Biosensor Platform for Rapid Detection of *E. coli* in Drinking Water

by

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ABSTRACT

The need for rapid, specific and sensitive assays that provide a detection of bacterial indicators are important for monitoring water quality. Rapid detection using biosensor is a novel approach for microbiological testing applications. Besides, validation of rapid methods is an obstacle in adoption of such new bio-sensing technologies. In this study, the strategy developed is based on using the compound 4-methylumbelliferyl glucuronide (MUG), which is hydrolyzed rapidly by the action of *E. coli* β-D-glucuronidase (GUD) enzyme to yield a fluorogenic product that can be quantified and directly related to the number of *E. coli* cells present in water samples. The detection time required for the biosensor response ranged from 30 to 120 minutes, depending on the number of bacteria. The specificity of the MUG based biosensor platform assay for the detection of *E. coli* was examined by pure cultures of non-target bacterial genera and also non-target substrates. GUD activity was found to be specific for *E. coli* and no such enzymatic activity was detected in other species. Moreover, the sensitivity of rapid enzymatic assays was investigated and repeatedly determined to be less than 10 *E. coli* cells per reaction vial concentrated from 100 mL of water samples. The applicability of the method was tested by performing fluorescence assays under pure and mixed bacterial flora in environmental samples. In addition, the procedural QA/QC for routine monitoring of drinking water samples have been validated by comparing the performance of the biosensor platform for the detection of *E. coli* and culture-based standard techniques such as Membrane Filtration (MF). The results of this study

indicated that the fluorescence signals generated in samples using specific substrate molecules can be utilized to develop a bio-sensing platform for the detection of *E. coli* in drinking water. The procedural QA/QC of the biosensor will provide both industry and regulatory authorities a useful tool for near real-time monitoring of *E. coli* in drinking water samples. Furthermore, this system can be applied independently or in conjunction with other methods as a part of an array of biochemical assays in order to reliably detect *E. coli* in water.

DEDICATION

I would like to dedicate this dissertation to my parents specially my mother. Without their unconditional love, support, patience, and encouragement, this would not have been possible.

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CHAPTER 1

BACKGROUND

1.1 Introduction

Despite significant improvements in water treatment and disinfection processes, there are still concerns about the microbiological safety of drinking water. To protect and maintain water quality from the source to the tap, it is critically important to consider a rapid method to identify indicator and pathogenic bacteria in drinking water. The standard techniques used for the detection and enumeration of Total Coliforms (TC) and Fecal Coliforms (FC) require 18 to 24 hours to obtain results. This delay in providing information regarding water quality makes it difficult to make timely decisions for protecting public health. Another limitation of these techniques is the inability to detect viable but non-culturable bacteria (George et al. 2000). Hence, rapid, sensitive and specific assays that provide a near real-time detection of bacterial indicators are of primary importance for monitoring microbiological quality of water.

Direct enzyme-based assays circumvent the time consuming cultivation period and enable the exploitation of a range of enzyme substrates to both improve sensitivity and practicality of the detection of bacterial cells (Bascomb 1988; Manafi et al. 1991). Moreover, the abundance of fluorogenic substrates available and the fast developments of biosensing technology allowed the application of fluorescence techniques to study bacterial activities in various water systems. Rapid assays to estimate the GUD activity of *E. coli* have been performed without any cultivation step where direct measurements

of GUD activity were successfully applied to river, sea and waste water samples (Farnleitner et al. 2001; Garcia‐Armisen et al. 2005; Fiksdal and Tryland 2008; Nikaeen et al. 2009). The GUD is a specific marker for *E. coli* and 4-methylumbelliferone-β-D-glucuronide (MUG), a sensitive substrate for determining the presence of *E. coli* in a sample. Approximately 97% of *E. coli* strains have GUD activity and almost all other coliform bacteria lack this enzyme (Caruso et al. 2002). The hydrolysis of MUG releases fluorescent 4-methylumbelliferyl (4MU) and the intensity of the measured fluorescent signal is proportionate to the amount of enzyme present, showing a correlation to the number of *E. coli* present in the sample (Fiksdal and Tryland 2008). However, current procedures are laboratory-based and require bench-top fluorometers for the measurement of fluorescence resulting from the enzyme–substrate reaction. In our previous study, we have developed bacterial enzymatic-biochemical signatures and shown the utility of a custom designed opto-electronic biosensor platform for the detection of *E. coli* and other bacterial cells in biofilm samples (Elzein et al. 2013). The biosensor detects bacterial enzymatic response of specific fluorogenic substrates.

1.2 Objectives

The main objective of this study is to develop a rapid detection method to analyze samples on a direct GUD assay for *E. coli* in drinking water samples. The hypotheses of this study are: 1) the opto-electronic biosensor can be used independently or in conjunction with other methods as part of MUG-based assays to reliably detect *E. coli* in water, 2) the presence of non-target bacteria will not impact the specificity of MUG for

the detection of *E. coli*, 3) the standard curve generated using laboratory reagents would be similar to the standard curve generated using tap water. The specific objectives of the study are to develop procedural quality assurance (QA) and quality control (QC) for routine monitoring of drinking water samples and to validate the performance of the biosensor platform for the detection of *E. coli* by culture-based standard techniques. This study is aimed to provide both industry and regulatory authorities a useful tool for real-time monitoring of *E. coli* in drinking water samples.

CHAPTER 2

LITERATURE REVIEW

2.1 Detection of *E. coli* in Drinking Water: Current Methods and Emerging Approaches

E. coli is the most common coliform among the intestinal flora of warm-blooded animals and its presence may be related to fecal contamination. Therefore no *E. coli* can be present in drinking water. The U.S. Environmental Protection Agency (EPA) has approved several methods for coliform detection such as the multiple-tube fermentation (MTF) and the membrane filtration (MF) techniques (Rompré et al. 2002).

MTF is labor intensive since many dilutions have to be employed for each water sample. This method is extremely time-consuming, requiring 48 hours for presumptive results, and necessitates a subculture stage for confirmation, which could take up an additional 48 hours. The results of the MTF technique are expressed in terms of the most probable number (MPN) of microorganisms present (Rompré et al. 2002). This number is a statistical estimate of the mean number of coliforms in the sample. As a consequence, this technique offers a semi-quantitative enumeration of coliforms. However, the precision of the estimation is low and depends on the number of tubes used for the analysis. Many factors may significantly affect coliform bacteria detection by MTF, especially during the presumptive phase. Interference by high numbers of non-coliform bacteria (Evans et al. 1981; Means and Olson 1981; Seidler et al. 1981) as well as the inhibitory nature of the media (Elzein 2005) have been identified as factors contributing to underestimates of coliform abundance.

MF consists of filtering a water sample on a sterile filter with a 0.45 µm pore size which retains bacteria, incubating this filter on a selective medium and enumerating typical colonies on the filter. The main concern about MF is its inability to recover stressed or injured coliforms. A number of chemical and physical factors involved in drinking water treatment, including disinfection, can cause sublethal injury to coliform bacteria, resulting in a damaged cell unable to form a colony on a selective state (Rompré et al. 2002).

However both methods have limitations, such as duration of incubation, organisms' interference, lack of specificity to the coliform group and a weak level of detection of slow-growing or stressed coliforms. Hence, the principal challenges for the development of new coliform detection methods are to improve the specificity of the method, which could eliminate the time-consuming confirmation step, to take into account stressed and injured cells and to reduce the analysis time (Rompré et al. 2002).

Based on the enzymatic properties of coliforms, a defined substrate method was developed to overcome some limitations of the MTF and MF techniques. Unlike these methods, which eliminate the growth of non-coliform bacteria with inhibitory chemicals, the defined substrate technology is based on the principle that only the target microbes (TC and *E. coli*) are fed and no substrates are provided for other bacteria. A defined substrate is used as a main nutrient source for the target microbe(s). During the process of substrate utilization, a chromogen or a fluorochrome is released from the defined substrate, indicating the presence of the target microorganisms.

One of the recent enzyme-substrate technologies that has been approved by the EPA is the IDEXX Quanti-Tray, which provides easy, rapid and accurate counts of coliforms, *E. coli*, enterococci and Pseudomonas aeruginosa. This method is designed to give quantitated bacterial counts of 100 mL samples using IDEXX reagent products. The IDEXX Quanti-Tray is a semi-automated quantification method based on the standard method MPN (IDEXX 2011b; a).

The Colilert 18/Quanti-Tray is based on defined substrate technology and is a colorimetric, specific and selective method for the detection and enumeration of GUD of Escherichia coli and GAL of coliforms in drinking water. U.S. EPA approves the use of IDEXX Colilert-18 for the detection and enumeration of FC in water and wastewater samples when used the Quanti-Tray system and incubated at 44.5° C. For the detection of β-D-galactosidase (GAL), Colilert-18 utilizes the chromogenic nutrient indicator ortho-nitrophenyl-β-D-galactopyranoside (ONPG) which produces a distinct yellow color after incubation at 44.5 ±0.2°C at 18 hours and up to 22 hours when hydrolyzed by GAL. After the incubation, the positive wells, which are yellow, will be counted and using the MPN table the bacterial number will be reported per 100 ml of sample. The IDEXX Quanti-Tray provides 95% confidence limits better than 5- or 10-tube MPN and 95% confidence limits better than or comparable to MF (ISHA 2010; IDEXX 2011a; b; 2013).

In conclusion, tests based on the defined-substrate technology using chromogenic and fluorogenic substrates are applicable for the detection and enumeration of coliforms and *E. coli* in drinking water and have improved the sensitivity of these methods. These tests are easy to use and give a more rapid and more accurate estimate (especially in the

presence of chlorine residual) of indicators of bacteriological contamination of waters than classical identification methods. In all cases, enzymatic methods require less labor intensive and consequently their cost in terms of commercial value is lower (Rompré et al. 2002).

2.1.1 Application of Rapid Enzymatic Assays for Bacterial Detection

The biochemical tests used for bacterial identification and enumeration in classical culture methods are generally based on metabolic reactions. For this reason, they are not fully specific and many further tests are sometimes required to obtain accurate confirmation. The use of microbial enzyme profiles to detect indicator bacteria is an attractive alternative to classical methods. In addition, reactions are rapid and sensitive. Therefore, the possibility of detecting and enumerating coliforms through specific enzymatic activities has been under investigation for many years now (Rompré et al. 2002).

Rapid enzymatic assays are based on bacterial hydrolysis of added substrates and detection of hydrolysis products that are released into the medium. Enzymatic activity is determined (1) in the water sample itself after addition of substrate or (2) after collecting the cells by filtration, transfer of the filtered cells to an assay solution and addition of substrate (Farnleitner et al. 2001). The activity of all micro-organisms that contain the actual enzyme is measured, but the assay does not give information at the single cell level. Enzymatic activity is determined at fixed intervals and calculated as released hydrolysis product per time unit. Correlation curves between enzyme activity and culturable bacterial numbers can then be established. These assays avoid a cultivation

step and utilize the GALase activity of coliforms and GLUase activity of *E. coli*. They are simple to perform and do not require expensive instrumentation. Such assays are in demand for risk assessment of water supply systems for early warning of high FC concentrations, for example by monitoring of raw water quality (Tryland et al. 2001), assessment of treatment efficiency, monitoring of finished water quality as well as recreational waters. They also have been recently exploited for daily monitoring of beach water quality (Lebaron et al. 2005). Less than four hours was chosen as 'rapid' by Noble and Weisberg (Noble and Weisberg 2005) in a recent review of rapid detection technologies for bacteria in recreational waters.

GAL and GUD properties of TC and *E. coli* have been exploited on freshwater (Berg and Fiksdal 1988; Tryland and Fiksdal 1998; George et al. 2000; Fiksdal and Tryland 2008) and seawater (Davies et al. 1995) samples in rapid assays without any cultivation steps. George et al. (2000) finalized a protocol based on the fluorogenic substrates 4-methylumbelliferyl-β-D-galactopyranoside (MUGal) and 4-methylumbelliferyl-β-D-glucuronide (MUG) for a direct enzymatic detection of FC in freshwaters in 30 min. These methods allow a rapid and direct estimate of the level of microbiological contamination of surface water.

