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Autonomous QUerying And PATHogen Threat Agent Sensor System (AQUA PATH)

Monitoring Source Waters with Geospatially Wirelessly Networked Distributed Sensing Systems

Clint B. Smith, Andmorgan R. Fisher, Alex T. Ly,
and Michael J. Anderson

July 2020

Geospatial Research Laboratory

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Final report

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Under ERDC Center-Directed Research Project 219, “Wireless AuTonomous Contaminant Hazard Monitoring Access Network (WATCHMAN)”

Abstract

Contaminants serve as health risks to recreational water, potable water, and marine life that result in undocumented effects on population exposure. In many areas of the world, the concern lies in contaminated drinking water, which would immediately effect social and economic order. As research advances for innovative solutions, the deployment of automated systems for source water monitoring could reduce the risk of exposure.

Water quality monitoring typically involves sample collection and analyses that are performed in a laboratory setting. These results are normally presented after an 18–48 hr period. This report details the prototyped Autonomous QUerying And PATHogen threat agent sensor (AQUA PATH) geoenabled system that is able to detect the presence/absence of pathogenic bacteria indicators in source waters and report these values in the field, in less than 30 minutes. The AQUA PATH system establishes rapid field data collection and reports assessment of source waters bacterial loads at near shore inner coastal locations, which makes a leap forward compared to current presence/absence tests standards established by the EPA.

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Preface

This study was conducted for the U.S. Army Engineer Research and Development Center (ERDC) Geospatial Research Laboratory (GRL) under the Center Directed Research Program 219, "WATCHMAN: Wireless AuTonomous Contaminant Hazard Monitoring Access Network." The technical monitor was Dr. Clint Smith.

The work was performed by the Data Signatures Analysis Branch (DSAB) (TR-S) of the Research Division (CF), ERDC-GRL. At the time of publication, Ms. Jennifer Smith was Branch Chief, Mr. Jeffrey Murphy was Division Chief, and Mr. Terrance Westerfield was the Technical Director for Geospatial Research and Engineering. The Deputy Director of ERDC-GRL was Ms. Valerie Carney and the Director was Mr. Gary Blohm.

The authors would like to acknowledge Dr. Kevin F. Harsh and Mr. Brian R. Schaible of Sporian Microsystems, Inc.; Dr. Michelle Berg of Nanosonic, Inc.; Dr. Jeremy Walker of the U.S. Army Program development Manager Petroleum And Water Systems (PdM PAWS), Dr. Ray Petrisek of HP Environmental, Inc.; and Mr. Greg Meiners of Shaw Environmental, Inc. for their contributions to this project.

The Commander of ERDC was COL Teresa A. Schlosser and the Director was Dr. David W. Pittman.

1 Introduction

1.1 Water quality analysis background

Water is vital for life and human existence in civil and military operations applications. Since 1905, the *Standard Methods for the Examination of Water and Wastewater* has represented "the best current practice of American water analysts." This comprehensive reference covers all aspects of water and wastewater analysis techniques that water quality analysts use to date (AWWA 1999, 2016). The standard is a joint publication of the American Public Health Association (APHA 1985), the American Water Works Association (AWWA 2016), and the Water Environment Federation (WEF 2016).

Water safety and quality are fundamental to human development and well-being. Providing access to safe water is one of the most effective instruments in promoting health and reducing poverty (WHO 2018). The mission of the world water community is to advance water quality improvement in order to sustain human life within a healthy ecological system. In the future, water quality techniques, specifically real-time reporting, may be innovative enough to support rapid monitoring and reporting. These innovative techniques could provide automated remote monitoring and reporting rather than the current method of on-site monitoring and reporting by individuals. Currently on a smaller scale, research and development efforts are attempting to show that monitoring and reporting of water quality can be done in the field, instead of the laboratory, with rapid unique testing and comparison of results to standards. If successful, this field technique could provide more rapid opening and closure (minutes versus hours or days) of recreational waters or the ability to determine critical threats to source waters, due to point and/or non-point source occurrences.

In a water quality report to Congress in 2001, the U.S. Environmental Protection Agency (EPA) revealed the waters surveyed by states, 44% of rivers and streams and 49% of lakes are impaired or threatened for one or more of the designated beneficial recreational uses, such as fishing or swimming (U.S. EPA 1998a; Grifith et al. 1999). Non-point sources, such as agricultural inputs that are difficult to track, were listed as the leading source of impairment in these surface waters and would benefit from the

Autonomous QUerying And PATHogen threat agent sensor (AQUA PATH) technology.

In 1990, 1.2 billion people lacked access to clean drinking water and 1.7 billion lacked access to adequate sanitation services. In 2001, the United Nations (UN) estimated that population growth alone would increase these numbers by nearly 900 million and there is no clear plan for satisfying these additional water needs (Gleick 1993). The 2017 UN world population clock estimate for global population in 2018 was 7.6 billion (United Nations 2017). The continuing urbanization and overall growth of the world's population is projected to add 2.5 billion people to the urban population by 2050, making the global population estimates between 9.7-10.1 B (United Nations 2014; United Nations 2017). Worldwide, more than 250 million new cases of water-related diseases occur each year, resulting in approximately 10 million deaths annually. In 2015, 2.1 billion people lacked safe water at home, while an estimated 844 million did not have basic drinking water services. Additionally, 159 million drink water directly from surface sources, such as streams or lakes, and in 2020, it is estimated that 2.3 billion people still will not have basic sanitation services (WHO 2018). Three of the most important barriers to effective biological monitoring are time, cost, and the lack of information to make informed decisions.

The largest impact to contaminated waterways are pathogenic bacteria from point and non-point sources. Pathogenic bacteria exist at soil surfaces as a result of practices such as spreading liquid manure on agricultural lands or use of wastewater for irrigation. Rainfall is a major factor affecting vertical and horizontal movement of bacteria in soil.

Surface water runoff carries bacteria downstream causing serious threats to ground and surface waters. *Escherichia coli (E. coli)*, the primary indicator organism for fecal pollution, can survive in semiarid areas and increases the potential of contamination (Abu-Ashour 2000). Solo-Gabriele et al. (2000) showed that *E. coli* can enter rivers in large pulses during storms. After storm events, *E. coli* numbers return to baseline levels and their densities in the water vary in a cyclical pattern, which correlate with tidal cycles. The highest concentrations of *E. coli* are observed during high tide, whereas the lowest are observed at low tide. The ability of *E. coli* to multiply in the soil was found to be a function of

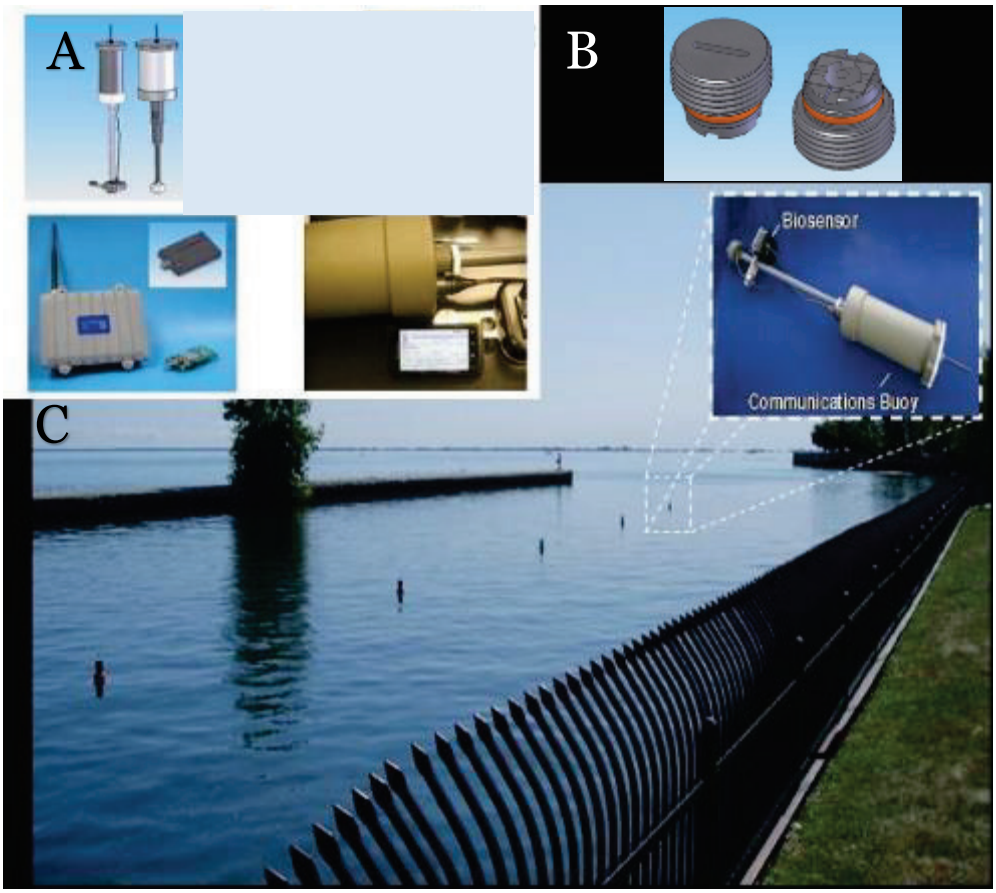
soil moisture content and presumably due to the ability of *E. coli* to survive in relatively dry soil (Solo-Gabriele et al. 2000).

Given the potential for growth in such systems, *E. coli* concentrations can be elevated above that expected from fecal pollution alone. Classical monitoring methods include the Multiple Tube Fermentation (MTF) and the Membrane Filter (MF) techniques in calculation of the Most Probable Number (MPN) analysis (APHA 1985; Federal Register 1975; Bergey's Manual 1994; TB-MED-577 2005). New techniques based on the classical methods include the Colilert® method by IDEXX Inc. that originated in 1994 and was approved by the EPA as a standard test method for water quality analysis (Bergey's Manual 1994). The fastest test parameters are 18 hr as opposed to the 48 hr classical MTF and MF methods. In 2012, the Colilert® 18 hr/Quanti-Tray® testing method became the new International Organization of Standards (ISO) Standard 9308-2:2012 as a method for Total Coliform and *E. coli* detection in the following types of waters: ambient water, surface water, ground water, and wastewater. IDEXX Inc. Inc. also has the Colisure® 16 hr presence/absence test method, also approved by the USEPA as a water quality standard.

1.2 Purpose

The purpose of this study was to develop a prototype water quality biosensor system and evaluate it to determine if it was possible to conduct in situ or in-field measurements of unsafe fecal pollution loads that can be found in source surface waters. The developed system was buoy based and designed for in situ bacterial level detection. This would measure and report harmful bacterial levels within rivers, lakes, and/or reservoirs in the field as opposed to the traditional methods of detection to retrieve a water sample and taking to a laboratory for analyses. The traditional methods are time consuming laboratory processes that can take from 18 hr to 48 hr to obtain results. This project aims at the development of a system that would speed up this analysis and formulate a new method to take minutes rather than hours or days of waiting for results. The bacterial detection system for water quality monitoring and reporting system is called the AQUA PATH Biosensing System (Figure 1).

Figure 1. Consortium of AQUA PATH Biosensing Systems. Clockwise from top left corner: A: Design sketches of the AQUA PATH Biosensing Buoy System (AQUA PATH Version I, AQUA PATH Version II), Gateway communications link and an example Cell Phone link with software application showing data collection, B: AQUA PATH Biosensing Buoy System replaceable Cartridge with thin film attached, C: AQUA PATH Biosensor Buoy systems deployed during initial field test and evaluation. Selfridge Air National Guard Base (SANGB), Lake St. Clair, Michigan, 2009. AQUA PATH Network Gateway Communications Box, and center photo: AQUA PATH Biosensing Buoy System with communications link through cellular data display.



1.3 AQUA PATH biosensing buoy system background

The AQUA PATH Buoy System was designed to be submersible with a custom housing comprised of optical detection mechanisms. This allowed for a sample of water to be collected and analyzed on board the deployed system and thus conduct measurements in the field. The system includes a very small low-power pump for stagnant water applications to help circulate water when necessary. The small pump was used to introduce and exchange sample water. The following design version, version II, of this part of the system was modified to use an improved pump to allow for better flow, which was not being filled in a timely manner without the pump. The communications integrated into the buoys are capable of

reconfigurable local sensor networking and satellite data transmission. Potential application areas can be seen for this autonomous system for municipal water and wastewater testing, tactical field use, homeland security, agriculture, and food production.

The AQUA PATH Biosensing Buoy System was designed specifically for improvements towards innovative technology and rapid improvement of biological identification, quantification, and reporting for water quality assessment of source water supplies or source waters used for recreational purposes. The AQUA PATH Biosensing Buoy System was co-designed with the technical guidance of the United States Government (USG) U.S. Army Corps of Engineers (USACE) Engineer Research and Development Center (ERDC) Geospatial Research Laboratory (GRL) and Sporian Microsystems Inc. from the U.S. Army Small Business Innovative Research (SBIR) Program. Phase I of the program began in 2004 when Sporian Microsystems, Inc. demonstrated the proof-of-concept for the systems. Between 2004 and 2008, the AQUA PATH Biosensing Buoy System was developed further between Sporian Microsystems, Inc. and the USG to improve its ability to function in the field versus the laboratory setting. In 2008, after the SBIR Phase II/III funding ended, reimbursable funding from CCDC-GVSC (formerly RDECOM-TARDEC) and ERDC 219 – now the FLEX-4 or Future Innovation Fund Program funds (FIF) were utilized to continue development efforts of the technology. The in-house funded effort focused on using the systems to assist the Army water monitoring and purification efforts to aid in reducing the use of costly ultrafiltration filtration on 1500 to 3000 gallon per hour purification systems by monitoring the bacterial load within raw source water supplies. Nanosonic, Inc. was also contacted for collaboration on the development and design of the thin film sensor components of the AQUA PATH Version II Biosensing Buoy System. Contracted Reference Items (EPA IAA W81EWF12065519), W81EWF1032406, MIPR1DDAT0R0253, W81EWF10559110 and W81EWF11326925, W9132V-12-P-0008, W9132V -12-P-0016, MIPR S85014125, MIPR S850-14-031.

