated classification of bacterial particles in flow by multiangle scatter measurement and support vector machine classifier. Cytometry. 369-379.

Renneberg, R., Pfeiffer, D., Lisdat, F., Wilson, G., Wollenberger, U., Ligler, F., Turner, A.P.F. 2008. Frieder scheller and the short history of biosensors, Biosensing for the 21st Century, vol. 109. Springer-Verlag Berlin, Berlin. 1-18.

Rider, T.H., Petrovick, M.S., Nargi, F.E., Harper, J.D., Schwoebel, E.D., Mathews, R.H., Blanchard, D.J., Bortolin, L.T., Young, A.M., Chen, J.Z., Hollis, M.A. 2003. A B cell-based sensor for rapid identification of pathogens. Science 301, 213-215.

Rocha-Gaso, M.I., March-Iborra, C., Montoya-Baides, A., Arnau-Vives, A. 2009. Surface Generated Acoustic Wave Biosensors for the Detection of Pathogens: A Review. Sensors 9, 5740-5769.

Ruan, C., Wang, H., Li, Y. 2002. A bienzyme electrochemical biosensor coupled with immunomagnetic separation for rapid detection of *Escherichia coli* O157:H7 in food samples. Transactions of the ASAE 45, 249-255.

Seo, K.H., Brackett, R.E., Hartman, N.F., Campbell, D.P. 1999. Development of a rapid response biosensor for detection of *Salmonella* Typhimurium. Journal of Food Protection 62, 431-437.

Shabani, A., Zourob, M., Allain, B., Marquette, C.A., Lawrence, M.F., Mandeville, R. 2008. Bacteriophage-Modified Microarrays for the Direct Impedimetric Detection of Bacteria. Analytical Chemistry 80, 9475-9482.

Skottrup, P.D., Nicolaisen, M., Justesen, A.F. 2008. Towards on-site pathogen detection using antibody-based sensors. Biosensors & Bioelectronics 24, 339-348.

Su, X.L., Li, Y.B. 2005. A QCM immunosensor for *Salmonella* detection with simultaneous measurements of resonant frequency and motional resistance. Biosensors & Bioelectronics 21, 840-848.

Sung, Z.R. 1976. Turbidimetric measurement of plant-cell culture growth. Plant Physiology 57, 460-462.

Tanji, Y., Furukawa, C., Na, S.H., Hijikata, T., Miyanaga, K., Unno, H. 2004. *Escherichia coli* detection by GFP-labeled lysozyme-inactivated T4 bacteriophage. Journal of Biotechnology 114, 11-20.

Thevenot, D.R., Toth, K., Durst, R.A., Wilson, G.S. 2001. Electrochemical biosensors: Recommended definitions and classification. Analytical Letters 34, 635-659.

Trinci, A.P.J. 1972. Culture turbidity as a measure of mold growth. Transactions of the British Mycological Society 58, 467-473.

Tu, S.I., Golden, M., Fett, W.F., Gehring, A., Irwin, P. 2003. Rapid detection of outbreak *Escherichia coli* O157 and *Salmonella* on alfalfa sprouts by immunomagnetic capture and time-resolved fluorescence. Journal of Food Safety 23, 75-89.

Turpin, P.E., Maycroft, K.A., Bedford, J., Rowlands, C.L., Wellington, E.M.H. 1993. A rapid luminescent-phage based MPN method for the enumeration of *Salmonella* Typhimurium in environmental-samples. Letters in Applied Microbiology 16, 24-27.

Ulitzur, S., Kuhn, J. 1987. Introduction of *lux* genes into bacteria, a new approach for specific determination of bacteria and their antibiotic susceptibility. Bioluminescence and

chemiluminescence: new perspectives. Wiley Interscience, Bristol, United Kingdom. 463–472.

Updike, S.J., Hicks, G.P. 1967. Enzyme electrode. Nature 214, 986-988.

Vaughan, R.D., Carter, R.M., O'Sullivan, C.K., Guilbault, G.G. 2003. A quartz crystal microbalance (QCM) sensor for the detection of *Bacillus cereus*. Analytical Letters 36, 731-747.

Vaughan, R.D., O'Sullivan, C.K., Guilbault, G.G. 2001. Development of a quartz crystal microbalance (QCM) immunosensor for the detection of *Listeria monocytogenes*. Enzyme and Microbial Technology 29, 635-638.

Vo-Dinh, T., Cullum, B. 2000. Biosensors and biochips: advances in biological and medical diagnostics. Fresenius Journal of Analytical Chemistry 366, 540-551.

Volpe G., Palleschi G. 2003. New biosensors for microbiological analysis of food. Detecting pathogens in food. Woodhead Publishing Ltd., Abington Hall, Abington, Cambridge, UK. 294-345.

Wachtel, M.R., Whitehand, L.C., Mandrell, R.E. 2002. Association of *Escherichia coli* O157:H7 with preharvest leaf lettuce upon exposure to contaminated irrigation water. Journal of Food Protection 65, 18-25.

Wendel, A.M., Johnson, D.H., Sharapov, U., Grant, J., Archer, J.R., Monson, T., Koschmann, C., Davis, J.P. 2009. Multistate Outbreak of *Escherichia coli* O157:H7 Infection Associated with Consumption of Packaged Spinach, August-September 2006: The Wisconsin Investigation. Clinical Infectious Diseases 48, 1079-1086.

Willford, J., Mendonca, A., Goodridge, L.D. 2008. Water pressure effectively reduces *Salmonella* Enteritidis on the surface of raw almonds. Journal of Food Protection 71, 825-829.

Wyatt, P.J. 1969. Identification of bacteria by differential light scattering. Nature 221, 1257-1258.

Wyatt, P.J., Jackson, C. 1989. Discrimination of phytoplankton via light-scattering properties. Limnology and Oceanography 34, 96-112.

Wyatt, P.J., Phillips, D.T. 1972. Structure of single bacteria from light-scattering. Journal of Theoretical Biology 37, 493-501.

Ye, J., Liu, Y., Li, Y. 2002. A chemiluminescence fiber-optic biosensor coupled with immunomagnetic separation for rapid detection of *E. coli* O157:H7. Transactions of the Asae 45, 473-478.

Yu, S.L., Ko, K.L., Chen, C.S., Chang, Y.C., Syu, W.J. 2000. Characterization of the distal tail fiber locus and determination of the receptor for phage AR1, which specifically infects *Escherichia coli* O157:H7. Journal of Bacteriology 182, 5962-5968.

Zhao, J.X., Jedlicka, S.S., Lannu, J.D., Bhunia, A.K., Rickus, J.L. 2006. Liposome-doped nanocomposites as artificial-cell-based biosensors: Detection of listeriolysin O, vol. 22. 32-37.

Ziegler, C. 2000. Cell-based biosensors. Fresenius Journal of Analytical Chemistry 366, 552-559.

Zordan, M.D., Grafton, M.M.G., Acharya, G., Reece, L.M., Cooper, C.L., Aronson, A.I., Park, K., Leary, J.F. 2009. Detection of Pathogenic *E. coli* O157:H7 by a Hybrid Microfluidic SPR and Molecular Imaging Cytometry Device. Cytometry Part A 75A, 155-162.