2.2 Biosensor Definition

Biosensing is the process of detecting cellular and biological activities through molecular or ionic interaction, binding, transformation, and products. In this process, a biosensor functions as a detection tool that requires the amount of analyte be transduced

into a measurable signal. To be useful in the cellular context, it is desired that the sensor follow some simple criteria such as selectivity, sensitivity, reproducibility, and ease of signal transport and delivery (Elzein 2005).

2.2.1 Bacterial Cell Enzymes as Biosensing Materials

Bacteria possess several enzymes that are important in the metabolic processes and are considered as the biocatalytic recognition elements in microbial biosensors (Davies et al. 1995). Microbial biosensors are less sensitive to inhibitors present in the sample, more tolerant to pH and temperature variations, and generally have a longer lifetime (Mello and Kubota 2002). The use of enzymes in a living system may overcome the problems of selectivity and slow response times in the detection process (Davies et al. 1995).

In enzyme-based fluorescence biosensors, it is necessary that the fluorogenic substrate should diffuse through the solution matrix and reach the enzymatic reaction site. The enzyme recognizes specific target sites of the fluorogenic substrate and selectively catalyzes the covalent linkage of substrate-fluorophore molecules, thereby triggering an emitted fluorescence signal, which can be picked up by the fiber optic transducer. The resulting response of the biosensor to the addition of a substrate is determined by the concentration of fluorescence product of the enzymatic reaction and controlled by the rates of two conjugated processes, substrate enzymatic conversion and product diffusion in the bulk solution (Evtugyn et al. 1998). Bacterial enzymes should be permeable to the substrate and non-reactive in reaction media. It is desirable to have uniform, oriented

mono layers with defect free and molecules that are closely packed in such a way that enzymes assume an orientation with their active sites facing the target substrate molecules in the solution phase (Phadke 1992).

Viable microbial cells have a number of advantages as biological sensing materials over purified enzymes in the fabrication of biosensors. Their enzymes are present ubiquitously and are able to metabolize a wide range of chemical compounds. In intact microbial cells, enzymes remain active, and stable, which allows viable microbes to adapt to adverse conditions and develop the ability to degrade different molecules with time (Elzein 2005).

Over 90% of the enzymes known to date are intracellular (D'souza 2001). In this respect, the utilization of whole cells as a source of intracellular enzymes has been shown to be a better alternative to purified enzymes, which are more expensive, in various industrial processes (Bickerstaff Jr 1997). Whole cells also provide a multipurpose catalyst especially when the process requires the participation of a number of enzymes in sequence (D'souza 2001). Viable cells are gaining considerable importance in the fabrication of biosensors (Burlage and Kuo 1994; Riedel et al. 1998; Simonian et al. 1998). The major limitation to the use of whole cells is the diffusion of substrate and products through the cell wall resulting in a slow response as compared to enzyme-based sensors (Rainina et al. 1996).

The enzymatic detection of the indicator of fecal contamination *E. coli* via its marker enzymes GUD and the marker for coliforms, GAL, have been widely used (Kilian and Bülo 1976; Edberg and Kontnick 1986; Berg and Fiksdal 1988; Rice et al. 1990;

Hattenberger et al. 2001). Considering that *E. coli* is employed as an indicative microorganism of wastewater and sewage contamination, the GUD-based assays and rapid tests are important in environmental monitoring where test simplicity and speed coupled with high sensitivity are critical features.

GUD is an enzyme which catalyzes the hydrolysis of β-D-glucopyranosiduronic derivatives into their corresponding aglycons and β-D-glucuronic acid. Although this bacterial enzyme was discovered first in *E. coli*, its relative specificity for identifying this microorganism was not apparent until Kilian and Bulow (1976) surveyed the Enterobacteriaceae and reported that glucuronidase activity was mostly limited to *E. coli* (Kilian and Bülo 1976). The prevalence of this enzyme and its utility in the detection of *E. coli* in water were later reviewed by Hartman (1989) (Feng and Hartman 1982). GUD-positive reactions were observed in 94–97% of the *E. coli* isolates tested (Kilian and Bülo 1976; Feng and Hartman 1982; Edberg and Kontnick 1986), while Chang et al. (1989) found a higher proportion of GUD negative *E. coli* (a median of 15% from *E. coli* isolated from human fecal samples) (Chang et al. 1989). In contrast, GUD activity is less common in other Enterobacteriaceae genus, such as *Shigella* (44 to 58%), *Salmonella* (20 to 29%) and *Yersinia* strains and in Flavobacteria (Kilian and Bülo 1976; Feng and Hartman 1982; Hartman 1989; Frampton and Restaino 1993). GAL catalyzes the breakdown of lactose into galactose and glucose and has been used mostly for enumerating the coliform group within the Enterobacteriaceae family.

2.2.2 Biosensors Types and Signal Transaction Methods

Various signal transduction techniques in the different biosensor types can be described as calorimetric, acoustic, electrochemical, and optical (Surface Plasmon and Fiber Optics) (Elzein 2005). The strategy developed in this study is based on a series of fluorescence assays using specific fluorogenic substrate. The biochemical properties and the enzymatic machinery of bacterial cells are used as the biological sensing elements in the biosensing process. The generated fluorescence signals are detected using a custom designed fiber optic based biosensor. This strategy relies on the rapid detection of bacterial biochemical activities in drinking water.

CHAPTER 3

DEVELOPMETNT OF A BIOSENSOR PLATFROM FOR RAPID DETETION OF *E. COLI* IN DRINKING WATER

3.1 Abstract

The need for rapid, specific and sensitive assays for the detection of bacterial indicators are important for monitoring water quality. In this study, the strategy developed is based on using the compound 4-methylumbelliferyl-β-D-glucuronide (MUG), which is hydrolyzed rapidly by the action of *E. coli* β-D-glucuronidase (GUD) enzyme to yield a fluorogenic product that can be quantified to the number of *E. coli* cells present in water samples. The detection time required for the biosensor response ranged from 20 to 120 minutes, depending on the number of bacteria in the reaction vial. The specificity of the MUG based biosensor platform assay was examined by pure cultures of non-target bacterial genera such as *Klebsiella, Salmonella, Enterobacter* and *Bacillus* and also non-target substrates: 4-methylumbelliferyl-β-D-galactopyranoside (MUGal), or L-Leucine β-Naphthylamide Aminopeptidase (LLβ-N), to identify patterns of enzymatic activities of *E. coli*. GUD activity was found to be specific for *E. coli* and no enzymatic activity was detected by other species. Then, fluorescence assays were performed for the detection of *E. coli* to generate standard curves. In addition, the sensitivity of rapid enzymatic assays was investigated and repeatedly determined to be less than 10 *E. coli* cells per reaction vial concentrated from 100 mL of water samples. The applicability of the method was tested by performing fluorescence assays under pure and mixed bacterial

flora in environmental samples. The results of this study showed that the fluorescence signals generated in samples using specific substrate molecules can be utilized to develop a bio-sensing platform for the detection of *E. coli* in drinking water. Furthermore, this system can be applied independently or in conjunction with other methods as part of an array of biochemical assays in order to reliably detect *E. coli* in water.

3.2 Introduction

β-D-glucuronidase (GUD) activity is characteristic of most strains of *E. coli* and, hence, has been widely used to monitor the presence of this indicator organism in environmental samples, particularly water (Berg and Fiksdal 1988; Edberg et al. 2000; Caruso et al. 2002). Approximately 97% of *E. coli* strains have GUD activity and almost all other coliform bacteria lack this enzyme (Caruso et al. 2002). Studies have shown that *E. coli* can preserves an active metabolism without being able to grow on culture media (Roszak and Colwell 1987; George et al. 2001). However, GUD-based assays for the detection of *E. coli* includes the important fraction of viable but non-culturable (VBNC) organisms that would be missed by culture-based methods (Fiksdal and Tryland 2008; Servais et al. 2009). MUG is a specific substrate for determining the presence of *E. coli* in a sample. The hydrolysis of MUG releases fluorescent 4-methylumbelliferone (4MU) and the intensity of the measured fluorescent signal is proportionate to the amount of enzymatic activities, showing a correlation to the number of *E. coli* present in the sample (Fiksdal and Tryland 2008).

Many chromogenic and fluorogenic substrates exist for the specific detection of bacterial enzymatic activities, and various commercial tests based on these substrates are available. To detect the presence of GUD in *E. coli*, the following chromogenic substrates have been previously used: indoxyl-β-D-glucuronide (IBDG) (Brenner et al. 1993), the phenolphthalein-mono-β-D-glucuronide complex and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Glu) (Watkins et al. 1988). Although there are several fluorescence-based glycoside enzyme substrates available, substrates based on 4MU have been more extensively used in diagnostic microbiology for the detection of bacterial enzymes (Dahlén and Linde 1973; Feng and Hartman 1982; Bascomb 1988; Manafi et al. 1991; Chilvers et al. 2001). Rapid assays to estimate the GUD activity of *E. coli* have been performed without any cultivation step where direct measurements of GUD activity were successfully applied to river, sea and waste water samples (Farnleitner et al. 2001; Garcia‐Armisen et al. 2005; Fiksdal and Tryland 2008; Nikaeen et al. 2009). However, current procedures are laboratory-based and require bench-top fluorometers for the measurement of fluorescence resulting from the enzyme–substrate reaction.

Isopropyl β-D-Thiogalactoside (IPTG) is known to be a noncompetitive inducer, i.e. non-hydrolysable substrate by GAL (Herzenberg 1959) but the possible effect of MetGlu on GLUase activity has not been tested previously. GAL and GUD are well known to be inducible enzymes (Herzenberg 1959; Pardee and Prestidge 1961). IPTG has been used commonly in cloning procedures that require induction of β-galactosidase activity, but recently have been used for induction of β-glucuronidase activity (Liu et al. 2012).

In our previous study, we have developed method for detecting bacterial enzymatic-biochemical signatures and have shown the utility of a custom designed opto-electronic biosensor platform for the detection of *E. coli* and other bacterial cells in biofilm samples (Elzein et al. 2013). In this study, rapid assays for the detection of in water were developed by using the compound MUG, which is hydrolyzed by the specific GUD enzyme to yield a fluorogenic product that can be quantified to the number of *E. coli* cells in water samples. At the ASU Environmental Microbiology laboratory, the biosensor instrumentation was assembled and customized for detecting the response of bacterial enzymatic machinery to the added specific fluorogenic substrate to rapidly determine bacterial water quality. In order to optimize the assay, fluorescent reagents were optimized to determine the detection limit and the working concentration range for the fluorescence assays. The present study introduces a biosensor designed to directly analyze samples for GUD activities for developing a rapid detection method for *E. coli* cells in water samples. The results obtained were substantiated by culture-based assays indicating comparable data.

3.3 Materials and Method

All experiments were conducted under laboratory conditions using aseptic techniques. First, all assays were optimized using pure chemical reagents and standards. Standard curves were generated to evaluate each assay working range of concentrations and detection limits. In the second step of optimization, increasing concentrations of pure *E. coli* culture which were obtained from American Type Culture Collection

(ATCC, Manassas, VA) were used as a model for bacterial biochemical and cultural assays. For confirmation, culture-based assays were performed to determine Colony Forming Unit (CFU)s per reaction before and after the assays in duplicate using selective agar media and to make sure the bacteria were viable. To rapidly quantify *E. coli*, the activity of GUD was exploited using the soluble fluorescent substrate MUG and measured the resulting fluorescence by the BDS1000 fluorescence detector (Figure 1). The results have been also compared and evaluated with the performance of the reference instrument Aqualoq benchtop fluorometer (Horiba, Kyoto, Japan), the only simultaneous absorbance and fluorescence system for water quality analysis, which measures both absorbance spectra and fluorescence excitation-emission matrices.