The AQUA PATH Biosensing Buoy System's technology was developed to require lower power (running on eight D-cell batteries) and was intended to detect pathogenic bacteria presence or absence in less than 30 min with a unique thin film design. The system is geoenabled for providing location, water quality parameter data collection, and provides onboard storage of data for on-site analysis or sharing with other users or sensor networks in

a dynamic environment. This project was geared for demonstration for in-field data collection and was based on a preliminary basis to show the process is promising. But, this would still need further development and advancement as the project continued to allow for a definitive study in terms of replication and exploring the full array of interferences, such as additional testing on introduction of bubbles in the system, organic matter, salinity, turbidity, etc.). Some evaluation began to look into the remote monitoring capability, which shows promise, but by no means provides the full evaluation of this portion of the project.

The AQUA PATH Biosensing Buoy System establishes a new effort to provide field data rather than laboratory data, to aid in providing a rapid assessment of source waters' bacterial loads at near shore inner coastal locations including fresh waters, brackish waters, and/or salt waters and via a remote wireless network for digitally derived data dissemination. This system demonstrated the ability to detect the presence/absence of pathogenic bacterial indicators common to water quality bacterial pollution that could harm drinking water supplies and access to natural resource supplies for recreational use. The system's analysis is estimated in the test and evaluations to be around 1,000 (CFU/mL) (EPA standard: 100 CFU/mL), but further testing would be necessary to verify these numbers. At a minimum, the AQUA PATH Biosensing Buoy System could distinguish the presence/absence of these harmful bacteria in less than 30 min. Therefore, the system is more comparative to the EPA presence/absence testing rather than the numerical specific comparative testing. There would need to be further research and development in the future for numerical-specific comparative testing.

The novelty of this system is a signal conditioning circuits and sensor control board comprised of firmware/hardware. This provides a unique ability to allow the biosensor to act autonomously while reporting (less than 30 min) for levels of pathogenic bacteria in source waters. This distinctive system design provides a new measuring technique using novel thin films, advanced electronic hardware and software to capture improved and rapid data collection (less than 48 hr) within the water quality community for total bacterial detection. This type of water resource data collection technique or method has yet to be demonstrated anywhere commercially to the best of our knowledge. Additionally, the AQUA PATH Biosensing Buoy System, test set ups, and exercise participation are shown in the Appendix. The development of the prototype to show feasibility of

this is an achievement in the field of environmental sensing related to presence/absence of bacterial loads for monitoring/reporting within source waters.

Many innovations in smart sensor-based sensing systems over the past decade have provided new technological advancements that aim to provide the ability to closely monitor and map natural environmental occurrences via small business investments. The ability to fully implement this type of technology has not yet been successful within the water quality community, specifically in the bacterial detection community. Current water quality monitoring typically occurs when samples are collected, analyses are performed within a laboratory setting, and, at best, results are presented after an 18-24 hr period (or more likely 48 hr) following the *Standard Methods for the Examination of Water and Wastewater* guidelines [The standard is a joint publication of the American Public Health Association (APHA 1985), the American Water Works Association (AWWA 2016), and the Water Environment Federation (WEF 2016)]. As research advances in the developed world, there will be more innovation towards deploying a number of automated and digital sensing systems from which the scientific community can benefit. One day, these advancements could be utilized to monitor water quality parameters within a reservoir or water resource for a recreational area. Remote monitoring of distribution systems or source waters will provide a method to monitor the risk for contamination without taking samples to a laboratory and monitoring directly in the field. Currently, the United States Geological Survey (USGS) and the National Oceanic Atmospheric Administration (NOAA) have mastered digital mapping and distributed sensor monitoring within the scientific community. Each has their own missions for measuring and monitoring earthquake activity, oceanic tsunami alerts, and river water levels on a national and global scale, respectively.

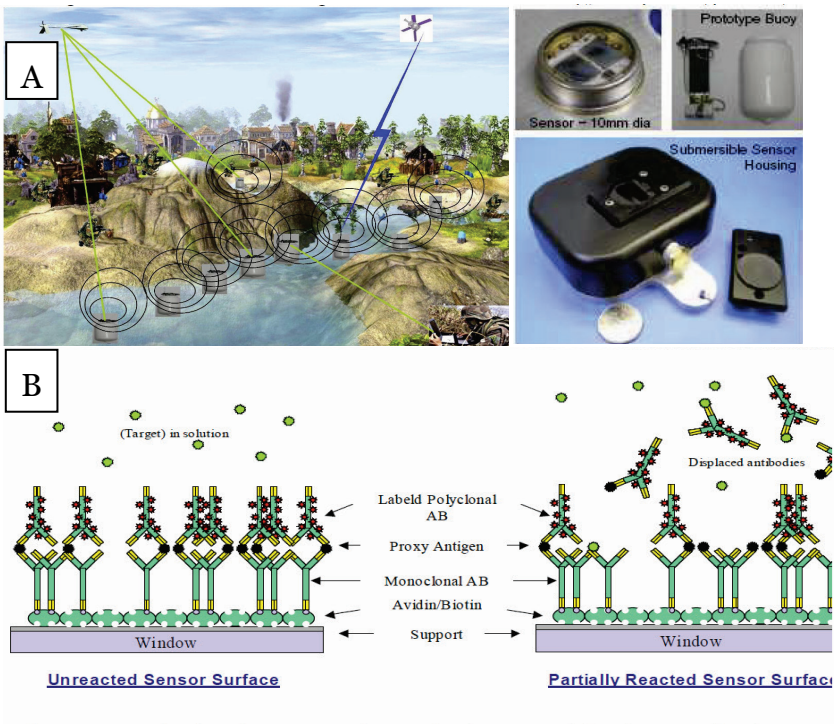
The end goal for this research and development effort is to push remote monitoring technology towards this new technique specifically related to biological detection. This would allow for USACE-ERDC to collaboratively aid other federal agencies, such as the USGS and the U.S. Department of Agriculture (USDA) in monitoring the U.S. and global waterways for safe water quality parameters. Advancements, such as the AQUA PATH Biosensor Buoy System, can provide faster opening and closures of recreational waters and determination of critical threats to source waters

due to non-point source occurrences related to bacterial related contamination.

Inside the water quality assessment community, rapid assessment of biological material within the environment has been a challenge for the past century. Although this technology does attempt to achieve this goal of rapid detection (minutes rather than hours or days), it has yet to reach its full potential and merely brings about the paradigm of change with new technology to achieve such goals. The technology employed in this assessment includes a biosensor-equipped buoy system for rapid detection of waterborne pathogens, such as *E. coli* and/or Coliforms (non-specific), indicating the presence of pathogenic bacteria, and reporting using a geospatial wireless networked system. The AQUA PATH sensing technology developed is based on an optic-electrical sensor utilizing state-of-the-art thin film inserts comprised of stabilized covalent bacterial binding mechanisms housed in a floating buoy equipped with radios for wireless network transmission. This technology is unique to the development of the system and Sporian Microsystems Inc. holds the patent on the AQUA PATH Biosensing Buoy System's biosensor construct manifold and sensor electronics. The original glass thin films were developed, tested, and redesigned to a non-glass thin film. These non-glass thin films and their coatings were developed in collaboration with the USG and Nanosonic Inc. The team chose the non-glass thin films because of their ability to be designed for improved stability of the embedded bacterial detection targets or antibody materials. Test results, shown later, provide data towards improved environmental stability, reuse applicability, and longer shelf life than use with glass based thin films (Anderson et al. 2012). Figure 2A and B show a conceptual design for use of the system and a breakdown of the biosensor itself as initially thought out and designed using an antibody based fluorescent sensing mechanism. The biosensor uses a light source via LED (xyz, nm) to excite or fluoresce the fluorophore (Labeled Polyclonal AB in Figure 2.B). When fluorophore is present, it will emit light of a different wavelength (nm). This wavelength (nm) light is detected and is reported as voltage/time, specifically in the units of volts/milliseconds (V/ms). The biosensor is always emitting a fluorescent signal. The biosensor is "ON" as long as the biosensor is stabilized, and when it comes into contact with targeted bacteria of interest, it loses its fluorescent intensity. The loss of intensity is calculated by the computer on board and reports the detection remotely, which would be a signal recognized by a software set limit as "OFF" or

when the fluorescent signal has diminished passed a threshold designated by studying the bound target of the biosensor with the bacteria of interest. This project began this analysis, but needs further inquiry on this matter to provide enough replication of the research to be able to set these threshold limits as compared to the natural environmental bacterial load parameters. A standard curve would need to be tested and evaluated thoroughly to establish these numbers respectably.

Figure 2. A. Conceptual design of the AQUA PATH waterborne pathogen mesh network, tactical use. B. The basic MDE formulation used in the preliminary studies: a sandwich immunoassay labeled with a fluorescent marker.



The biosensor was designed to effectively secure, monitor, and maintain a safe water supply, however the results did not reflect this design. The detection level was 10 times too high. The device was intended to provide the following:

- Constant, persistent in situ monitoring and identification of water bacterial contamination for water quality; knowing the thin film detection mechanism will still need advancing for improved performance regarding persistent monitoring techniques.
- Early detection of threat and threat type to the water supply.

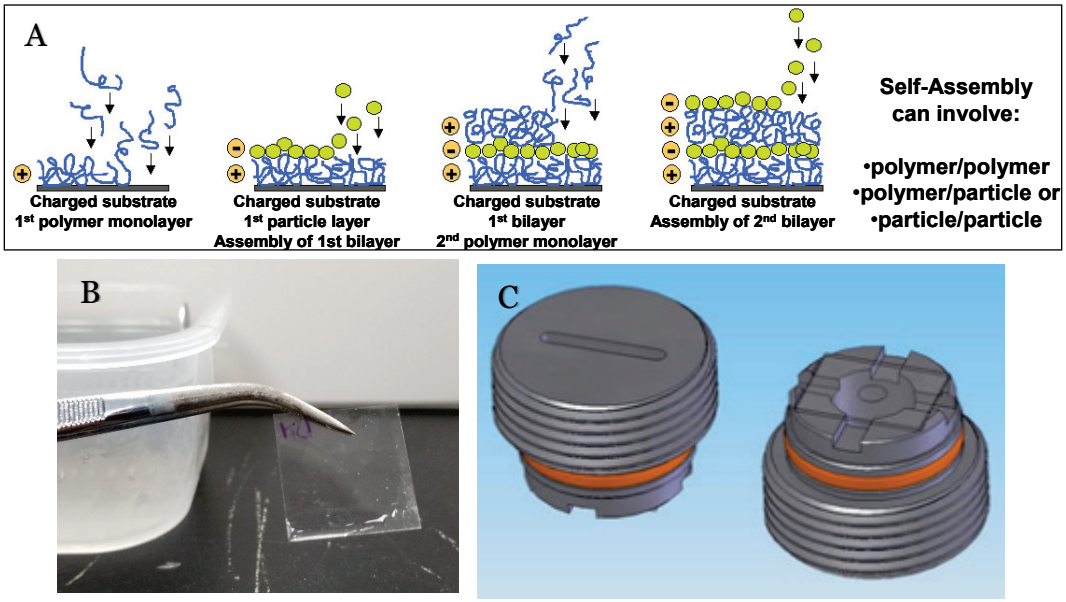
1.4 Objectives

The objectives of this research include developing a remotely distributed biosensor system for the detection of bacteria in the field and providing improvement of different sensor components. This would produce a biosensor with a significantly reduced cost, time of analysis associated with potable water monitoring, and providing a new capability to report the data operationally in the field digitally versus analog to allow for deployment ability at a global scale and survive harsh environmental conditions.

1.5 Approach

At the outset, thin films for the biosensors were co-designed first with Sporian Microsystems, Inc. on glass thin films with specialized coating mechanisms called Molecular Detection Elements (MDEs). After the initial testing took place and weaknesses were discovered in the glass based thin films, a second thin film was designed with Nanosonic, Inc. for a more flexible thin film with improved stability and completed biosensing element and coating. The Nanosonic thin film are self-assembled and surface functionalized for bacterial detection (Figure 3).

Figure 3. A. Self-Assembly model of the Nanosonic Inc. thin film. Each thin film/chip is a 1" x 1" flexible chip with proprietary NanoSonic surface functionalization (top). B and C. Nanosonic Inc. flexible thin film with proprietary surface functionalization for bacterial detection mechanism (bottom).



Data was collected after exposing various *E. coli* concentrations in non-chlorinated water to provide a baseline that the biosensor was operating correctly to detect bacteria in the water. Targets of interest were *E. coli* (ATCC strain 25922) encompassing a broad serotype of the *E. coli* into the biosensor. Additionally, a salt water bio-indicator was developed using *Enterococcus faecalis* to test the biosensors ability to detect bacteria found in salt water sources.