Figure 1: Custom Designed Touch Screen Biosensor BDS1000

The assay was performed by adding 3 mL of a representative water sample to 0.1 mL of the MUG and 0.9 mL of 0.1 M N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] Buffer (HEPES), (VWR, Chester, PA) at pH 8.0 in a 4.0 mL

reaction cuvette. The cuvette was then placed in the Custom Designed Biosensor BDS1000 at Arizona State University (ASU) Environmental Microbiology laboratory. Each set of assays consisted of a negative control of 3.0 mL of 0.1 X Phosphate Buffered Saline (PBS) containing 0.9 mL of 0.1 M HEPES and 0.1 mL of 8.52 mM MUG. Assays were performed in triplicate by simultaneously processing three aliquots of *E. coli* suspension in three separate cuvettes and examined using the biosensor. The enzymatic activity data were collected for less than 120 minutes and after a desired linearity was achieved (R^2 = 0.90 or higher), the fluorescence signals were subtracted from the blank signal and reported as the final results. Raw biosensor data were analyzed for correlation between different parameters in order to confirm the functionality of the biosensor.

3.3.1 Custom Designed Touch Screen Biosensor BDS1000

The biosensor instrumentation was assembled and customized at ASU Environmental Microbiology laboratory for detecting the response of bacterial enzymatic machinery to the added specific fluorogenic substrates. The optical and spectrometer components for the biosensor were obtained from Ocean Optics (Model # HR 2000, Ocean Optics, Dunedin, FL). The Light-Emitting Diode (LED) based light source provided filtered excitation light at specific wavelengths to allow single excitation, single emission detection of a specific fluorophore in each enzymatic assay. The excitation light spectrum appeared to have a ± 10 nm range around the peak maxima. This allowed all fluorescence assays to be carried out at a single excitation wavelength (350 ± 10 nm). The fluorescence signals were collected as Relative Fluorescence Unit (RFU) at single

excitation, dual emission wavelength (SEDEW) settings. Readings were taken by placing cuvettes in the reaction chamber of the biosensor.

3.3.2 Stock Culture Preparation

Pure culture of *E. coli* (ATCC 25922) was grown in Tryptic Soy Broth (TSB) (Becton, Dickinson, Sparks, MD). Log phase bacterial stocks were prepared by incubating the bacterial suspension at 37°C in a C24 shaker-incubator (New Brunswick Scientific, Edison, NJ) at 150 rpm. The log phase bacterial cultures were stored at 4°C for at least 24 hours and then used for assays. Bacterial stocks were diluted in 0.1 X PBS in a range of 10-10^8 CFU counts per mL of *E. coli*.

3.3.3 Fluorometric Assay Reagents

Methylumbelliferone (MUF-β) Standard Preparation

For the GUD assays, substrate stock was prepared by placing 0.030 g MUG (Sigma Chemical Co., St. Louis, MO) in a sterile 15 mL centrifuge tube and completely dissolved in 5.0 mL of pure ethanol. After all the crystals were completely dissolved, an amount of 5.0 mL of sterile distilled water (DI water) was added to the homogenized solution. The tube was capped and labeled as 8.52 mM stock MUG substrate solution. The solution was protected from light and stored at 4°C. For every test, 0.1 mL of this substrate was used.

Preparation of 0.1M HEPES Buffer, pH 8.0

HEPES buffer was prepared by dissolving 23.83g of HEPES in 500 mL of autoclaved DI water. The pH of HEPES solution was adjusted to 0.8 using sodium hydroxide solution. The final volume was adjusted to 500 mL with DI water and sterilized using 0.2 µm filters. For each assay, 0.9 mL of this buffer was used. The solution was protected from light, and kept at room temperature. The HEPES buffer solution was prepared fresh before performing the assays.

3.3.4 Culture-Based Assays

At the start and end of each assay, samples of *E. coli* were enumerated by plate count using Membrane Filtration (MF). This was done by filtering 1 mL of the appropriate dilution through a 0.45 µm membrane (Millipore SAS, Billerica, MA) and plating them on Brilliance (Oxoid LTD, Basingstoke, England) or mEndo (Becton, Dickinson and company) media followed by incubation at 37°C for 24 hours. This step was performed to achieve CFUs before and after the assay and to make sure the bacteria were viable and culturable.

3.3.5 Development of Standard Curves using GUS Reporter Kit

The calibration curves for MUG were generated by using a GUD reporter kit which was purchased from Marker Gene Technologies, Inc., (Eugene, OR), and Aqualog fluorometer were employed. A series of MUG concentrations were prepared by taking different volumes of the GUS assay buffer, GUD extraction buffer, and blank solution

(extraction buffer) were utilized for this assay (Table 1). This step was taken in order to determine the optimum MUG response concentration to the added number of *E. coli* cells.

Table 1: Assay Conditions for Generating MUG Calibration Curve

[MUG]	**GUD Assay Buffer (0.1 mM MUG)**	**GUS Extraction Buffer**	**Enzyme or Blank Solution**
0.08 mM	80 µL	10 µL	10 µL
0.06 mM	60 µL	30 µL	10 µL
0.04 mM	40 µL	50 µL	10 µL
0.02 mM	20 µL	70 µL	10 µL
0.01 mM	10 µL	80 µL	10 µL

A black 96-well plate and Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT) was also employed to create the calibration curve for comparison. Four wells were allocated for each concentration of MUG. The 96-well plate was incubated at ~38°C for 10 min, and then placed in the plate reader at room temperature. The fluorescence intensity values were averaged and compared to the blank.

3.3.6 Specificity of MUG Assays for the Detection of *E. coli*

3.3.6.1 Impact of Non-Target Substrates on the Detection of E. coli

The impact of different substrates, 4-methylumbelliferyl-β-D-galactopyranoside (MUGal) and L-Leucine β-Naphthylamide Aminopeptidase (LLβ-N) aminopeptidase on the detection of *E. coli* was investigated.

a) Methylumbelliferyl-β-D-Galactopyranoside

In the galactosidase assays, substrate stock was prepared by completely dissolving 15 µmol of MUGal (Sigma Chemical Co.) in 0.2 mL of Dimethyl Sulfoxide (DMSO) which was purchased from Mallinckrodt Baker Inc, Paris, KY. Then the solution was diluted to 10 mL with 0.5 X PBS at pH 7.3 and prepared according to the method of (Maddocks and Greenan 1975). The solution was protected from light and stored at 4°C. For every test, an amount of 100 µL of this MUGal stock was used.

b) L-Leucine β-Naphthylamide Aminopeptidase (LLβ-N)

Stock solution (40 mM) LLβ-N substrate (Sigma-L1635) was prepared in analytical grade ethanol and the pH was adjusted to 7.5 using HEPES buffer. For the proteolytic enzymatic assays, 0.100g LLβ-N was weighed and dissolved in 9.75 mL of pure ethanol in a sterile 15 mL centrifuge tube. The content was mixed at room temperature until dissolved. The tube was capped and labeled as 40 mM LLβ-N Stock substrate solution. The solution was protected from light and stored at 4°C.

3.3.6.2 Impact of Non-Target Bacteria on the Detection of E. coli

The specificity of the MUG based biosensor platform assay was examined by using pure and mixed cultures of non-target bacterial genera. All bacterial cells were

obtained from ATCC. *Enterobacter*, *Bacillus*, *Klebsiella*, and *Salmonella*, cultures were grown in TSB. A volume of 1.0 mL of each strain was taken from pure stocks and suspended in 9.0 mL of the corresponding broth media. The bacterial suspensions were incubated in a shaker-incubator (New Brunswick Scientific C24, Edison, NJ) (150 RPM at 37°C) to achieve a log phase bacterial culture which ranged between 3-8 hours, depending on the bacteria used.

3.3.7 Sensitivity (Detection Limit) of Rapid Enzymatic Assays

The applicability of the biosensor method was tested using both environmental and tap water.

Tap Water:

In order to investigate the sensitivity of the assay, 1000 mL of tap water was collected and 1 mL of 1% w/v solution of Sodium Thiosulfate at was added to dechlorinate the water sample prior to the assay. After complete mixing, an amount of 200 mL of water was taken from the 1000 mL of the water sample and approximately 30-40 *E. coli* cells were added to the sample. The mixture was vortexed and divided into two 100 mL parts. The first part was analyzed by filtering 100 mL through a 0.45 µm membrane and plating it on Brilliance or mEndo media, followed by incubation at 37°C for 24 hours. This step was performed to achieve CFU counts so as to be compared with the other 100 mL after the concentration. The second 100 mL sample was concentrated in 250 mL centrifuge tubes at 1500 × G for 15 minutes. Using a Pasteur Pipette, the supernatant was carefully aspirated to a final volume of 3 mL above the pellet. Extra

care was taken to avoid aspirating *E. coli* cells during this step. The centrifuge tube was vortexed vigorously until the pellet was completely resuspended. At the end, a pipette was used to collect any residual volume that dripped down to the bottom of the tube to ensure that as much of the sample volume was recovered as possible. After transferring 3 mL of the sample from the centrifuge tube to the cuvette, MUG and HEPES were added to the sample.

Lake Water:

The water samples were taken from different points of Tempe Town Lake, transported to the laboratory on ice for processing within 8 hours and tested for the presence of *E. coli*. The water was first analyzed by filtering 1 mL of the appropriate dilution through a 0.45 µm membrane and plating on mEndo and Tryptic Soy Agar (TSA) media (Becton, Dickinson, Sparks, MD), followed by incubation at 37°C for 24 hours. This step was performed to determine CFU counts. The MUG was added to the 3 mL of water sample in order to examine the specificity of the MUG assays for detection of *E. coli*. Each subsequent reading as the blank providing baseline fluorescence intensity, then, 3 mL of the tap water was taken and approximately 100 *E. coli* cells were added to the sample and the previous steps were followed to take readings.

3.3.8 IPTG Effect on the Environmental Samples

Impact of different concentrations of IPTG on GUD activities was investigated in this study. According to Liu et al. 2012, the induction condition for the optimum production of the β-glucuronidase gene (AtGUS) protein is at ~0.5 µM IPTG (Liu et al.

2012), hence, two different concentrations of IPTG have been used for the assessment as following:

0.5 µM IPTG:

Stock solution (20 mM) IPTG (Sigma-16758-1G) was prepared in sterile nonopure deionized water. An amount of 23.83 mg IPTG was weighed and dissolved in 5 mL sterile nonopure deionized water in a sterile 15 mL centrifuge tube. The content was mixed at room temperature until dissolved. The tube was capped and labeled as 25 mM IPTG Stock substrate solution. The solution was protected from light and stored at -20°C. For every test 100 µL of this added to the samples.

1 µM IPTG:

Stock solution (40 mM) IPTG was prepared in sterile nonopure deionized water. An amount of 47.66 mg IPTG was weighed and dissolved in 5 mL sterile nonopure deionized water in a sterile 15 mL centrifuge tube. The content was mixed at room temperature until dissolved. The tube was capped and labeled as 25 mM IPTG Stock substrate solution. The solution was protected from light and stored at -20°C.

IPTG effect has been studied both on tap and environmental water samples and results were compared for the GUD activities in the absence of IPTG. The environmental water samples were taken from different points of consolidated canals in Mesa, AZ, transported to the laboratory on ice for processing and tested for *E. coli* presence within 8 hours. The water was analyzed by filtering 1 mL of the appropriate dilution through a 0.45 µm membrane and plating on mEndo and TSA media, followed by incubation at 37°C for 24 hours. This step was performed to achieve CFU counts. The MUG was

added to the 3 mL of water sample in order examine the specificity of the MUG assays for detection of *E. coli*. Each subsequent reading as the blank providing baseline fluorescence intensity, then, 3 mL of the tap water was taken and approximately 100 *E. coli* cells were added to the sample and the previous steps were followed to take readings.

3.4 Results and Discussion

The feasibility of the direct assay for sensitive biochemical detection of *E. coli* was evaluated using serially diluted cultivated bacteria. Figure 2 provides the results obtained for *E. coli* concentrations at approximately 10, 100, and 1,000 CFU/ mL in the reaction cell. The direct MUG assay analyzed with BDS1000 showed a good linearity of the fluorescent signal with increasing number of *E. coli* (Figure 2). As concentrations of bacteria increase, more MUG molecule are broken per unit of time and higher fluorescence intensity is measured. The fluorescence intensity (arbitrary units) is directly related to the number of bacteria and MUG concentrations. In addition, the reaction time needed to detect *E. coli* was directly proportional to the bacterial cell numbers (Figure 2). Using our biosensor, all optimized assays resulted in positive linear response of fluorescence signals in the range of bacterial concentrations of 10-10^8 *E. coli* per mL. This is comparable with the sensitivity of the previously reported hand-held confocal fluorescence detector FLUO SENS SD (ESE GmbH, Stockach, Germany), which showed increasing trend of the fluorescent signal with increasing number of *E. coli* in a range of 10–10^8 CFU per mL of the water sample (Wildeboer et al. 2010). However, the hand-

held sensor-based assay required incubation of samples with the substrate for 30 min prior to the assay (Wildeboer et al. 2010).

Bacterial biochemical activities for all assays, if present, generally appear to increase with time and with increase in concentration of the bacterial cells (Figure 2). As time passes, bacteria have more time to cleave MUG molecules. For a fixed number of bacteria, the fluorescent signal increases with time as bacteria continue to hydrolyze MUG molecules over time (Figure 2). This increase in activity might be explained by the adaptation and survival of *E. coli* to the environmental conditions such as water quality parameters, reaction buffers and substrate consumptions in the reaction cell (cuvette). The detection time required for the biosensor response versus the culture methods ranges from 20 to 120 minutes and 24 to 48 hours, respectively. The sensitivity of the method is such that it enables rapid detection, well within the 4 hours which is the period defined as rapid (Noble and Weisberg 2005). No increasing trend in the relative progression of GUD activities was noted and data is not plotted here.

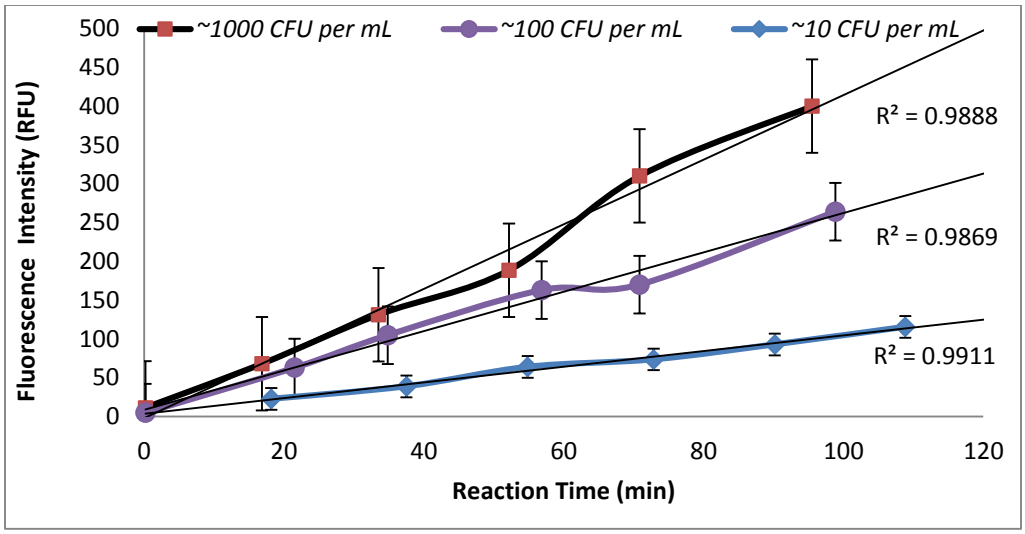

Figure 2: Time Series Hydrolyses of MUG by Different Concentrations of *E. coli*

Since overnight cultures of *E. coli* cells were stored at 4°C, the cells were at stationary phase prior to use. The high levels of activity observed in some cultures may indicate that their starved metabolic state lead to an increase in bacterial enzymatic activities hydrolyzing the fluorogenic substrate rapidly. Caruso et al. (2002) reported that full development of enzymatic activities start at lag phase and is required for the enzyme expression.

3.4.1 Calibration Curves and Comparison Study

Comparison study was performed between BDS1000 and Aqualoq fluorometer. The calibration curves were used to determine the sensitivity of the enz-bio assay using the reference instrument, Aqualog fluorometer (Figure 3). GUD assay fluorescence signals produced a linear response with a correlation coefficient higher than 0.90 (Figure 3). The lower detection limit was 0.01 mM of MUG as determined by three standard

deviations of the blank, however the results for MUG at 0.01, 0.05 and 0.08 mM are plotted in Figure 3. Though, the optimum MUG response was found at a concentration of 0.08 mM. This result was confirmed for the ~10 to 1000 CFU per mL of *E. coli*. As a result, MUG at concentration of 0.08 mM was selected as the working substrate concentration. The assay range and sensitivity for the enzyme using the BDS1000 was identical to the results obtained when measuring the same samples with the Aqualog benchtop fluorometer. This optimum concentration was confirmed using Synergy H1 Hybrid Multi-Mode Microplate Reader. The resulting graph for the different concentration of MUG substrate is shown in Figure 4. Linearity obtained for this calibration curve ranged from 0.01mM to 0.08mM at ~10000 CFU/mL (Figure 4).

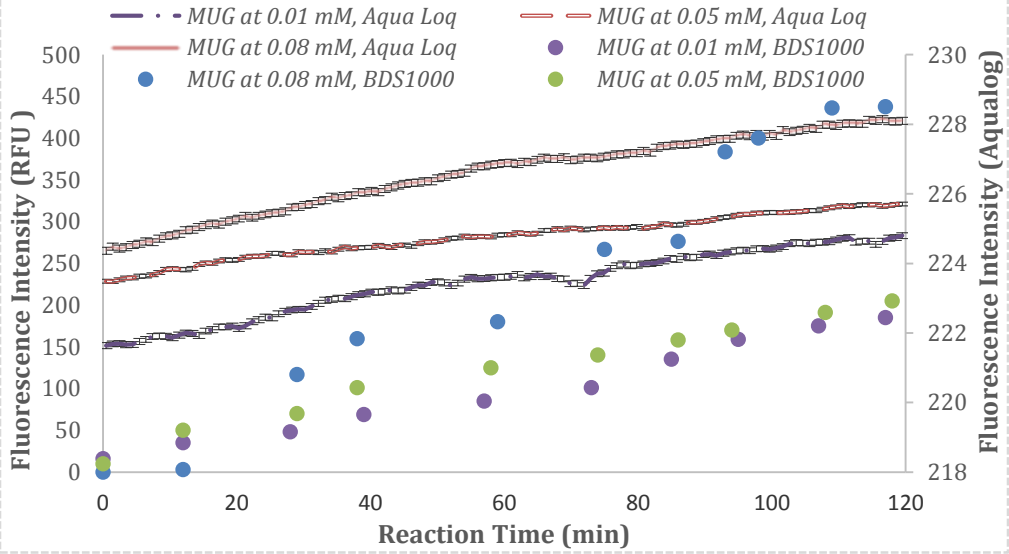

Figure 3: Comparison of the MUG Calibration Curves by Using BDS1000 and Aqualog Fluorometer

Note: Comparing the calibration curve using the BDS1000 and Aqualog benchtop fluorometer; graphs show representative data for three independent experiments; the fluorescence intensity is arbitrary units; note the different scales of the two instruments.

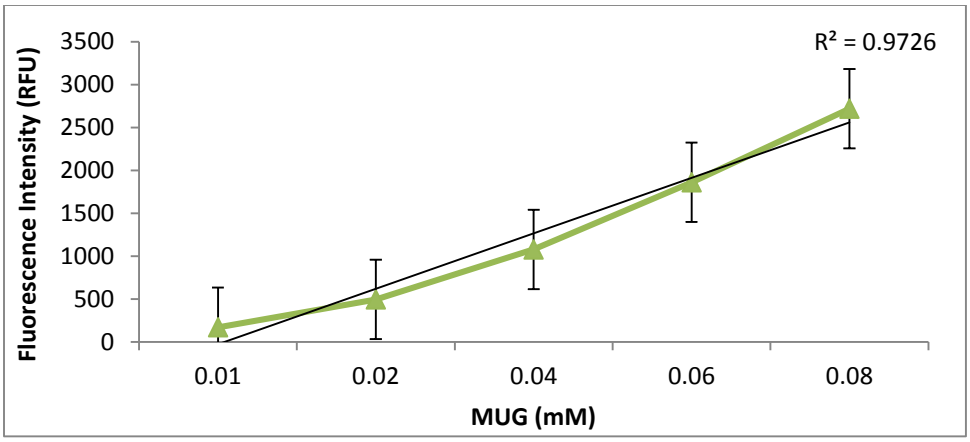

Figure 4: MUG Calibration Curve using 96-well Plate Reader

3.4.2 Specificity of MUG Assays for the Detection of Non-Target Bacteria and Substrates

In this study, the specificity of the MUG assay was examined by using pure and mixed cultures of non-target bacterial genera such as *Klebsiella, Salmonella, Enterobacter*, *Bacillus* and *E. coli* (Figure 5). The Fecal Coliform (FC) group mainly consists of *E. coli* and *Klebsiella* (Edberg et al. 1997). No GUD activities were observed for the non-target bacteria and this finding is in the agreement with the previous research that "species of *Klebsiella* do not normally express GLUase activity" (Brenner et al. 1993). Furthermore, studies have been performed on the GUD activities of the Enterobacteriaceae and *E. coli* and confirmed that GUD activity was mostly limited to (Kilian and Bülo 1976). In the Enterobacteriaceae genus, only 20% to 29% of the *Salmonella* isolates tested showed GUD positive activities (Kilian and Bülo 1976; Massenti et al. 1980; Feng and Hartman 1982; Frampton and Restaino 1993).

In addition, the specificity of the MUG to *E. coli* was assessed by performing experiments using non-target substrates, MUGal and LLβ-N, to detect other enzymatic

activities of in pure cultures (Townsend and Chen 2002; Kim and Han 2013). GAL, catalyzes the breakdown of lactose into galactose and glucose and has been used mostly for enumerating the coliform group within the Enterobacteriaceae family. Chromogenic substrates such as MUGal were used to detect the presence of GAL produced by coliforms (Rompré et al. 2002). The results showed that no galactosidic or proteolytic enzyme activities were detected in (Figure 6). In Figures 5 and 6, assays performed using 0.08 mM of MUG and spiked water samples contained 100 CFU/mL of each type of bacteria.

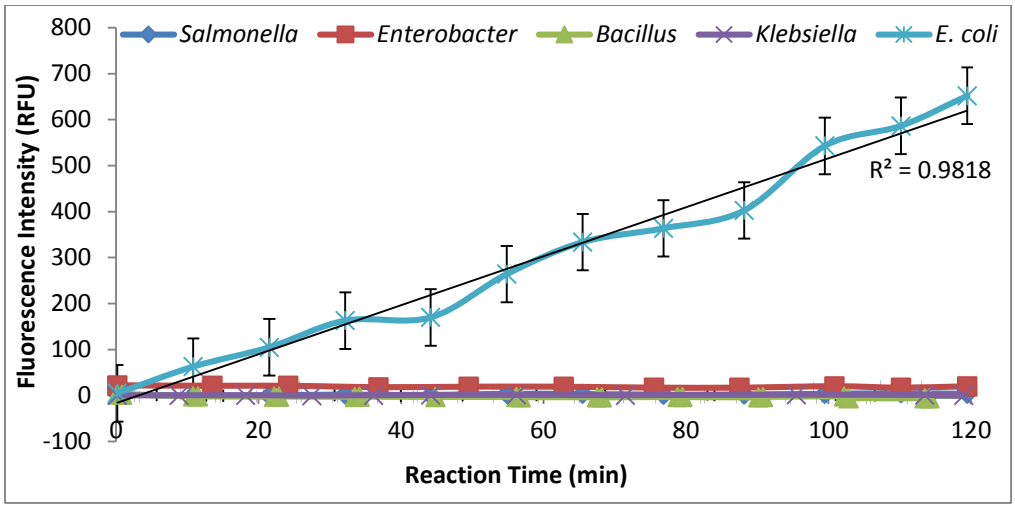

Figure 5: Specificity of MUG Assay on Pure Cultures of Non-target Bacterial Genera