An in-house evaluation was conducted of the AQUA PATH Biosensor Systems and their collection end point thin film cartridge tests were conducted. The systems were tested in laboratory and field experiments. To be more specific, the thin films were designed to be a flexible, conductive sensory platform usable within the biosensor construct and for efficiency with its use with the opto-electrical components of the AQUA PATH Biosensing Buoy System. The flexible plastic carriers were designed to work as a conductive application comprised of a surface modification. The surface attachment chemistry was used for this work in the fabrication of the thin film, which was a novel approach to production. The reusability of the thin films used on the biosensor cartridge insert were investigated and the long-term stability of the sensor was evaluated, *i.e.* storage and robustness (Anderson et al. 2012). Metrics evaluation of internal communications of sensors were completed and provided the AQUA PATH Biosensing Buoy System the ability to show or release an alarm for positive detection of pathogen indicators of interest.

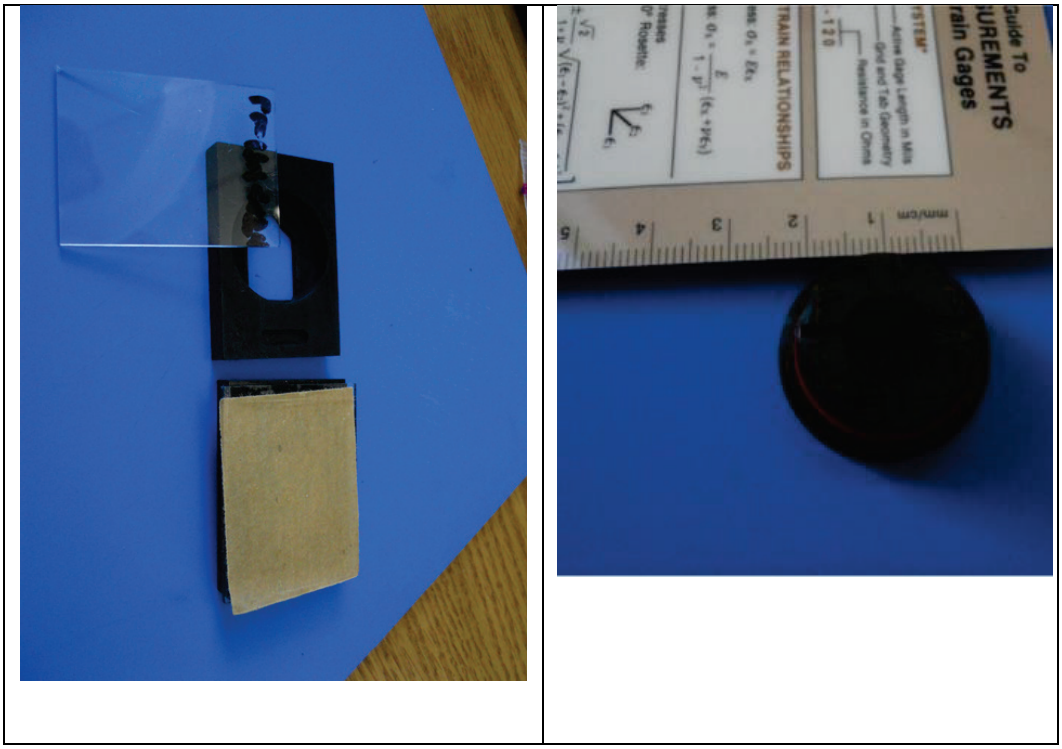
2 System Development and Methods

The AQUA PATH Biosensing Buoy System's biosensor thin films were evaluated in the development for their ability to detect bacteria. The thin film bio-detection capability study began by looking at the thin film's ability to provide a flow of water across its biosensor to allow for attachment of pathogenic bacteria indicators. Essentially, would the biosensor work to recognize and/or detect the bacteria of interest for water quality analytics. The thin film needs to be comprised with a correct coating to maximize contact of the biosensor with the available source water samples taken in by the system's pumps. The thought was to increase the thin film's ability to be as close to the water molecules as possible and this is done by adding a hydrophilic coating. Nanosonic, Inc. was selected to develop the thin films and also address the maximization of the ability of the thin film to interact with the environment it was presented with to essentially detect pathogen bacteria indicator microorganisms found in source waters.

2.1 Hydrophilic coating evaluation: Abrasion resistant coating for acrylic window of biosensor cartridge

Samples were received from Sporian Microsystems, Inc. as shown in Figure 12 to include individual parts for the cell assembly as well as an assembled cell (square cell shown on the left). An additional cell component for a different detection system was received from the sponsor and is also shown in Figure 4. This cell will require a 13 mm diameter circular sensor cell (shown on the right).

Figure 4. Pieces of water sensor cell as received from Sporian Microsystems Inc. (left) and circular sensor cell (right).



Nanosonic, Inc. applied an abrasion resistant coating to the rectangular acrylic window and evaluated the sample for percent transmission, haze before and after Taber abrasion (ASTM D1044) (American Society of Testing and Materials, International (ASTM); D1044 refers to the standard letter and number for Standard Test Method for Resistance of Transparent Plastics to Surface Abrasion), contact angle before and after abrasion and scratch and Peel according to ASTM D3359 (Standard Test Methods for Rating Adhesion by Tape Test).

The percent transmission for the HybridSil® abrasion resistant coating as measured at NanoSonic is approximately 90.2% (commercial value of uncoated acrylic is 92%) and is shown in Figure 5. The wavelength region of interest for the sensor system (680 nm – 702 nm) is circled in red. The coated samples were then abraded for 500 cycles using 250 g weights and sample weights; haze measurements were measured before and after each cycle of abrasion (Figure 6). The haze values are reported as a percentage. As shown in Table 1, the haze values are significantly lower for the acrylic sample coated with the HybridSil® Hydrophobic material. After 500 cycles, the haze was only 4% for the coated sample compared to the uncoated acrylic which was 10%. Scratch and peel rating results for the

coated sample are also reported as a '5B,' which indicates no significant delamination of the coating from the surface after the tape is peeled from the scratched surface. This indicates excellent material adhesion to the substrate surface and can indicate coating durability.

Figure 5. Transmission trace for abrasion resistant coating applied to acrylic window of sensor cell assembly. The targeted transmission range for the optical sensing lies approximately within the red circle (680 nm – 702 nm) and averages >90% for the coating system.

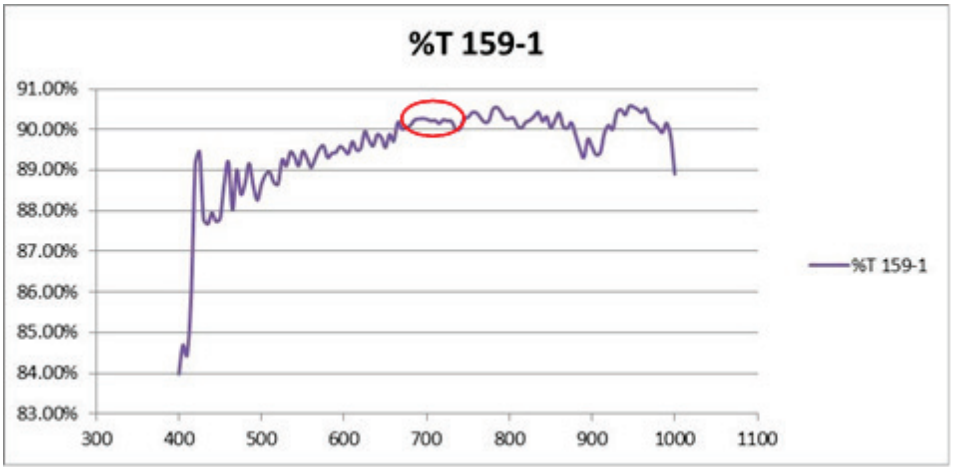


Figure 6. Water droplet on the surface of the acrylic window before Taber abrasion (left) and after 500 wear cycles (right).

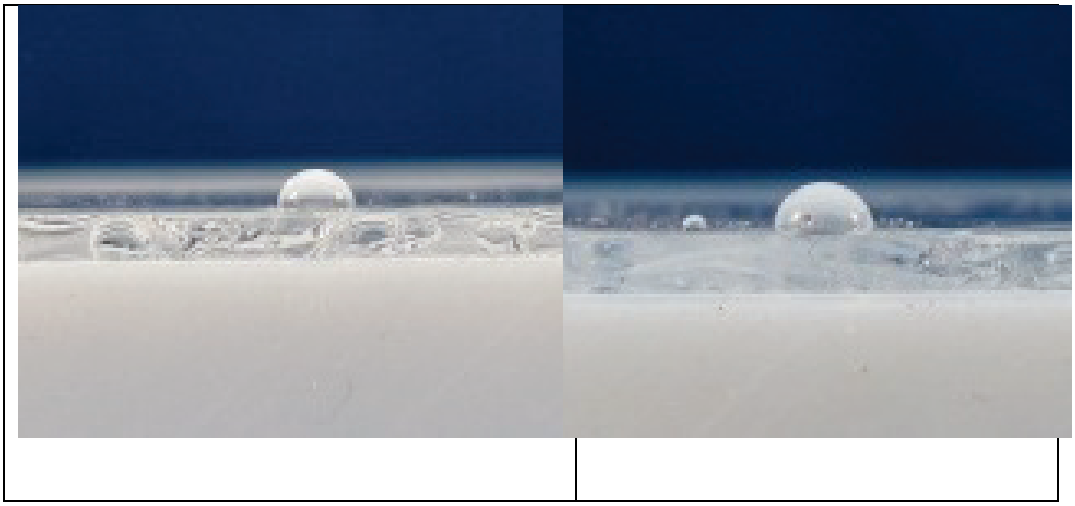


Table 1. Haze and scratch/peel data for HybridSil material on acrylic.

Haze % pre-Taber	Haze after 100 cycles	Haze after 500 cycles	Initial Weight	Weight 100 cycles	Weight 500 cycles	Scratch/ Peel rating
1.6374	1.24078974	4.1954764	3.61	3.61	3.611	5B
0.0633	0.63246619	10.313782	11.745	11.745	11.744	NA

From these analyses, Nanosonic, Inc. recommended the application of HybridSil® abrasion resistant material as an appropriate abrasion resistant/protective coating for the acrylic 'windows' used in the manufacture of the biosensor cell. The coating did not impact the transmission of the acrylic material in the operational wavelength range and improved abrasion resistance through Taber abrasion tests. The material is chemically compatible with the acrylic substrate material and exhibited excellent adhesion to the window surface.

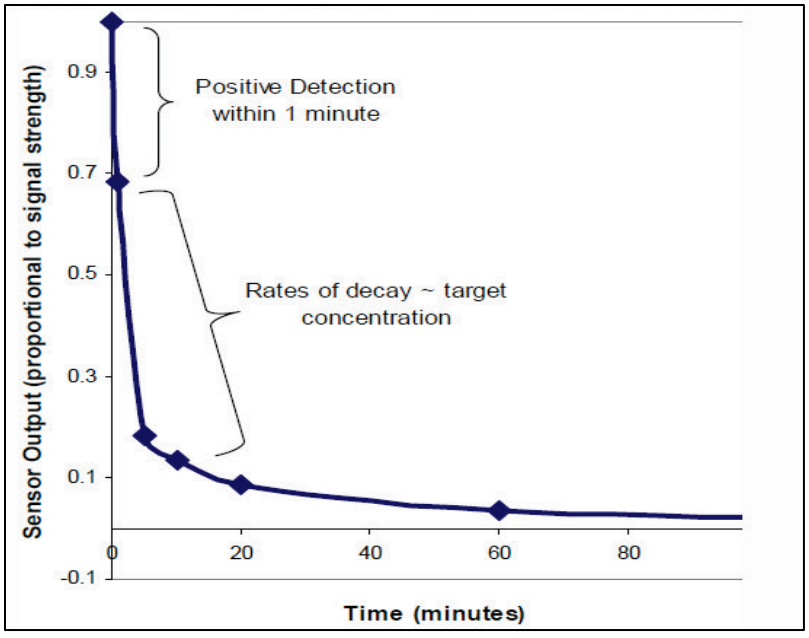
Employing the HybridSil® abrasion resistant material could replace the current adhesive paper that must be peeled from the acrylic surface prior to sensor cell installation. The optimal goal would be applying the coating to cells prior to cell assembly as a step in the manufacturing process.

3 Test and Evaluation

3.1 AQUA PATH Biosensing Buoy System

It is important to point out that the first laboratory testing of the biosensor configurations and elements were conducted at Sporian Microsystems, Inc. laboratory in Boulder, CO before the systems were provided to the Army ERDC-GRL for further evaluation. Figure 7 shows the initial results on the bench controlled laboratory test results of the AQUA PATH Biosensing Buoy System conducted by Sporian Microsystems, Inc.

Figure 7. Preliminary testing results from Sporian Microsystems Inc: Sensor output in response to 1000 CFU/mL detection range shown from 1-60 minutes upon positive sensor interaction with bacterial detection. The MDE biosensor was targeting for *E. coli*. This experiment was a controlled environmental setting on a bench in a laboratory. It demonstrated the first initial scientific testing of the system for proof of concept of the system. Further field evaluations were the next phase to test the system for detection of bacterial targets of interest in source waters, mainly *E. coli* and Coliforms. Both *E. coli* and Coliforms are the common indicator bacteria of fecal bacterial pollution in source waters.