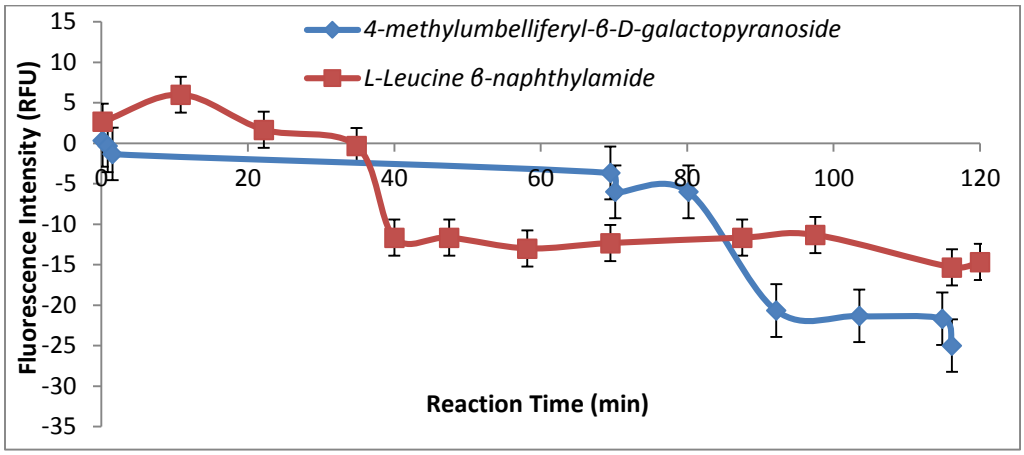

Figure 6: Impact of Non-target Substrates on the Detection of *E. coli*

3.4.3 Sensitivity Determination of Different Environmental Water Samples

The applicability of the method was tested using environmental and tap waters. No fluorescence seen by enzymatic assays (Figure 7), since no *E. coli* was present as tested using mEndo plates in both types of sample but other types of bacterial colonies

were present on TSA plates. When both samples were spiked by adding the same number of *E. coli* cells to each sample, the tap water sample showed more fluorescence generation than the environmental sample. The lower fluorescence signal in the environmental samples could be due to either the inhibition of enzymatic activity by other types of bacteria or chemical contamination. Studies have shown that in the enzymatic assay, microorganisms other than *E. coli*, or algae or plants, may contribute to GUD activities but their possible interference on enzyme determination depends on their concentrations (Davies et al. 1994). This is greater when they are present in high numbers or in conditions of low contamination, while the interference becomes negligible in heavily polluted conditions (Davies et al. 1994; Tryland and Fiksdal 1998). However, in our study interference by these compounds would be detected in the blank reading and thus subtracted from the sample reading. In addition, the presence of copper at a concentration of 1ppm in Tempe Town Lake could inhibit the enzymatic reaction of *E. coli*. Nevertheless, the blank values found in the samples studied were very low, suggesting that no significant interference from non-GUD sources in the water samples existed. The fluorescence measurements carried out on serial dilutions of *E. coli* cultures have shown a sensitivity threshold of less than 10 *E. coli* cells per reaction vial concentrated from 100 mL of water samples. Assays were performed using 0.08 mM of MUG and spiked water samples containing 100 CFU/mL *E. coli*. A distinct signal above background was obtained even at the minimum detection limit, demonstrating the high sensitivity of the BDS1000 that was comparable to the sensitivity of the hand-held fluorescence detector, where the detection limit was less than 10 CFU/mL using river

water samples (Wildeboer et al. 2010). Moreover, this detection limit and the rapid response of the biosensor should be sufficient to meet the requirement of most of the monitoring standards for environmental water samples.

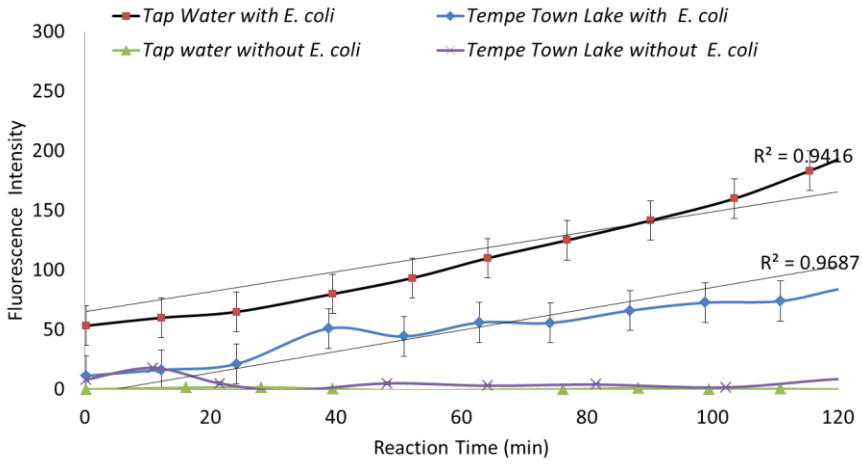

Figure 7: Application of Biosensor in Environmental Samples

3.4.4 IPTG Effect on Tap Water and Environmental Samples

No *E. coli* with green sheen were detected on mEndo plates in the environmental samples, but other type of colonies from non-target bacteria were present on TSA plates. There was no obvious change in the level of GUD activity after the addition of inducer IPTG neither in tap water nor in environmental samples (Figures 8 and 9). However, starting point of GUD activities was higher by adding IPTG. The results for addition of 0.5 µM IPTG and 1 µM IPTG to the samples were compared and higher enzymatic activity was observed using 0.5 µM (data not shown) which confirmed previous study which states that the induction condition for the optimum production of the AtGUS

protein is at ~0.5 µM IPTG (Liu et al. 2012). All the data points are the average of three replicates of each sample. Spiked water samples contained 100 CFU/mL *E. coli* and 100 µL of 0.5 µM IPTG. Spiked water samples contained 100 CFU/mL *E. coli* and 100 µL of 0.5 µM IPTG (Figures 8 and 9).

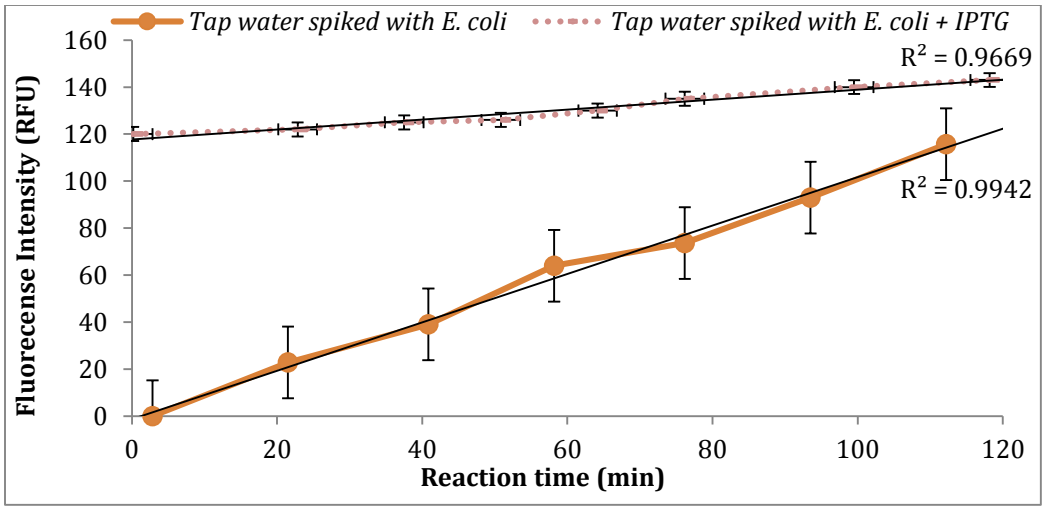

Figure 8: IPTG Effect on Tap Water

Note: All the data points are the average of three replicates of each sample.

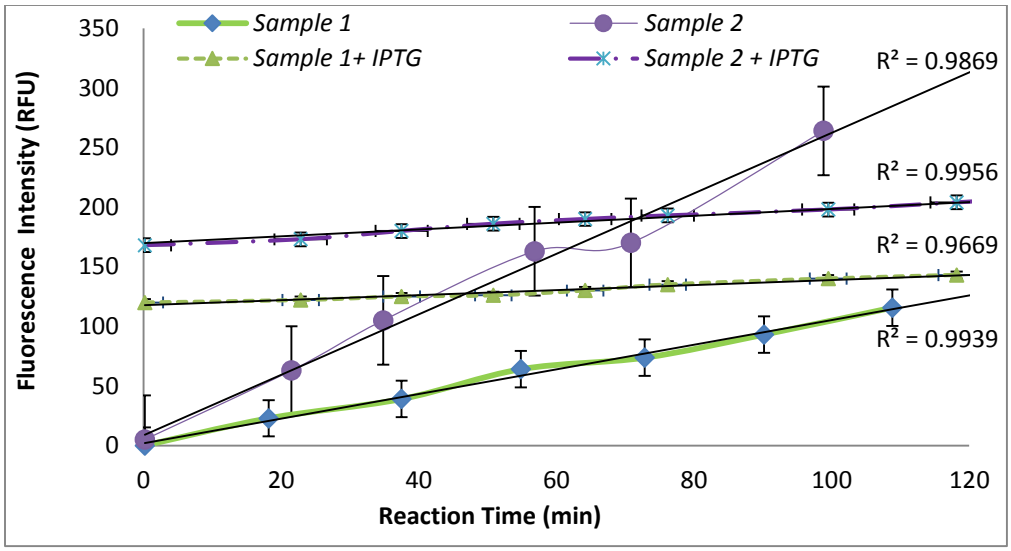

Figure 9: IPTG Effect on Environmental Samples

Note: Samples were collected from different points at the middle and head of consolidated canals in Mesa, AZ. At least duplicate samples were taken from each location. All the data points are the average of three replicates of each sample.

3.5 Conclusions

In the present study, a rapid procedure has been developed by incorporating a biochemical reaction in a biosensor fluorescent detector. Rapid assays for the detection of *E. coli* were developed by using MUG, which is hydrolyzed by the specific *E. coli* GUD enzyme yielding a quantifiable fluorogenic product that directly proportional to the number of *E. coli* cells in water samples. The system is based on monitoring the response of bacterial enzymatic machinery to the added specific fluorogenic substrates.

The data obtained in this study demonstrate that biosensor BDS1000 can be used to directly (without processing or concentration steps) analyze the presence of *E. coli* in drinking water samples. Biosensors that are capable of simple and rapid detection of *E. coli* will allow utilities to respond to water quality issues in a timely manner. To the best

of our knowledge, enzymatic and physiological processes of *E. coli* have not been investigated to develop biosensors to rapidly detect *E. coli* in water samples. The biosensor in this study can be used independently or in conjunction with other methods as a part of an array of biochemical assays in order to reliably detect *E. coli* in water. In addition, the specific substrate molecule used in the design of this biosensor can be utilized as a platform to monitor bacterial quality in water samples.

CHAPTER 4

QUALITY CONTROL AND QUALITY ASSURANCE FOR THE APPLICABILITY OF A NEW BIOSENOR IN RAPID DETECTION OF E.COLI IN DRINKING WATER

4.1 Abstract

Rapid detection using biosensor is a novel approach for microbiological testing applications. Validation of rapid methods is an obstacle in adoption of such new bio-sensing technologies. Therefore, establishing a Quality Assurance and Quality Control (QA/QC) for the new biosensor will demonstrate accuracy and reliability of the new method and generate acceptable precision to detect indicator bacteria in drinking water. In this study, first, different fluorescent reagents and assay conditions such as different temperatures, holding time, *E. coli* strains, dissolving agents, and quality of substrates from different 4-methylumbelliferyl glucuronide (MUG) vendors have been evaluated for the assay optimization and documentation. On the other hand, the procedural QA/QC for routine monitoring of drinking water samples has been created for validating the performance of the biosensor platform for the detection of *E. coli* by culture-based standard techniques such as Membrane Filtration (MF). The key components of QA/QC for this project examined mainly include: reference instrument, methods comparison, NaCl and pH effects on the assay. The established procedural QA/QC for the biosensor will provide both industry and regulatory authorities a useful tool for near real-time monitoring of *E. coli* in drinking water samples.