The purpose of this experimental test plan was to validate the accuracy of the AQUA PATH Biosensing Buoy System biosensors detection limits used for the analysis of water quality against industry standard testing procedures. The end result is envisioned to be a state-of-the-art system with a detection requirement of one Colony Forming Unit (CFU) per 100 mL of any source water or treated water sample in less than 30 min. If

feasible, a detection rate of less than 5 min could be optimal or desired by driven requirements. The AQUA PATH Systems were designed for such an effort. This evaluation was completed by performing the experiment described below. The objective was to compare the AQUA PATH Biosensing Buoy System as a water quality biosensor with current and certified water quality methods from a third-party certified water quality laboratory to gain a greater insight into the detection limits of the AQUA PATH Systems. The experiment was conducted at GMU S&T Campus. The Certified Laboratory sent out a representative to collect the 70 total samples for the third-party analysis in accordance with standard testing procedures. Although there are other prototype biological detection systems in development, the team did not have access to these for test and evaluation comparison analyses. The AQUA PATH system was the first of its kind including a digital communications and reporting component and designed with the goal of the ability to remove the laboratory testing and conduct the analyses directly in the field for point source detection, monitoring, and more rapid reporting. The most rapid water quality test currently available for biological testing is the IDEXX Inc. 18-hour Colilert® system. The team did have access to this system and used it for test and evaluation comparison analyses. Table 2 describes the experimental plan including serial dilutions of the *E. coli* ranging from 100 to 100,000 CFU/mL. A stock 15 L solution was first made and then each subsequent serial dilution was added to the selected 5 gal containers.

Table 2. In-house experimental plan for serial dilutions evaluation.

To make 15L@:			Add this to the 15L	
	100,000	CFU/mL	15	mL's culture stock
	10,000	CFU/mL	1.5	mL's culture stock
	1,000	CFU/mL	1.5	mL of 1:10
	100	CFU/mL	1.5	mL of 1:100

The growth time of American Type Culture Collection (ATCC) strain 25922 *E. coli* occurred from 4-5 hr after a refresh culture that was made from stock 10^8 CFU/mL of live cells. A refresh of 8-12 hr generates 10^9 CFU/mL, but is a mix of live/dead cells.

3.2 Procedure for Molecular Detection Element (MDE) biosensor test

Principle: Testing of the AQUA PATH Biosensor Buoy System's biosensor MDE cartridges against estimated concentrations of *E. coli* ATCC strain 25922.

A 1 milliliter (mL) inoculation of overnight culture into Tryptic Soy Broth will grow into 10^8 CFU/milliliter (mL) in 4 hr at 37°C anaerobically based on the in-laboratory experimentation and colony counting computations.

Day of Experiment: (Test Held at the ERDC GMU Laboratory): Every sample was started and allowed to run 5 min with each biosensor submerged in 15 liters of various solutions (i.e. Phosphate Buffer Saline (PBS)), 18 MΩ purified water, and PBS with various inoculations of *E. coli* as shown in Table 2 above. At 5 min, when a spike event occurred, the solution was spiked and stirred in each 5 gal container for the experiment. The test was then started, after spiking, and let run for 60 min. Controls were run for approximately 30 min. Samples of tested water with either controls (no *E. coli*) or with the various serial dilutions of *E. coli* and the wild unknown sample containing local Pond water, taken from the S&T Campus Pond at GMU, comprised of all waterborne bacteria including *E. coli* were taken and labeled at the half way point of the test. Figure 8 shows the sampling set up with each AQUA PATH Biosensing Buoy System.

The biosensor cartridge or MDE was placed into clean PBS (PBS is the storage buffer for the MDE and labeled upon completion of each sample run. The experiments were continuous following the control sample collection. Inoculation samples started with the lowest and ran to the highest dilution for each sample course.

Figure 8. AQUA PATH Biosensing Buoy Systems Bacterial Cartridge Biosensor Challenge (Test and Evaluation) GMU S&T Campus Laboratory. From left to right. AQUA PATH Biosensing Buoy Systems along with a modified version of the floating Buoy that was meant for dropping down less than 4 inch diameter wells to 1500 Ft to test aquifer based water quality.

3.3 Results and discussion of the AQUA PATH Biosensing Buoy System

The data shown in Figure 7 (collected in the laboratory by the AQUA PATH Biosensing Buoy System) show the sensor cartridges demonstrated positive detection of the targeted capture agent *E. coli* at the various dilutions each was exposed. All samples showed decay in their profiles. The initial rate of detection was determined by analyzing the MDE, which measured a 90% rate of decay from target concentration interaction within 20 min. The sensor output (proportional to signal strength), Y-axis, to time in minutes, X-axis.

Further testing occurred within the laboratory setting as the prototyped systems were acquired by the government. The testing for data shown in Figures 10-16 was conducted by the government and used for comparison to results from a third party evaluation team on concurrent days of sampling analysis. The third party evaluation is discussed in the Section 3.4. The controls that were established for the next set of tests were reported as all negative when they were exposed to solutions with no *E. coli,* such as the phosphate buffered saline (PBS), distilled water or tap water as expected. The cartridges did respond to various serial dilutions

based on a logarithmic scale from 100 CFU/mL to 10^6 CFU/mL, as well as an unknown pond water sample. These data are shown in Figures 10-16. The data was processed during the experiment and the graphs depict the plot of time in minutes (X-axis) versus a change in voltage per unit time (V/ms) (Y-axis). The voltage per unit time is used in the calculation of the signal strength value by using the ratio of the total signal strength when the sensor is first measured in the biosensor system. The calculation is conducted by taking the dose exposure value V/ms and dividing this value by the biosensor reading during an initial measurement value in V/ms and reported as Signal (%). The initial measurement value is the value of when the biosensor has had no interaction with the environment and is considered the "Sensor ON" value. When exposure occurs, the "Sensor ON" value is designed to diminish as bacterial targets of interest bind to the biosensor thin film triggering a detection, shown in Figure 9 as the red line at 86%. The greater the percent change in signal strength demonstrates detection of the select target of interest, in this case *E. coli*.

Figure 9. AQUA PATH Biosensing Buoy System biosensor challenge, serial dilutions and exposure to *E. coli* in 15 liters of water (N=4).

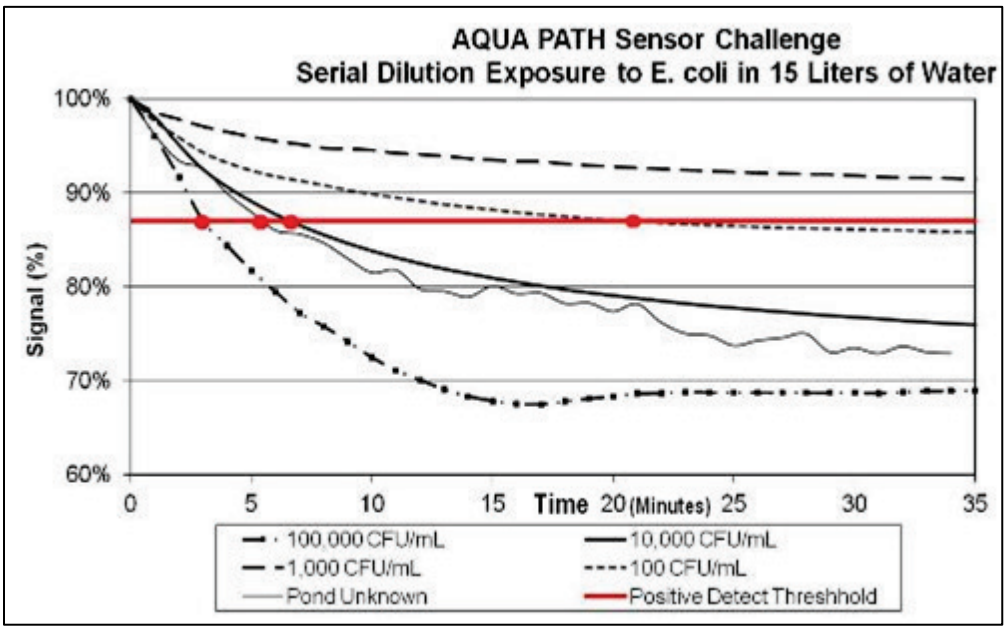


Figure 10. AQUA PATH Biosensing Buoy System In-Laboratory ERDC assessment data obtained directly from AQUA PATH Biosensing Buoy System: Calibration control phosphate buffered saline exposure only.

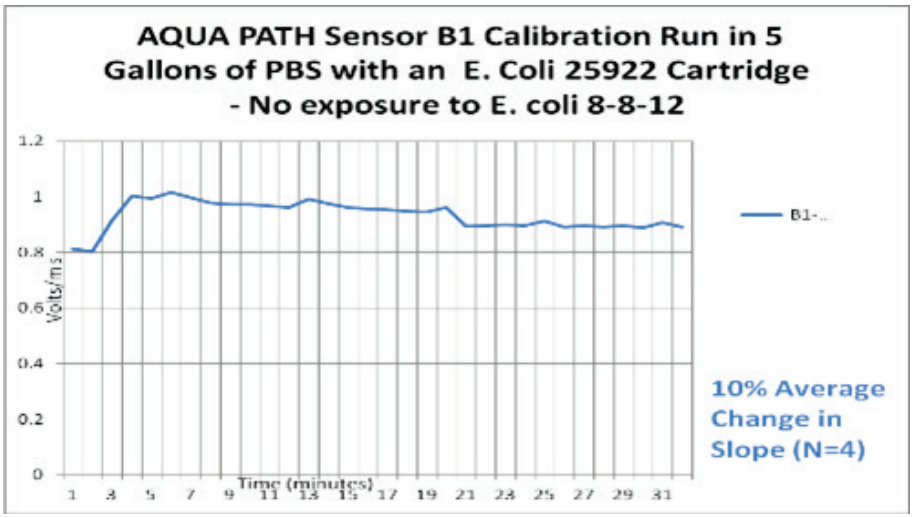


Figure 11. AQUA PATH Biosensing Buoy System In-Laboratory ERDC assessment data obtained directly from AQUA PATH Biosensing Buoy System Calibration control 18 MΩ water exposure only.

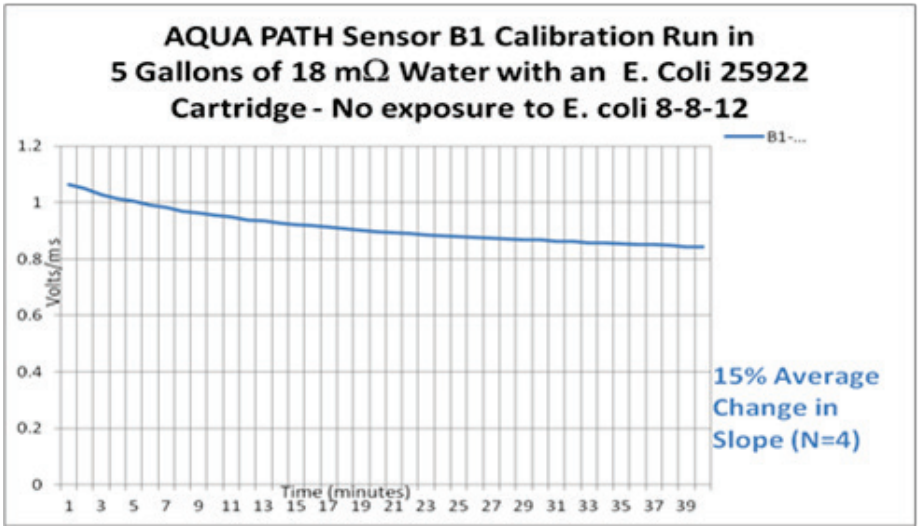


Figure 12. AQUA PATH Biosensing Buoy System In-Laboratory ERDC assessment data obtained directly from AQUA PATH Biosensing Buoy System: exposure to 100 CFU/mL *E. coli*.

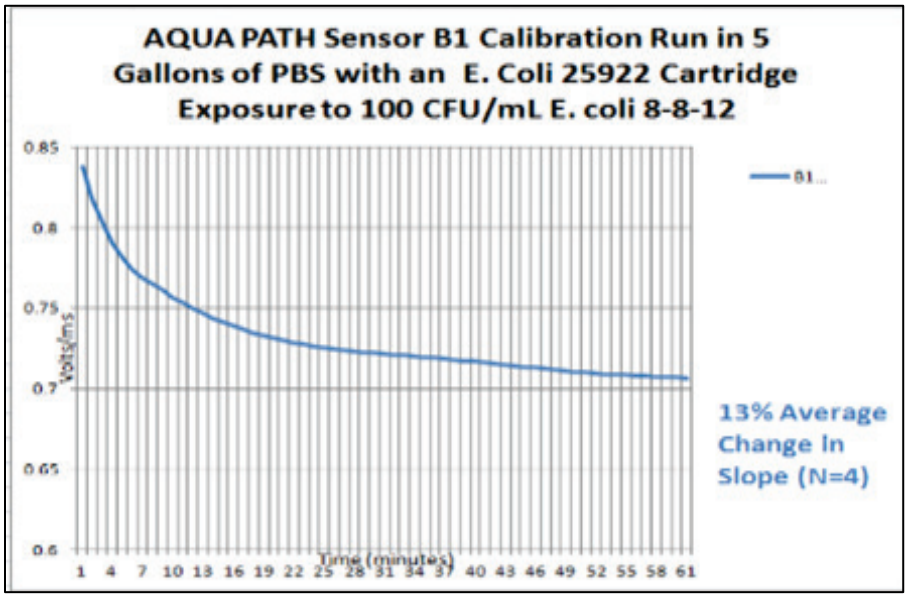


Figure 13. AQUA PATH Biosensing Buoy System In-Laboratory ERDC assessment data obtained directly from AQUA PATH Biosensing Buoy System: exposure to 1,000 CFU/mL *E. coli*.

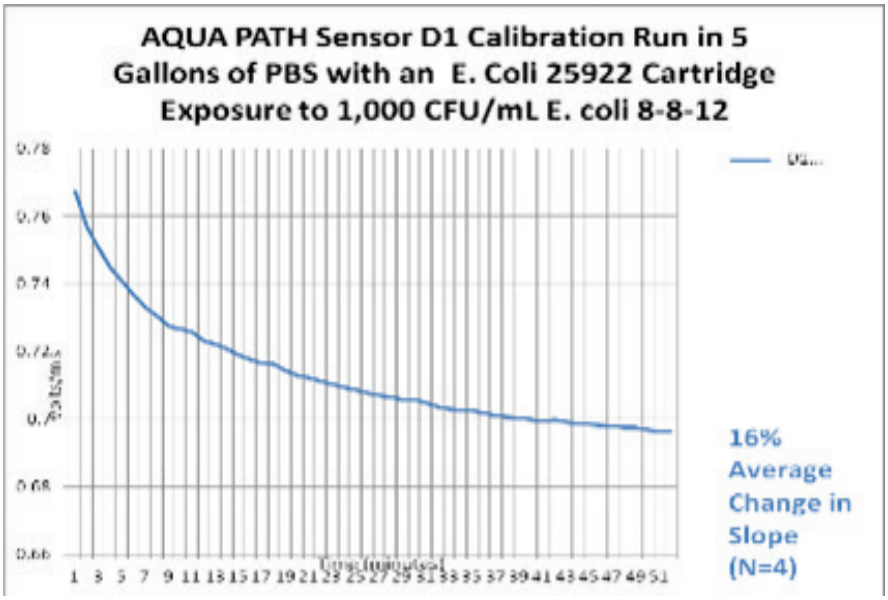


Figure 14. AQUA PATH Biosensing Buoy System In-Laboratory ERDC assessment data obtained directly from AQUA PATH Biosensing Buoy System: exposure to 10,000 CFU/mL *E. coli*.

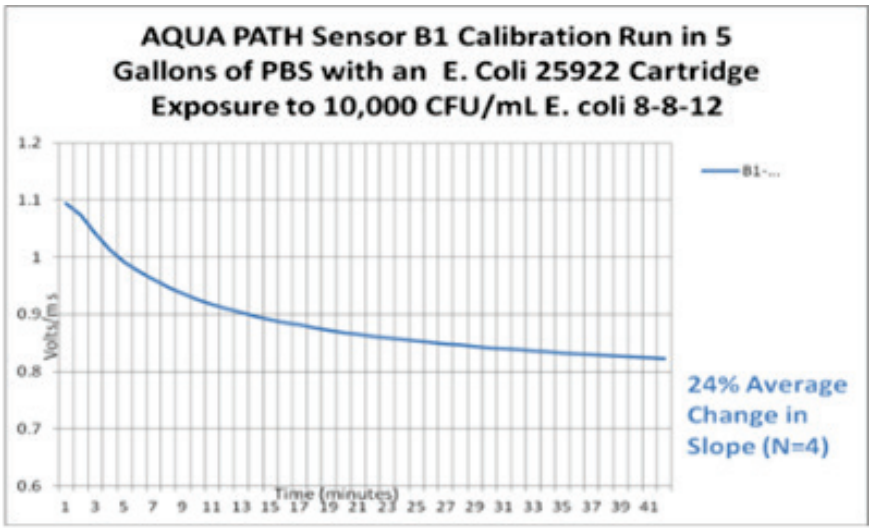


Figure 15. AQUA PATH Biosensing Buoy System In-Laboratory ERDC assessment data obtained directly from AQUA PATH Biosensing Buoy System: exposure to 100,000 CFU/mL *E. coli*.

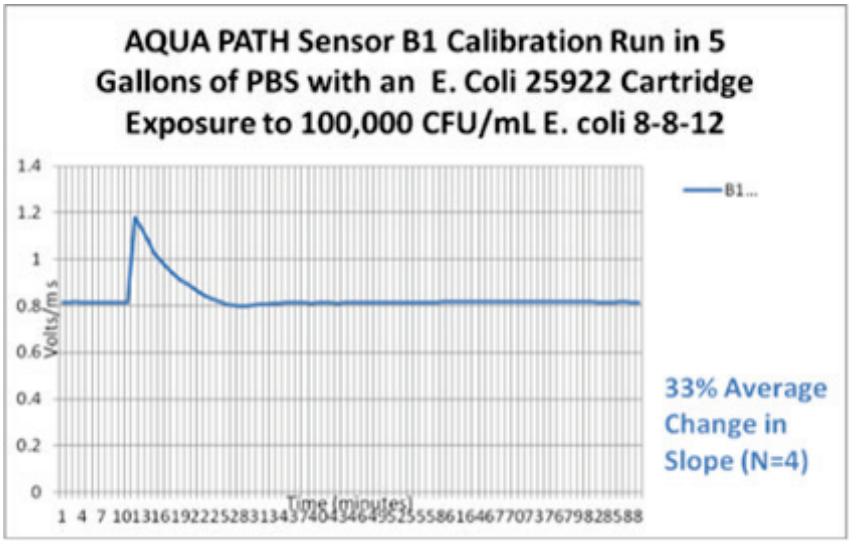
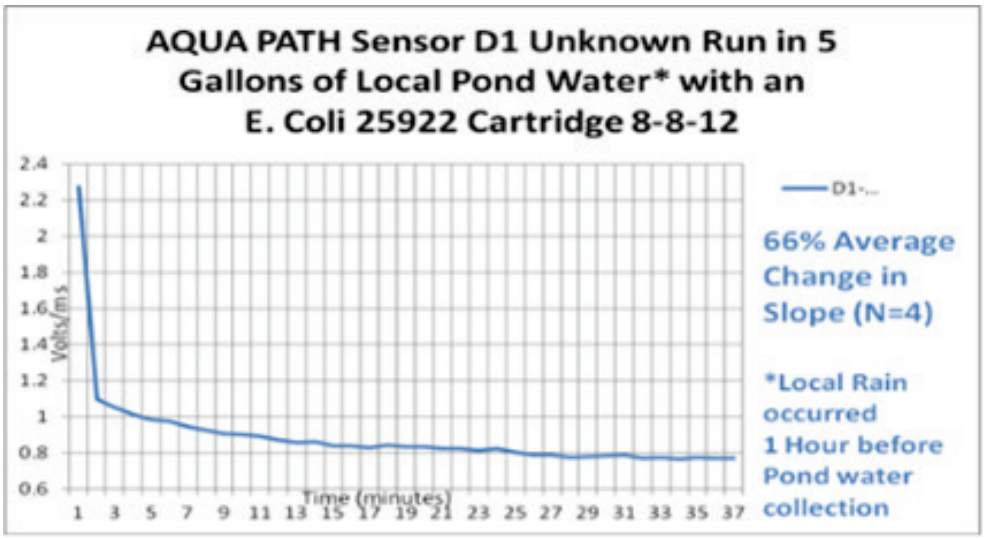


Figure 16. AQUA PATH Biosensing Buoy System In-Laboratory ERDC assessment data obtained directly from AQUA PATH Biosensing Buoy System: exposure to Local Pond Water containing natural coliforms and *E. coli*.



The team recognized the systems are not immune to false-positive detection. The following are some probable causes for false alarms:

- False Alarms could occur with slopes < 13% change in signal strength; sensitivity level is estimated to be ~100 CFU/mL
- A change in signal strength will indicate a response of change from the sensor providing a positive detection (> 16%) The lowest limit of detection in this set of experiments was 1,000 CFU/mL
- High Turbidity could cause false alarms.

Pre-settlement procedures could be used for turbid waters.

- More robust thin films for sensor construct could be used to reduce false alarm rate (robust in that a thin film that can survive environmental conditions, such that it could have greater temperature stability (~0°C to ~32°C or ~32°F to ~90°F) of air and/or water temperatures, humidity, salinity at a minimum). Also, robust in that it may have the ability to be reused multiple times within the system.

Preliminary in-laboratory testing of the new biosensor construct was evaluated over a course of a series of repeated experiments (N=4). Detection was demonstrated and shows promise of a functional biosensor, but would still need a more in-depth analysis to confirm the usefulness of the biosensor detection elements robustness for field testing. In assessing

the data from Figures 9-16, the results show the biosensor performed to detect the bacteria, however, the biosensor thin films stability were not at the optimum in stability. This is shown in Figure 18 where a baseline was set to be around 10% signal strength with exposure of the system to no bacteria using a PBS substrate. Figure 19 shows the progression of testing with purified water with a 15% baseline established. Figure 20 was the first demonstration of a bacterial dose of 100 CFU/mL of *E. coli* of which the signal strength reached a 13% slope value. This value is acknowledged as being less than the baseline and means the exposure interaction with the biosensor was not functioning to its maximum potential for detection and the biosensor thin film most likely degraded upon use for the tests. Regardless, as testing commenced with greater dosing of the bacteria to 1,000 CFU/mL, the signal strength only went up to 16%. The change was greater by only 1% and was not necessarily what would have been expected. This response prompts for additional investigation into the manufacturing of the thin film construct as well as post manufacturing stability. Figure 22 shows the results of a greater dose of bacteria at 10,000 CFU/mL of which the signal strength reached a 24% slope value. The biosensor demonstrates that bacterial interaction is occurring with the thin filmed active surface area to measure the signals. The signal strength number is increasing some, but not as much as expected still. Again, this is thought to be due to how the active surface components of the thin filmed biosensor is functioning and could be due to degradation before use. Figure 23 shows the highest value tested in this series of tests with a dose value of 100,000 CFU/mL and a signal strength of 33% slope value. Upon assessment of the known dose values of bacteria the study included looking at a dose of an unknown value, which was a collected pond water sample from a local pond. The pond received rainfall and runoff at 1 hr before the pond water sample was collected. Figure 24 shows this test of the biosensor with a signal strength of 66%, which indicates the bacterial binding occurred and when it did the numerical comparison to the previous testing indicates that the pond water sample was comprised of bacterial estimated numbers that were twice as high as the 100,000 CFU/mL dose.

Testing of the new construct of the AQUA PATH Biosensing Buoy System in August of 2012 showed positive outcomes and improvements from the first AQUA PATH design. Results from Figures 9-16 of the exposed biosensor thin films suggested that even though the thin films may have shown some degradation in storage before being used for testing, they still provided a

demonstration of binding and reporting the values within less than 60 min. In most cases, it is important to note that it was less than 30 min.

A change in signal strength indicates a response change from the sensor providing a positive detection (>16%). The trend shows the greater the concentration, the faster the detection time. The detection of the concentration is not fully understood, but would be useful for rapid responses needed for presence/absence water quality testing with the AQUA PATH Biosensing Buoy System. At a minimum, these initial tests demonstrate the current prototyped AQUA PATH Biosensing Buoy system could be used as a test to replace the 16 hr IDEXX Inc. Colisure® test. It was recommended that the new system go through a rigorous test and evaluation procedure to be able to have it listed as a test available to the military via the Army Test and Evaluation Command (ATEC) and civil via the EPA water and standards communities pending funding to support such an effort. The IDEXX Inc. Colisure® test is the classical test it was compared against. Discussions occurred with the U.S. Army Public Health Command, GVSC (TARDEC), the Quartermaster, G4, and ATEC for the possibility of this to be considered in the future. Figures 10-16 show results from the initial laboratory tests. Figure 10 demonstrates a control medium with no targeted *E. coli* sensing thin film or bacteria present in solution. This solution, phosphate buffered saline (PBS), was introduced to provide a base value of the systems detection mechanism functioning. Figure 11 demonstrates another calibration control solution to the system using 18 MΩ for a water exposure only test, again evaluating only the system sensor construct functionality and mechanism. The following subsequent tests did include *E. coli* and the various serial dilutions (Table 2) as shown in Figures 12-16, with Figure 16 showing exposure to the unknown waterborne bacterial load from the local Pond water sample (collected August 2012). Each graph shows an increasing percentage of slope along with each increase in concentration of known *E. coli* bacterial load and also with the greatest bacterial load from the unknown local Pond water sample. As noted on Figure 16, it did rain 1 hr before collecting the local Pond water sample. Local storm water runnoff, as in any typical case related to this, can cause the bactieral loads to increase significantly in surface waters.

3.4 Third-party laboratory analyses*

HP Environmental, Inc. was the third-party laboratory that tested the various solutions, biosensor cartridges, and controls regarding various exposures to *E. coli*. Figures 10-16 show data that reported directly from the systems as the tests were being conducted. The comparison third-party classical tests that took more than 18 hr or more to retrieve was not shown (it was agreed upon between the Government and HP Environment Inc. to provide the results of all comparison testing within one week of taking the samples for evaluation). Samples taken were from the same five gallon containers and the unknown Pond water samples. A shortened version of the experimental design, classical tests, and the data are provided in Figures 17-20. The certificate for conducting these studies were labelled for tracking the sample analyses: Certificate No.: 997 DCLS Lab ID: 00156: August 2012. Methods used were the SM9223 Colilert, *E. coli* SM9223 Colilert, Total Coliform, Membrane Filtration, Colilert, Biolog.

HP Environmental, Inc. assisted with sample collection that were taken back to a laboratory in Herndon, VA for analysis (Table 4). They conducted analyses and returned results within a week. Sample collections were limited by the following factors: (1) the number of AQUA PATH Biosensor Buoy Systems available, (2) the number of sample cartridges that were made for the testing, and (3) the limit of time to conduct the tests to enable the third-party to test the numerous samples within a reasonable time frame for data collection.