4.2 Introduction

Rapid detection using biosensor is a novel approach for microbiological testing applications. Microbiological testing can provide important information only if sampling plans and methodology are properly designed and performed. Validation of rapid methods is an obstacle in adoption of such new bio-sensing technologies. Therefore, a validated rapid method to detect indicator bacteria in drinking water is of primary importance for monitoring microbiological activities and water quality from the source to the tap. Establishing a procedural Quality Assurance and Quality Control (QA/QC) for the new biosensor will demonstrate accuracy and reliability of the new method and generate acceptable precision.

U.S. EPA states, "compliance monitoring is one of the key components the Agency uses to protect human health and the environment by ensuring that the regulated community obeys environmental laws/regulations through on-site visits by qualified inspectors, and a review of the information EPA or a state/tribe requires to be submitted" (USEPA 2005). Contrary to QC, which is a reactive system that emphases on legal requirements and focuses on statistically appropriate measurements, QA is a preventive approach that emphasizes operational procedures (Caprita and Caprita 2005). In order to insure that compliance monitoring standards are met, several procedures must be followed. Formal QA programs assess each laboratory's ability to process documentation. QA programs are also used to evaluate instrument and equipment maintenance and performance as well as quality of reagents.

In addition, by emerging new sophisticated detection methods in water quality analysis, the need for new monitoring technologies and the expertise levels of a microbiology QA/QC laboratory will increase. While biosensing technologies will allow for faster, sensitive detection capabilities, they increase the need for internal quality control and personnel who are adequately trained to ensure accuracy and proper interpretation of their results. Regulatory approval of molecular methods imply that strict QA/QC performance and inter-laboratory validation (Ziprin et al. 2008).

Appropriate QA/QC measures are not limited to biosensing technologies and are necessary by using any monitoring system to ensure reliability of the analytical data generated and increase confidence in the relevance of possible responses. Preliminary detection results should be confirmed because false-positives may be affiliated with monitoring instrumentation or improper reports. Measurements such as complete checking the result's QA/QC, resampling and repeating the analysis, and performing more-accurate or more precise alternative methods of analysis may be included as the confirmation process (Gullick et al. 2003).

In some molecular techniques such as PCR-based methods the QA/QC procedures include the integration of internal spiked sample controls and the sequencing of PCR products (Ziprin et al. 2008). In some other detection techniques such as Mass Spectrometry (MS) the necessary QA/QC can be more time consuming than that for some of the simpler analyses, however this step is very essential for confirmation of the results providing accurate identification of organic in select samples (Gullick et al. 2003).

The present study attempts to establish the ability to demonstrate quality control over the biosensor by creating a set of QA/QC requirements for the routine monitoring of drinking water samples and to generate an acceptable precision and recovery. The following sections will discuss the importance of the parameters that have been considered for establishing the biosensor procedure in the present study:

Reagents and Assay Conditions

Enzyme activities are subject to the physiological status of bacteria and that under nutritional and light stresses, a fraction of cells may gradually lose its culturability, although remaining metabolically active (Caruso et al. 2002). Besides, *E. coli* β-D-glucuronidase (GUD) activities are very sensitive to temperature. As reported by Caruso, that "the specificity and selectivity of the enzyme assays towards *E. coli* are strongly related to the temperature of incubation" (Caruso et al. 2002). In the present study, in order to assess different assay conditions and reagents, different temperature, holding time, *E. coli* strains, dissolving agents at different concentrations, quality of substrates from different 4-methylumbelliferyl glucuronide (MUG) vendors, water versus Phosphate Buffered Saline (PBS) and environmental samples have been evaluated for optimization and documentation.

NaCl and pH Effect on the assay

It is generally observed that the microbial growth is impacted by adding salt, therefore this factor has been also evaluated in this study. On the other hand, the alkalinity of the GUD assays have been reported previously by Caruso et al. (2002) in freshwater; however the present study investigates this parameter in drinking water.

Validation

Collilert-18 (IDEXX, Westbrook, ME) has been applied as a GUD validation tool prior to the assay in this research. Colilert-18 is a new standard in coliform/*E. coli* detection which is known as QC procedure based on IDEXX's patented Defined Substrate Technology (DST). When *E. coli* metabolizes Colilert-18's nutrient-indicator, MUG, the sample also fluoresces. This method is able to detect a single viable coliform or *E. coli* per sample and also eliminates false positive detection of non-target organisms (Bascomb 1988; Geary et al. 2011; IDEXX 2011a; b; 2013). The requirements mentioned above were adapted as a guideline for establishing QA/QC for the biosensor procedure.

4.3 Materials and Method

4.3.1 Different Reagents and Enzymatic Assay Conditions

E. coli cultures used for the assays kept at 4°C for the different time periods. Also, samples were divided into two different temperatures, room temperature ~24°C and 37°C. For every assay, samples were incubated at 37°C in a hot plate in 10 minutes intervals prior to each measurements.

The enzymatic activity measured for the sample aliquots from the same *E. coli* stock preparation with the substrate purchased from different MUG vendors such as, Sigma Chemical Co. (St. Louis, MO), EMD Millipore (Billerica, MA) and Bioworld (Dublin, Ohio) for the quality comparison. For the comparison, the substrate was dissolved in Dimethyl Sulfoxide (DMSO) and in ethanol according to the MUG suppliers' preparation instruction. In addition, *E. coli* was diluted in 10 mL of 0.1, 0.5

and 1 X PBS at pH 7.3 and the results were compared for GUD activities. Furthermore, two additional *E. coli* strains, ATCC 35218 and 11175 (Manassas, VA) were compared with the reference strain ATCC 25922.

4.3.2 pH Adjustment and NaCl Effect

Alkalinity of the sample was increased by adding NaOH to N-[2-hydroxyethyl] piperazine-N'-[2- ethanesulfonic acid] Buffer (HEPES) and adjusted to pH 8 or 9 before testing. Furthermore, samples were prepared by dissolving 5 g of NaCl in 1 L of the water sample and the results were compared with the samples without adding salt. Each set of assays consisted of 3.7 mL of a representative sample containing 5% NaCl.

4.3.3 Validation

Collilert-18 was used as a positive control for confirming each of *E. coli* strains with GUD activities before starting the assay. For each test, contents of one pack of collilert-18 was added to a 100 mL sample in a sterile, transparent, non-fluorescing vessel and then was capped and shaken. One mL of overnight culture of *E. coli* stock was added to the 100 mL of sample and then incubated at 37°C for 18 hours to confirm GUD activities. When *E. coli* metabolized colilert-18's nutrient-indicator, ortho-Nitrophenyl-β-galactoside (ONPG), the sample turned yellow under UV light.

4.4 Results and Discussion

In the present study, the procedural QA/QC for routine monitoring of drinking water samples have been validated for the performance of the biosensor platform for the detection of *E. coli* by culture-based standard techniques such as MF. The key components of QA/QC examined included: media preparation, *E. coli* cultures, triplicate sampling, blanks (method blank and negative control samples), holding time and condition, reference instruments, validation methods, different MUG reagents from different suppliers, the effects of NaCl, pH and documentation. Other considerations for the biosensor procedural QA/QC includes: lab equipment's quality control (such as annual micropipette and balance calibrations) and lab records. QC per each new lot of every reagent and standards of the assay such as the substrate and buffers have been also investigated. The optimization steps were performed in order to determine that under which assay condition, the highest GUD activity would be produced and then the optimum reagent concentration or assay conditions were selected and used as part of the new procedural QA/QC.

4.4.1 Reagents and Different Enzymatic Assay Conditions

Temperature Effect

The results proved that for the sample aliquots from the same *E. coli* stock preparation incubated at 37°C showed higher enzymatic activity than the samples kept at room temperature (Figure 10). This finding is in agreement with the previous studies that have shown increased selectivity related to the higher temperature which may have inhibited the growth of injured or stressed cells (Caruso et al. 2002). Assays performed

using 0.08 mM of MUG and spiked water samples contained 100 CFU/mL *E. coli* (Figures 10 to 13).

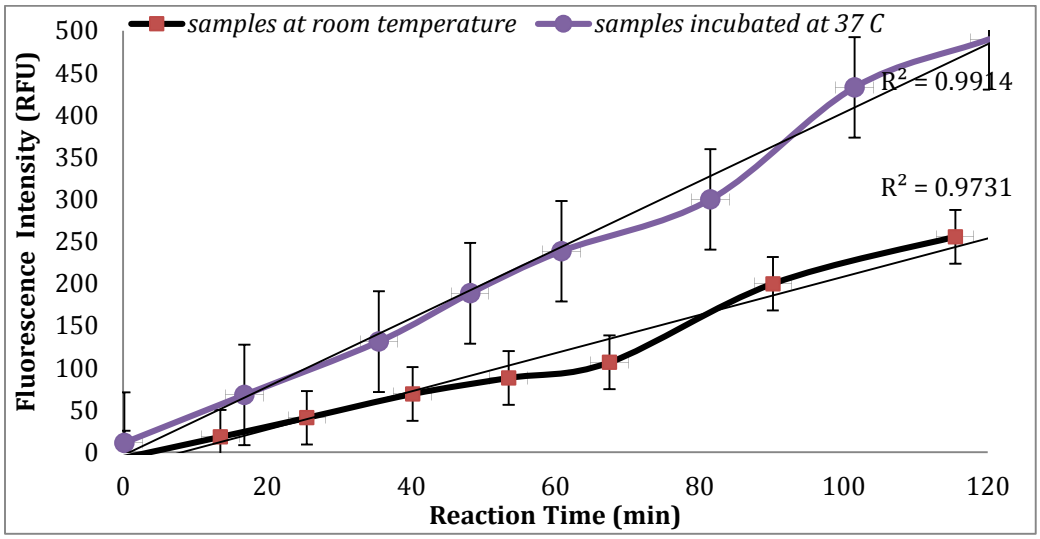

Figure 10: Incubation Effect on GUD activities

Note: Samples were incubated at 37°C prior to the measurements by placing cuvettes in the biosensor. The cuvettes were again incubated at 37°C for 10 min between each measurement.

MUG Quality

The results of the comparison between MUG purchased from different suppliers showed significant difference in fluorescent intensity (Sigma vs. EMD) and (Sigma vs. Bioworld). MUG purchased in May 2014 (lot# BCBH7903V) from Sigma resulted in non-reproducible data. MUG purchased from Bioworld produced similar fluorescent intensity as previously obtained by MUG purchased from Sigma prior to May 2014 (Figure 11).

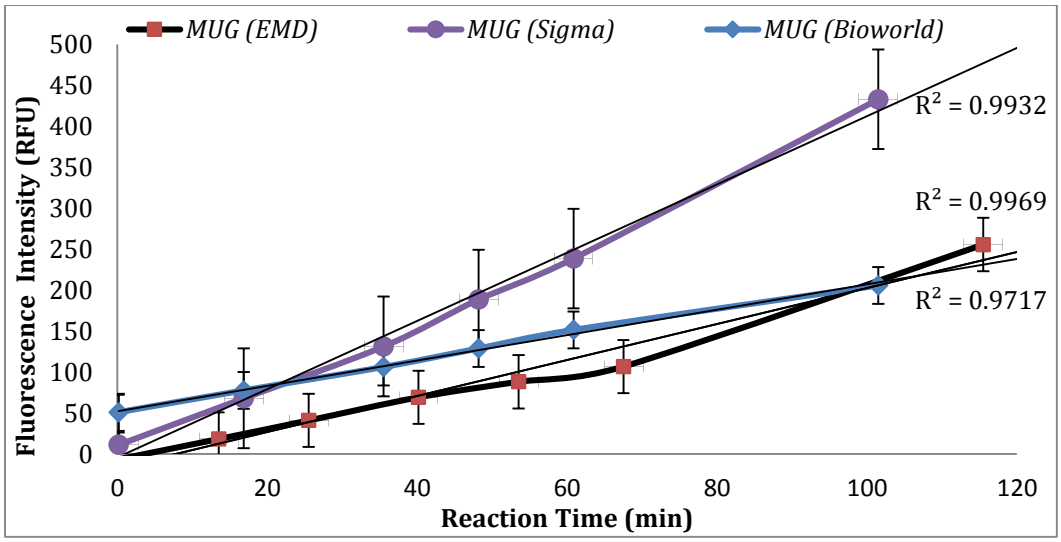

Figure 11: Comparison of MUG Quality from Different Suppliers

Note: For the control samples, no increasing trend in the RFU was noted and data is not presented here. All the data points are the average of three replicates.