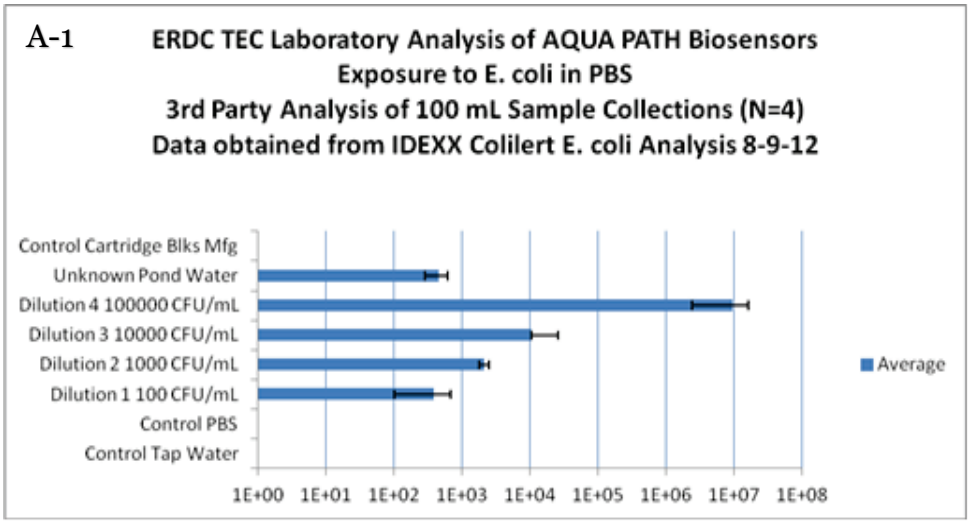
For Figures 17-20, the X-axis is log scale for reporting the number of *E. coli* specific 25922 or *E. coli* broad (Coliform) present in the aqueous solutions. The Y-axis consists of the various types of *E. coli* concentrations or controls. All errors are reported within one standard deviation.

The data (collected by HP Environmental, Inc.) show that the sensor cartridges demonstrated positive detection of the targeted capture agent *E. coli* specific 25922 or *E. coli* broad ((encompassing a broad range of Coliforms) at the various dilutions (concentrations) exposed to the AQUA PATH Biosensor Buoy Systems. The controls were all negative when exposed to solutions such as the PBS, tap water, or a blank cartridge as expected and were in agreement with in-house tests. The cartridges did

* Note: ERDC-TEC is now known as ERDC-GRL.

respond to various serial dilutions based on a logarithmic scale from 100 CFU/mL to 10^6 CFU/mL as well as the unknown pond water sample which also agreed with the in-house tests. The test arrangement can be seen in Figure 9. Data are shown in comparison of the systems to classical USEPA approved water quality standard tests including the IDEXX Inc. Colilert *E. coli* Test, IDEXX Inc. Colilert Coliform Test, Membrane Filtration *E. coli* Test, and Membrane Filtration Coliform Test, respectively.

Figure 17. A-1; B-1. Exposure to *E. coli* specific strain 25922 in PBS, 100 mL sample collections of solution. Sonicated Biosensor Cartridge, respectively. IDEXX Inc. Colilert analyses for both. The X-axis is log scale for reporting the number of *E. coli* strain 25922 present in the aqueous solution. The Y-axis consists of the various types of *E. coli* specific strain 25922 concentrations or controls.



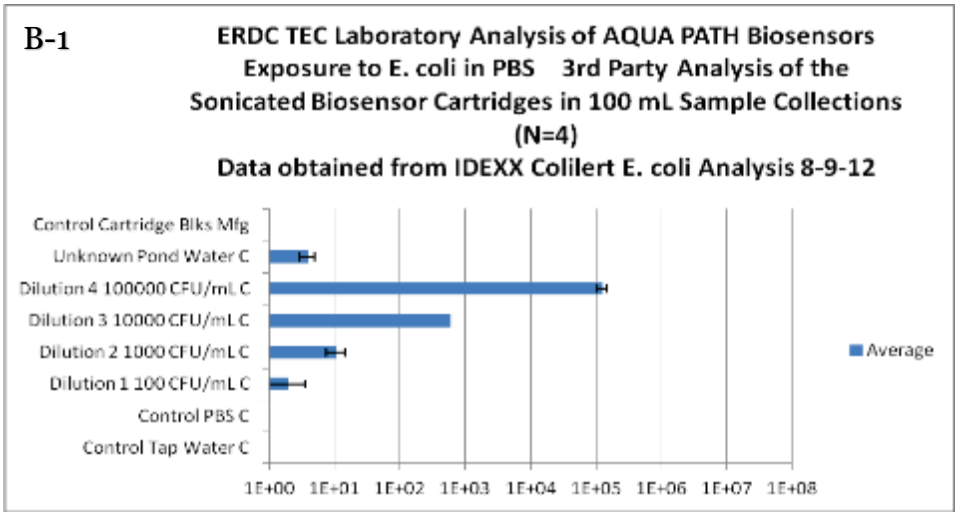
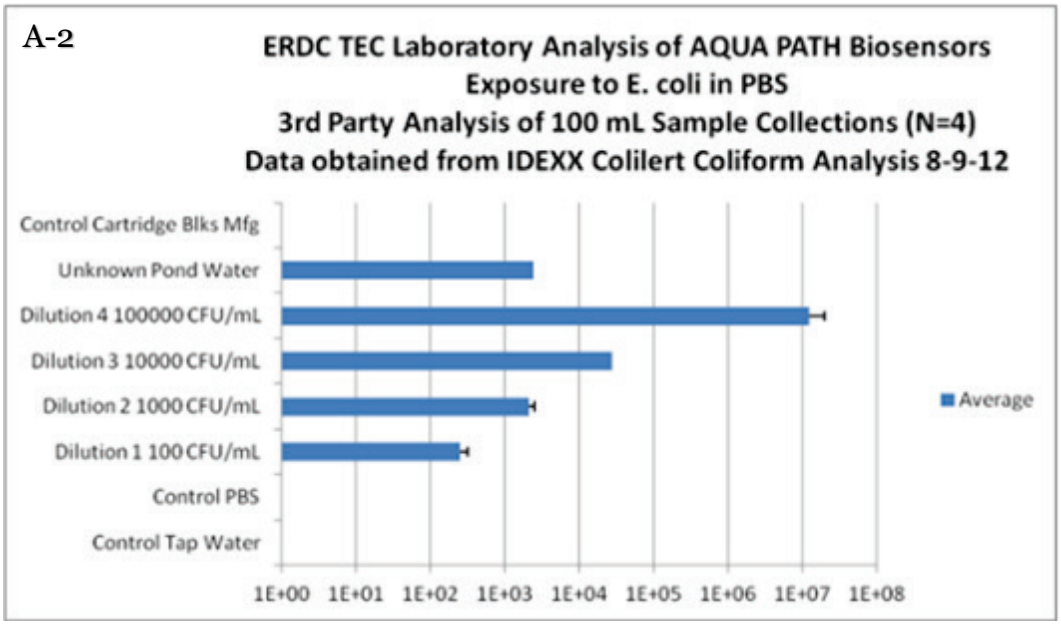


Figure 18. A-2; B-2. Exposure to *E. coli* broad (encompassing a broad range of coliforms) in PBS, 100 mL sample collections of solution. Sonicated Biosensor Cartridge, respectively. IDEXX Inc. Colilert analyses for both. The X-Axis is log scale for reporting the number of *E. coli* broad present in the aqueous solution. The Y-Axis consists of the various types of *E. coli* broad concentrations or controls.



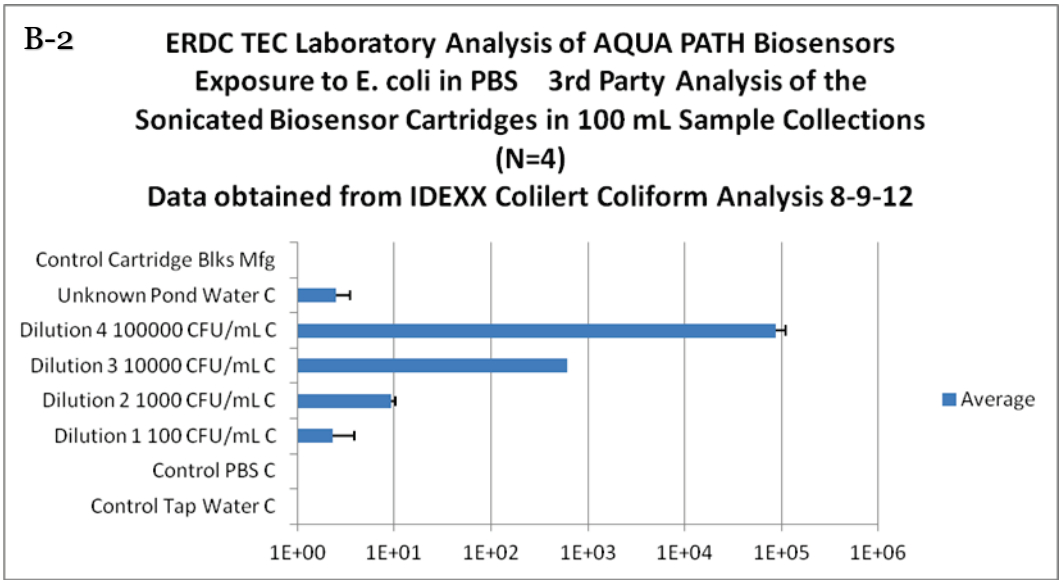
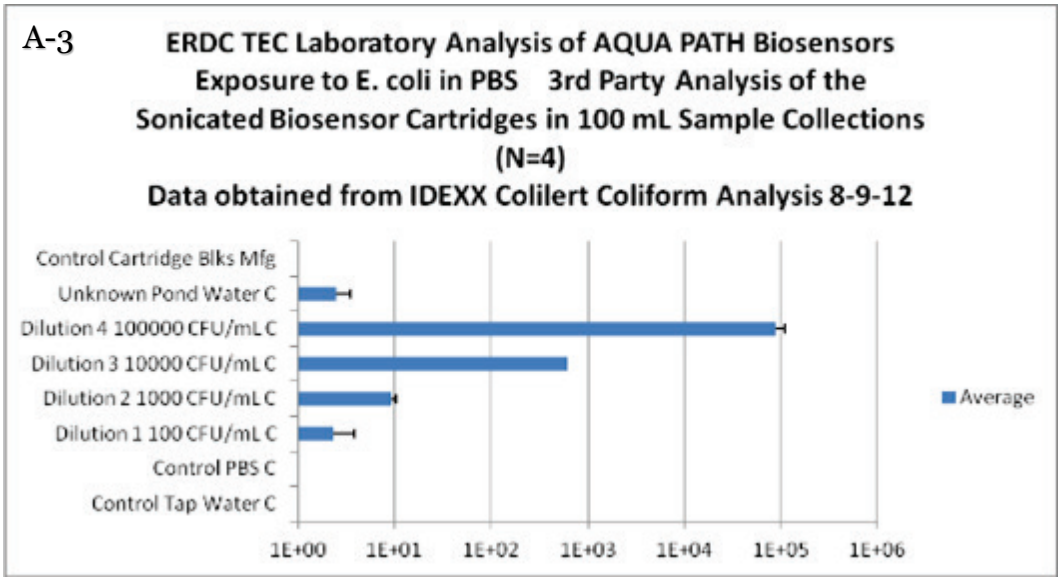


Figure 19. A-3; B-3. Exposure to *E. coli* specific strain 25922 in PBS, 100 mL sample collections of solution. Sonicated Biosensor Cartridge, respectively. IDEXX Inc. Colilert analyses for A-3 and Membrane Filtration B-3. The X-Axis is log scale for reporting the number of *E. coli* specific strain 25922 present in the aqueous solution. The Y-Axis consists of the various types of *E. coli* specific strain 25922 concentrations or controls.



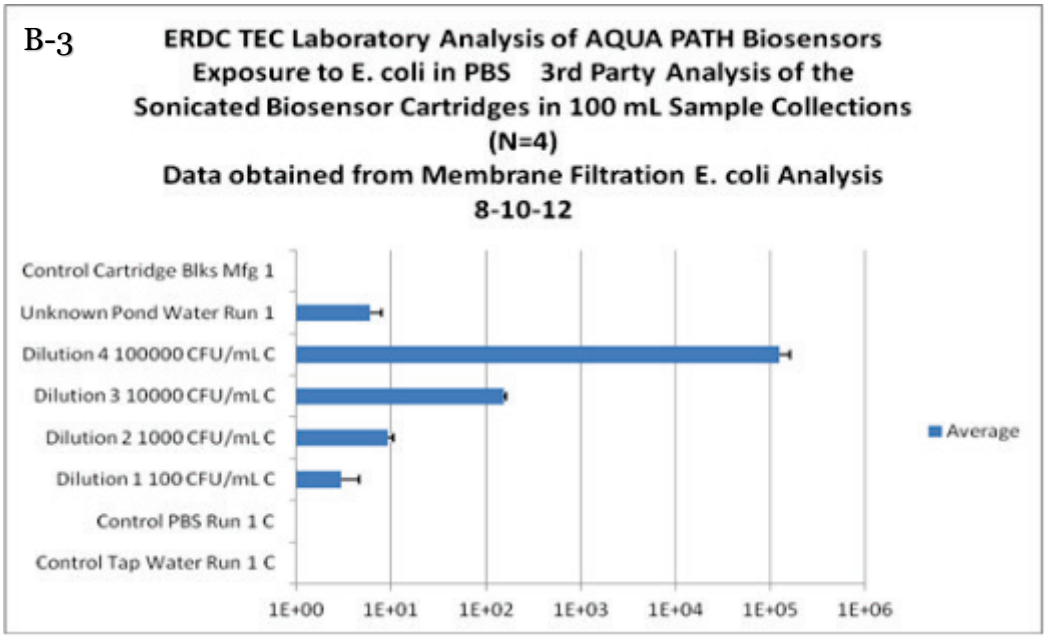
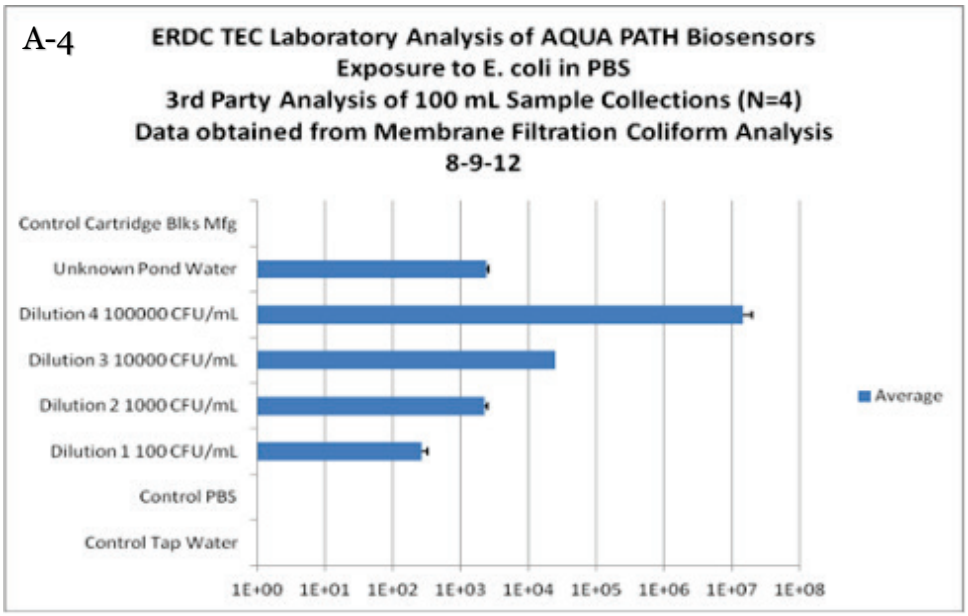
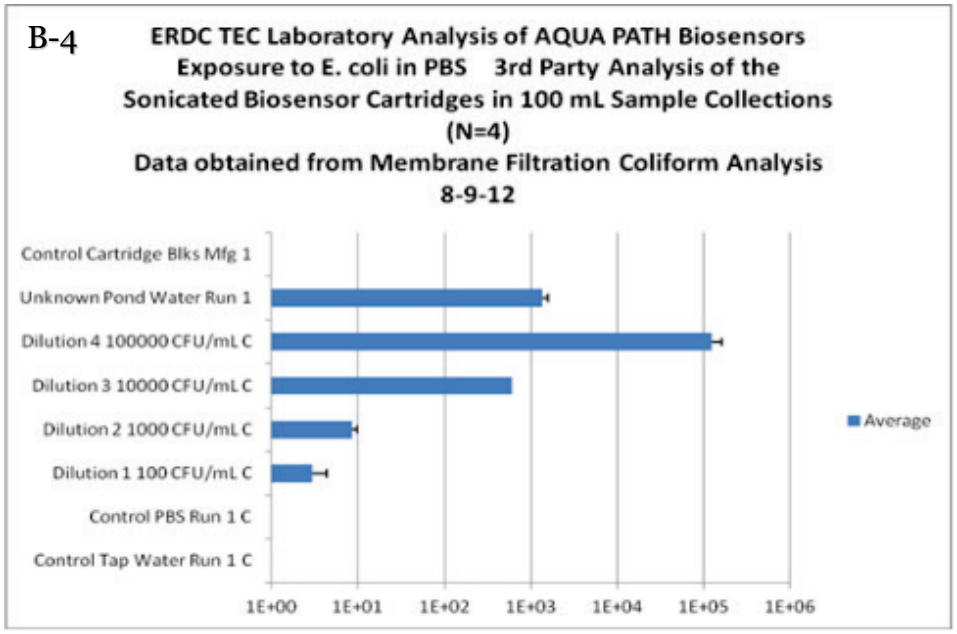


Figure 20. A-4; B-4. Exposure to *E. coli* broad (encompassing a broad range of coliforms) in PBS, 100 mL sample collections of solution. Sonicated Biosensor Cartridge, respectively. Membrane Filtration analyses for both. The X-Axis is log scale for reporting the number of *E. coli* broad present in the aqueous solution. The Y-Axis consists of the various types of *E. coli* broad concentrations or controls.





The results of the third-party laboratory tests and evaluations met and exceeded expectations. The AQUA PATH Biosensor Buoy Systems demonstrated that they were able to detect waterborne bacterial indicators. Not only did the systems do this, but this test showed, for the first time, that the systems are capable of remotely detecting bacteria and

reporting the monitored concentration values in under one hour and sometimes under 30 min based on the initial load of bacteria to the systems. The test best suited to compare the capability of the AQUA PATH Biosensing Buoy System is known in the water testing community as a presence/absence test. Further testing is needed to decipher a way to rapidly detect the confirmation of the quantity of bacteria more thoroughly or quantitative analytics of the systems. A future area to improve upon is the speed of bacterial load detection. The values achieved demonstrated rapid presence/absence of responses, but with non-specific binding occurring on the sensors thin films did occur. This means that the *E. coli* specific test cartridges evaluating the pond water samples had binding occurring to other Coliform bacteria other than for the specific *E. coli* strain 25922 it was designed for. This confirms, that for this project, the biosensor cartridge thin film components would need additional development to improve upon their ability to specifically test for a specific indicator bacteria and in doing this also maintain their stability to survive the natural conditions when exposed in the environment or maintained in storage. More work needs to be completed on improving selectivity and sensitivity of the biosensor mechanisms. Unfortunately, in the initial design used in this project it was thought that the antibody used would allow for surface interactions to provide a displacement of the target bacteria with protein to protein interactions. This interaction was recognized as a surface protein that was not as specific as it was first thought to be. The modification may need to be made at a greater level of biochemical interaction such that a genetically related interaction with the target of interest, greater than a protein to protein interaction between the biosensor antibodies or other marker, would need to be set at the target of interest to allow for a stronger binding interaction or trigger of the biosensor indicating a positive detection. In doing this, it would allow for remote monitoring to be greatly enhanced regarding the systems capabilities for the civil works and military missions.

3.5 MDE thin film stability and function testing in the laboratory

Originally, glass cover slips or glass thin films were developed for use with the biosensors MDE designed by Sporian Microsystems, Inc. and yielded baseline results for the initial prototyped AQUA PATH Biosensing Buoy System as shown in Figure 8. However, thin film designs have been overall improved upon over the past few decades, so the art of biological detection using thin films has demonstrated the ability to provide greater stabilization of the bio-detection capability within biosensor technology.

This also shows improved response to fluctuating temperatures of the environment and stability within natural waterways (Tvarozek et al. 1998; Anderson et al. 2012; Saxena and Bikas Das 2016). Upon testing with the MDE glass thin film, ERDC's in-house scientists and engineers collaborated with Nanosonic, Inc. to functionalize newly designed thin films. The results of the information provided in this study focuses mainly on the glass thin film MDEs.

The glass thin film material was used in this part of the study to contain a covalently linkable amine group. The amine groups were used to permanently attach an antibody to the glass thin film surface. The method and technique is applicable to plastic and possibly metal surfaces, but would need to be investigated further to confirm. Attachment chemistries also include carboxyl (-COOH), mercapto (-S), hydroxyl (-OH) and other linkage chemistries in addition to the amine (-NH3) group.

The glass thin films were cleaned by sonification in 50% methanol/water and then in chloroform. First, the glass thin films were cleaned in acid to remove all residue and water using piranha solution (70% sulfuric acid, 30% hydrogen peroxide (30% w/v) v/v). The glass thin film was rinsed vigorously in water and functionalized with 2% (3-Aminopropyl) triethoxysilane (APTES) in acetone (v/v) for 30 sec. The surfaces were then rinsed in water and dried at 37°C in an oven overnight.

Antibody attachment was accomplished using glutaraldehyde, an amine to amine cross-linking agent. A 1% v/v glutaraldehyde solution was applied and reacted for 90 min at 4°C with protection from light, washed with deionized (DI) water, and then an antibody was applied. The antibody was reacted for 90 min under the same conditions and then washed off. The surface was blocked using a solution of 0.2 M TRIS and the glutaraldehyde bond was made irreversible with 10 mM sodium cyanoborohydride. The reaction was conducted at room temperature for 60 min and the slides were washed with 1x PBS and stored in 1x PBS until use, which is used for preservation purposes. Other molecules, such as sugars or small molecules, could be stored in water or stored dry. Later, ERDC and Nanosonic, Inc. worked together to design a non-glass thin film to incorporate the antibody binding procedure in the build out of the thin film itself via a proprietary process to improve stability and possibly sensitivity of the thin films. These thin films bound to the biosensor manifold and the biosensor turned on upon binding with the bacterial

target of interest once the bacterial target flowed through the system and was exposed to the thin film comprised of the antibody detection mechanism. The glass thin films originally produced, reported via an opposite affect where the biosensor was always on and shut off when detection occurred. The new design proved to be more stable, bound to targets of interest and was reusable multiple times, yet still did not yield specific binding as required (Anderson et al. 2012). Some initial verification test attempts were run on the captured bacteria to provide data showing that non-specific binding was occurring. These tests included cross testing the cartridges against other known bacteria (i.e. a cartridge made for the detection of a specific *E. coli* tested against other consortiums of Coliform bacteria; data not shown). This metric allowed for the determination of specificity of the senor thin film cartridges. Verification was run for the design of the sensor thin film cartridges, (data as shown in, Anderson et al. 2012), showing various dye interactions such as with SYTO green, PicoGreen, or Vybrant Ruby staining and fluorescence microscopy techniques to validate binding of the bacteria and design of the thin films. The successful construction and demonstration of a loss of signal fluorescent biosensor was accomplished. The surrogate antigen was successfully generated using formaldehyde fixation and stained with both PicoGreen and Vybrant Ruby fluorescent dyes. The sensing surface was shown to exchange live target for surrogates and can be used for detection of *E. coli* target levels of 100 CFU/mL. Preliminary tests showed the sensors were sustainable for 300 sample reads at an interval of 1 min per point (Anderson et al. 2012).

3.6 Testing of specific binding agents in the laboratory and field

Initially, cell cultures were refreshed and grown in Tryptic Soy Broth (TSB) overnight the day prior to testing. On the day of testing, the culture was refreshed 1:10 into TSB and grown for four hours before spiking for testing. The four hour growth of a 1:10 culture of *E. coli* (ATCC #25922) produces a culture of approximately 10^8 CFU/mL. Dilutions were made 1:10 and the appropriate volume of dilution (1.5 mL) was used for establishing the required CFU/mL for testing. The 1.5 mL sample of cultures were briefly centrifuged to remove the excess TSB, and then stained with PicoGreen DNA dye for five minutes at 1x final concentration of dye. The samples were then pelleted again to remove excess dye and finally re-suspended in water before spiking. The cells fluoresced a strong green color (532 nm) upon 450 nm excitation after being dyed with PicoGreen. The Sensor cartridges were run for 60-120 min at the experimental conditions in order to achieve

known binding events. Images of the sensors prior to and after testing were taken using a Cy5.5 and Green Fluorescent Protein (GFP) filter set, which are compatible with the PicoGreen dye. Sensor data was recorded using the AQUA PATH Biosensor Buoy System and gateway to assess the capability of the system for detection and at what threshold and time a positive identification can be made.

The sensor constructs were tested without target in 1x PBS and in tap water to generate baseline fluorescence signals. The sensor's thin films were post stained with PicoGreen to allow visualization of the surrogate sandwich structure remaining on the sensor surface. The surfaces were tested between 60-120 min against a known concentration of target *E. coli* in 15 L of 1x PBS at room temperature. MDE glass thin films were imaged with a Cy5.5 filter prior to and after testing, with imaging using a GFP filter prior to testing to assess the binding ability of the MDE glass thin film sensor to the antibody bacterial ATCC target.

The antibody was successfully attached to glass thin film surfaces using silanization via ATPES and glutaraldehyde attachment. Constructs were stable for at least one month before use and stable for at least four months with target bound. Upon staining, all slides showed the ability to capture bacterial targets of interest from environmental water samples and thus have the ability to detect. All triplicates of all samples showed positive identification for *Enterococcus* and Coliform, respectively, however more work needs to be completed to help validate and verify specific binding of targets of interest. Non-specific binding was shown to occur with some of the cartridge tests. In other words, if the target of interest was *E. coli* specifically, the target would also capture other Coliform bacteria, which means the target cartridge or thin film sensor within the AQUA PATH Biosensing Buoy System was not as specific as originally designed. Additional testing and design factors would need to be completed to help provide a better target sensor thin film cartridge capable of detecting the biological or bacterial agent of interest specifically.