Dissolving Agents

Two dissolution agent, ethanol and DMSO were tested for the impact on the sensitivity of the assay using the sample aliquots from the same *E. coli* stock preparation. DMSO resulted in lower fluorescent intensity than ethanol (Figure 12). In addition, higher enzymatic activity was observed when lower strength of PBS was used (Figure 13).

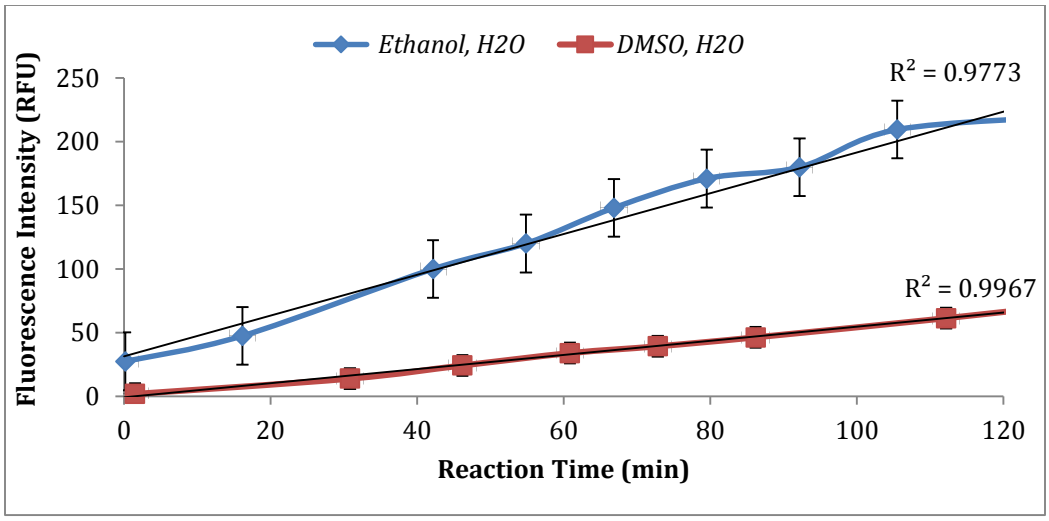

Figure 12: Comparison of Dissolution Agents for MUG Preparation

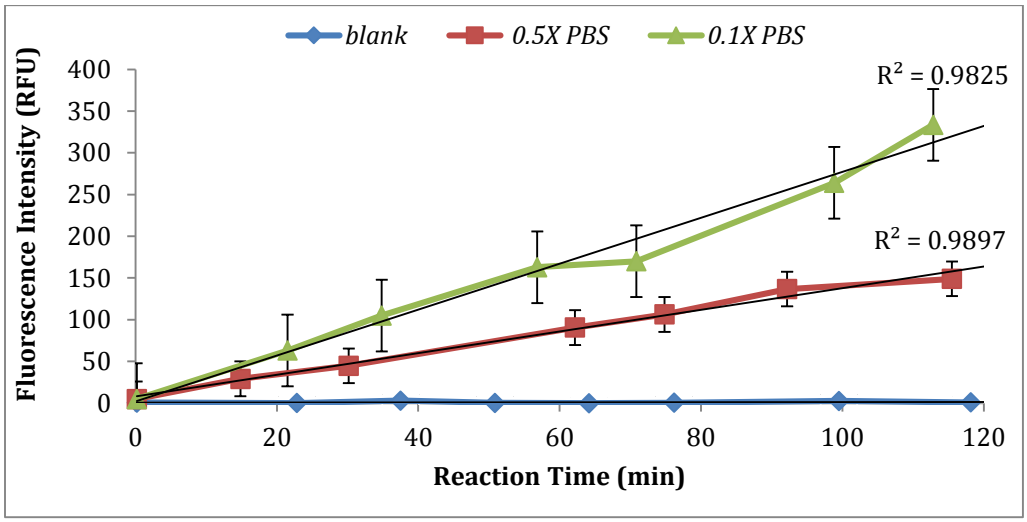

Figure 13: Impact of Buffer Strength on Fluorescence Intensity of MUG

Holding Time

Since overnight cultures of *E. coli* cells were stored at 4°C, the cells were at stationary phase prior to use. The high levels of GUD activity observed in some cultures may indicate that their starved metabolic state lead to an increase in bacterial enzymatic

activities hydrolyzing the fluorogenic substrate rapidly. Caruso et al., (2002) reported that full development of enzymatic activities start at lag phase and is required for the enzyme expression. The results confirmed the previous studies that *E. coli* stored more than a week and to a month at 4^{o}C have higher enzymatic activities hydrolyzing MUG substrate (data not shown).

Also, other reagents such as substrate stock and HEPES storage time play a very important role in the enzymatic assay and fluorescence generation. The quality of MUG substrate and HEPES buffer decrease after one week of the preparation date. For the MUG substrate, there is a possibility to be crystalized which causes a significant drop in the fluorescence generation. Therefore, it is suggested to prepare these per each time of use and keep them for no more than one week (data not shown). MUG and HEPES are light-sensitive chemicals so this also should be considered in their storage and use conditions. In summary, QC per each new lot of the reagents and standards recommended prior to use in order to insure that compliance monitoring standards are met. Table 2 represents the outline of QC for each new lot.

Table 2: QC for each New Lot Prior to Use

Reagents and Standards		
Bacterial Cultures	**Reagents –substrates/enzyme**	**Buffers – HEPES**
QC for media Reference strain -ATCC	QC for batch	QC for batch

pH Adjustment and NaCl Effect on the Assay

As seen in Figure 14, the sample aliquots from the same *E. coli* stock preparation yielded higher enzymatic activity at pH 9 and this is in agreement with previous studies reported by Caruso et al. 2002, addition of NaOH before the spectro-fluorometric measurement entails an increase in fluorescence (Caruso et al. 2002; Garcia-Armisen et al. 2005). Furthermore, pH 9 was suggested by Hoppe et al. 1993 as optimum pH value at which MU reaches its peak of fluorescence intensity (Hoppe 1993). Also, samples which contained 0.05% NaCl exhibited higher fluorescent measurements (Figure 14).

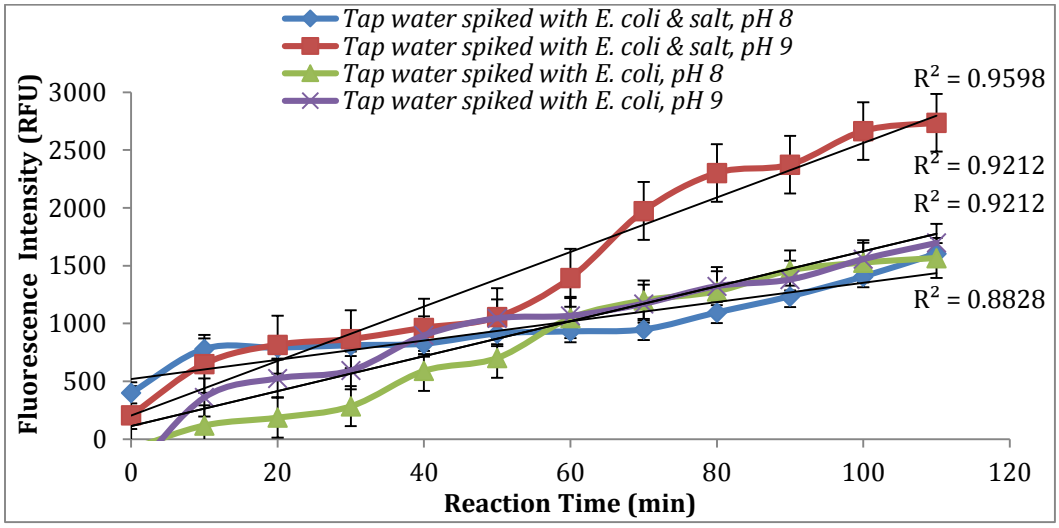

Figure 14: Different pH and NaCl Effect on the Assay

Note: Spiked water samples contained 1000 CFU/mL *E. coli* and 100 µL of 0.5 µM IPTG.

In this study, statistical analysis was performed to compare effect of different reagents concentrations and assay conditions on GUD activities. For each variable, the average of GUD activity [with lower and upper 95% Confidence Intervals (CI)] is

presented in Table 3. As far as the impact of incubation temperature, GUD activity increased remarkably over time by incubation of the samples at 37^{o}C, with 116 and 225 average relative fluorescence units (RFU) for 24°C and 37°C, respectively. Similar impact was recoded for dissolution agents, with 131 and 208 averages RFU for DMSO and ethanol, respectively. Other variables that impacted fluorescence intensity were pH (100 and 130 RFU for pH 8 and 9, respectively) and PBS strength (131 and 208 RFU for 0.5 X and 0.1 X, respectively). Additionally, MUG from different sources (EMD and Bioworld and Sigma) also showed variable level of fluorescent activity when tested at the same concentrations. The obtained data for all the different assay conditions provided the desired linearity (R^2 = 0.90 or higher) otherwise the assays were repeated. According to this analysis, as part of establishing the procedural QA/QC, for every assay, the baseline assay conditions were MUG purchased from Bioworld dissolved in Ethanol and samples incubated at 37°C prior to the assay. All the samples were diluted in 0.1 X PBS and the reaction pH was adjusted at 9.

Table 3: Effect of Different Experimental Conditions on Fluorescence Intensity

Parameter Study		Lower CI	Average	Upper CI
Incubation	37°C	166	225	283
	~24° (Room Temperature)	83	116	150
MUG Suppliers	Sigma	102	217	158
	EMD	82	118	153
	Bio World	140	175	210
MUG Dissolving Agents	Ethanol	152	208	263
	DMSO	98	131	164
PBS Strength	0.5 X	98	131	164
	0.1 X	152	208	263
pH Adjustment	8	79	100	122
	9	102	130	158

Note: The results have been obtained based on three replicates of each sample from three independent experiments; the units are RFU (arbitrary units) samples contained 100 CFU/mL *E. coli*.

Other Parameters Evaluation

GUD activities by three different *E. coli* cultures, ATCC 25922, 358218 and 1175, were validated using collilert-18 kit. The findings were in the agreement with the previous research (Maheux et al. 2008) and proved that *E. coli* 25922 produces the highest GUD activities (Figure 15). Furthermore, different units of turbidity and TSB added to the samples which resulted in decrease of GUD expression as turbidity increased (data not shown). On the other hand, the impact of *E. coli* stored in PBS or tap water at 4°C with subsequent dilutions in PBS or tap water under the same laboratory conditions were examined. As seen in Figure 16, storing *E. coli* in PBS with dilutions in water resulted in higher fluorescence measurements. Spiked water samples contained 100 CFU/mL *E. coli* (Figures 15 and 16).

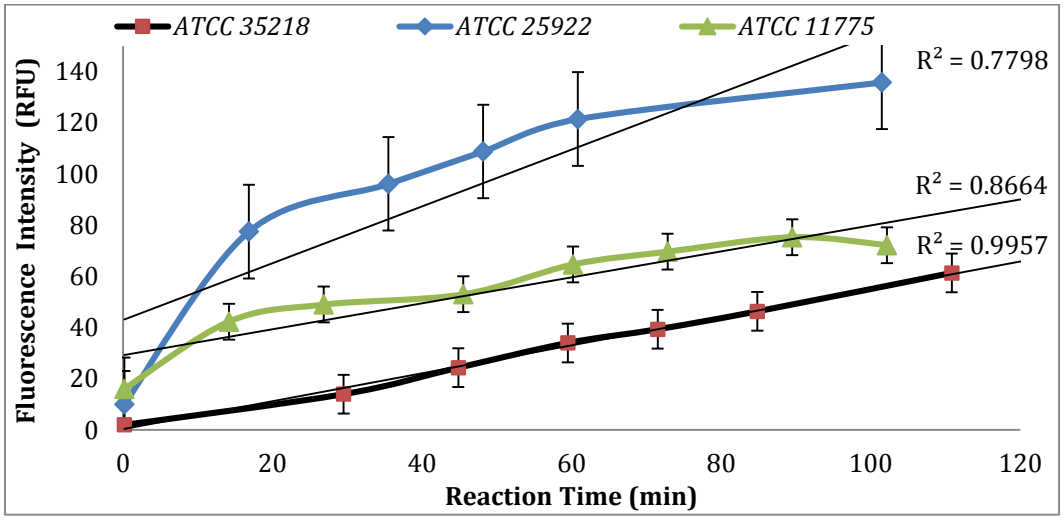

Figure 15: Time Series of Hydrolysis of MUG by Different Strains of *E. coli*

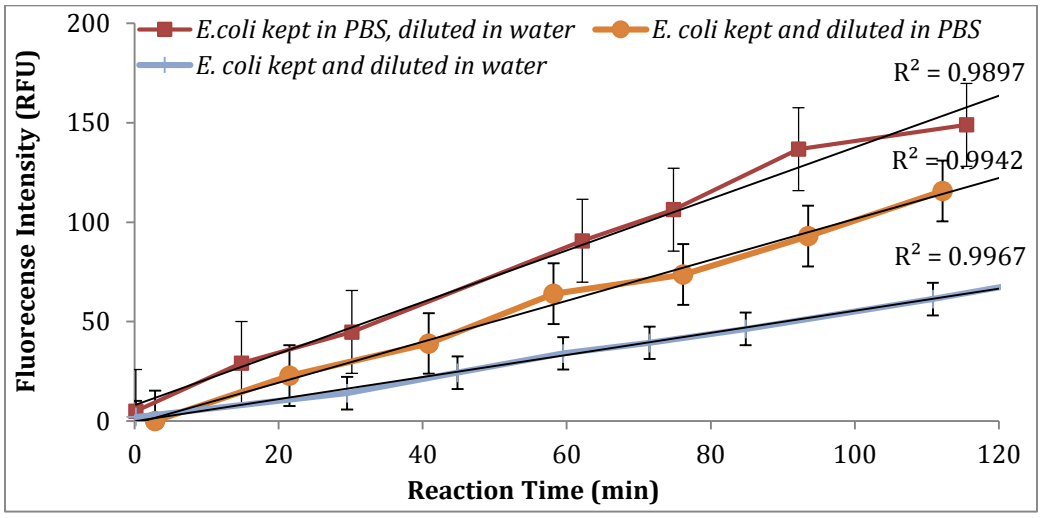

Figure 16: Effect of Storage Condition of *E. coli* on GUD activities

Note: Samples diluted in 0.1 X PBS and spiked tap water samples contained 0.05% NaCl.

4.5 Conclusions

A set of QA/QC requirements have been established for the routine monitoring of drinking water samples using biosensor BDS1000. The key components of QA/QC for this project examined included: reference instrument, methods comparison, MUG purchased from different suppliers, the effect of NaCl, temperature, pH and IPTG. This method was compared with accepted biochemical and reference microbiological procedures. Figure 17 indicates the summary of QA/QC for the biosensor procedure.

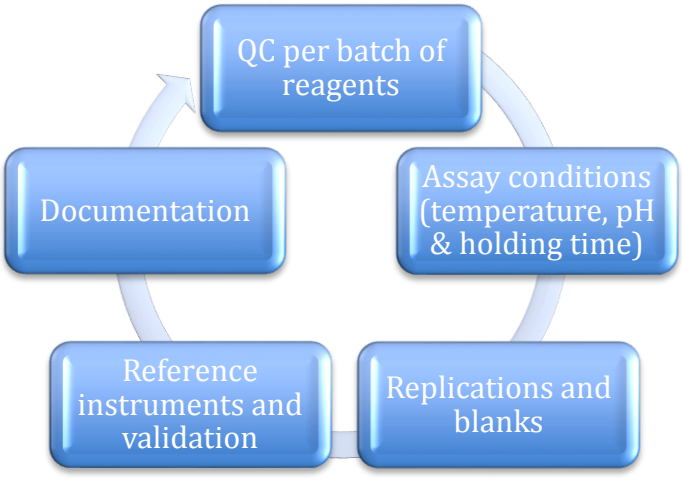

Figure 17: QC/QA for Biosensor Procedure

In addition, the specific substrate molecule used in the design of this biosensor can be utilized as a platform to monitor bacterial quality in water samples. The procedural QA/QC of the biosensor will ensure the quality data by both industry and regulatory authorities by using biosensor for near real-time monitoring of *E. coli* in drinking water samples.

Based on the lessons learned from these experiments, improvements for the procedural QA/QC can be made to enhance accuracy and reliability of the new method and to achieve acceptable precision to detect indicator bacteria in drinking water. Some other factors that should be incorporated into future experimental plans include the kinetic studies to identify binding preferences of GUD to better optimize the reaction. Additionally, it is essential to evaluate the performance of the biosensor with more reference instruments and improve the procedural steps for the sample processing to increase the sensitivity of the method and reproducibility of the results.

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APPENDIX A

REFERENCE INSTRUMENTS

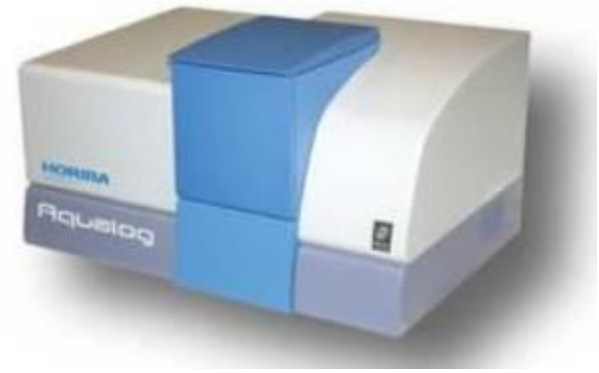

Figure A1: Aqualoq benchtop fluorometer

Figure A2: Synergy H1 Hybrid Multi-Mode Microplate Reader

APPENDIX B

CALIBRATION CURVES

A calibration curve was generated for 0.08 mM MUG for different concentrations of *E. coli* ranged between 10 to 10^8 CFU per mL (Figure B1). Synergy H1 Hybrid Multi-Mode Microplate Reader was employed for creating MUG calibration curves. The effect of incubation of 96- plates and also the extraction buffer have been assessed. The procedure for creating the calibration curve was followed according to Table 1. As seen in the Figures B2 and B3, the incubation of the samples increased the fluorescence generation and the effect of extraction buffer was not significant.

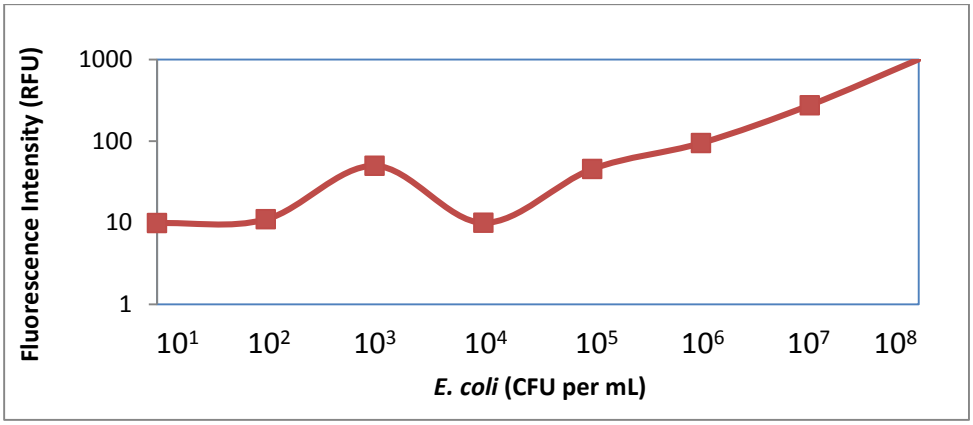

Figure B1: Correlation between the Concentration of *E. coli* and GUD Production

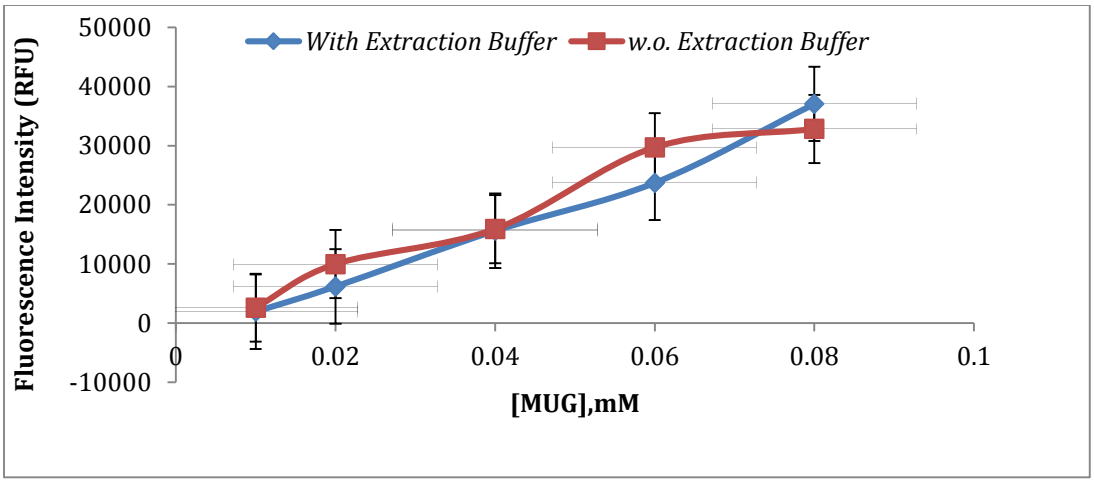

Figure B2: MUG Calibration Curve with Incubation at 37°C

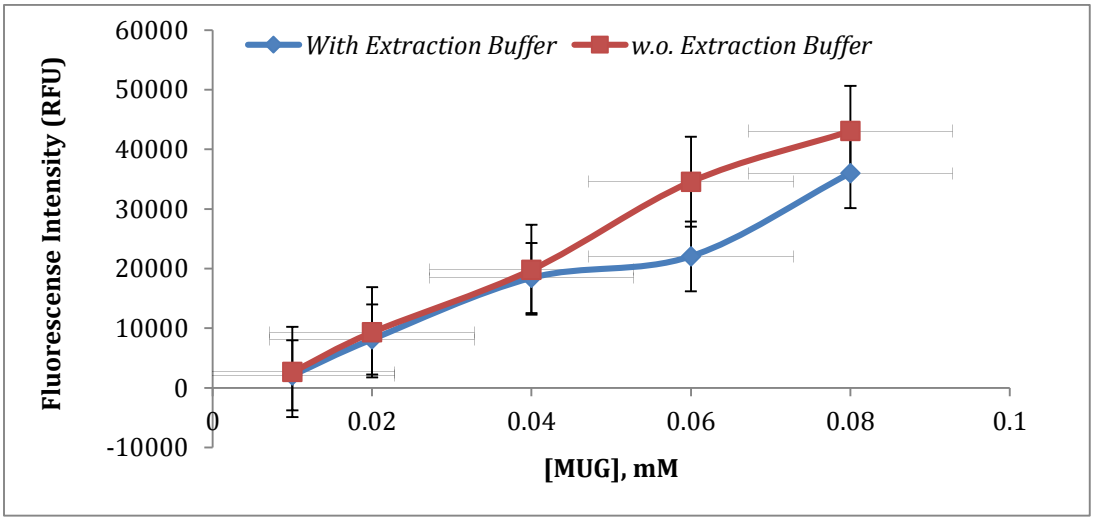

Figure B3: MUG Calibration Curve without Incubation at 37°C

BIOGRAPHICAL SKETCH

Nikou Hesari, native of Iran, received her Bachelor of Science in Water Engineering from University of Tabriz, Iran in 2009 and her Master of Science in Natural Disaster Engineering Management from University of Tehran in 2011. Immediately after graduation, she moved to U.S and started her Ph.D. in the fall of 2011 at ASU in civil, environmental and sustainable engineering. In May 2015, she got selected as the Outstanding Graduate Student from School of Sustainable Engineering and the Built Environment, ASU.