The attachment method used is a simple and robust technique that holds great promise for use in fluorescent work. Background fluorescence decreased or quenched as there were no additional linkages, proteins, or layers to bind dyes or fluoresce. The glass thin films constructed are thermodynamically stable, providing a future platform for attachment of a wide variety of capture molecules which include, but are not limited to:

sugars, proteins, nucleic acids, phage display, molecular imprinted polymers (MIPS), other antibodies, or single domain antibodies (Ligler and Taitt 2008; Turner et al. 2014). This new design could be utilized within the constructs thus far of the AQUA PATH's Biosensing Buoy System electro-optical architectural design. Some modifications to the electro-optical manifold/architecture can be completed in-house to accommodate the new sensor design. Once complete, the in-house design can be re-evaluated pending further support.

MDE glass thin film sensors were tested to confirm and characterize the binding and detection ability of the cartridges constructed by Sporian Microsystems, Inc. within the AQUA PATH Biosensing Buoy System's detection area (Figures 21-34). Sensor functionality was confirmed on a Leica fluorescent microscope and Olympus digital camera for image capture using Cy5.5 and GFP filter sets. Exposures for each sensor to target bacteria was a minimum of 30 min, pre- and post- as designated in the figures.

Figure 21. Buoy B4 Sensor testing against distilled water with Cy5.5 filter.

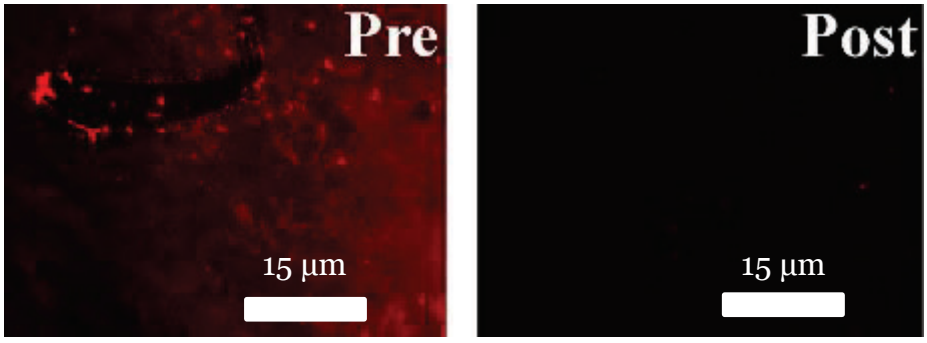


Figure 22. Buoy D1 Sensor testing against distilled water with Cy5.5 filter.

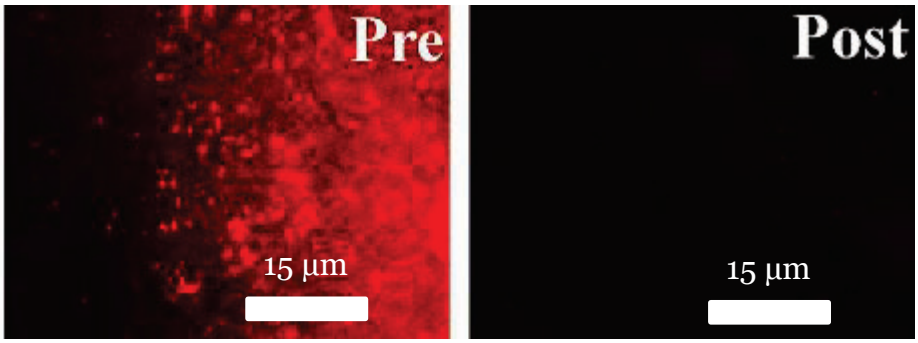


Figure 23. Buoy B2 tested in 1x PBS with Cy5.5 filter.

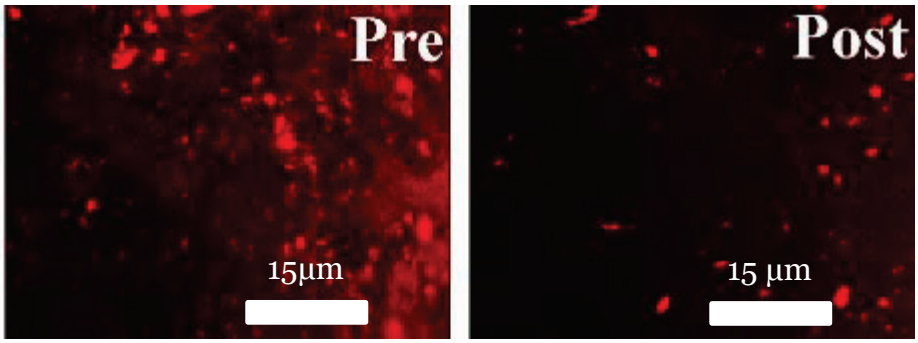


Figure 24. Buoy B4 tested in 1x PBS with Cy5.5 filter.

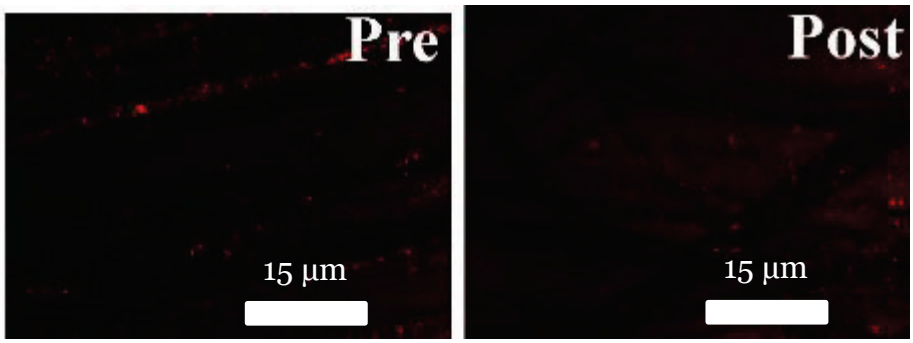


Figure 25. Buoy D1 tested in 1x PBS with Cy5.5 filter.

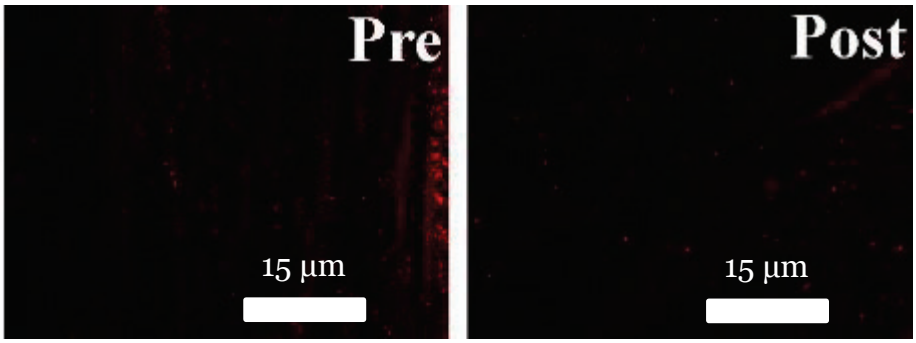


Figure 26. (a) Buoy B4. (b) Buoy D1. Images of the sensor using Cy5.5 filter (red) and GFP filter (green) after 100 CFU/mL testing. (red dots are dead cells and green dots are live cells).

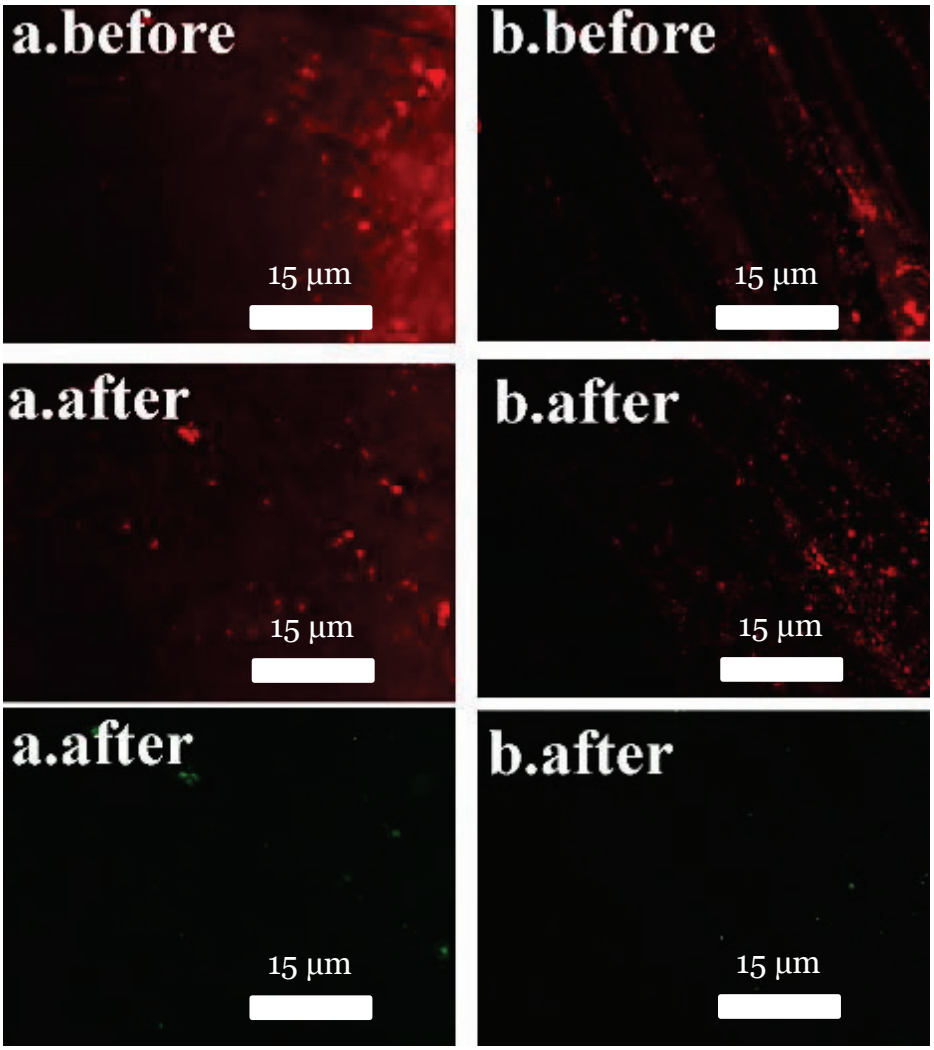


Figure 27. (a) Buoy B4. (b) Buoy D1. Images of the sensor using Cy5.5 filter (red) and GFP filter (green) after 1,000 CFU/mL testing. (red dots are dead cells and green dots are live cells).

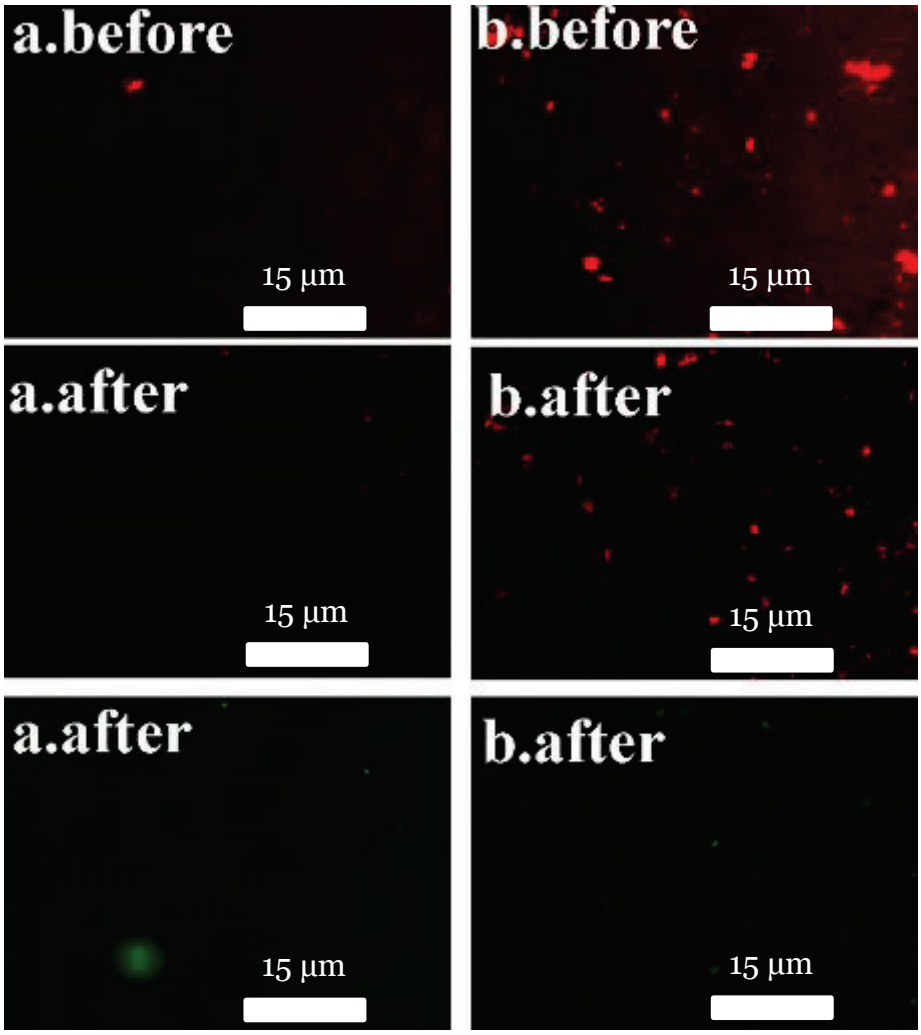


Figure 28. a1-3: Buoy B4. b1-3: Buoy D1. 1) Cy5.5 filter of the sensor before testing. 2) Cy5.5 filter of MDE after 10,000 CFU/mL testing. 3) GFP filter of the sensor after 10,000 CFU/mL testing. (red dots are dead cells and green dots are live cells).

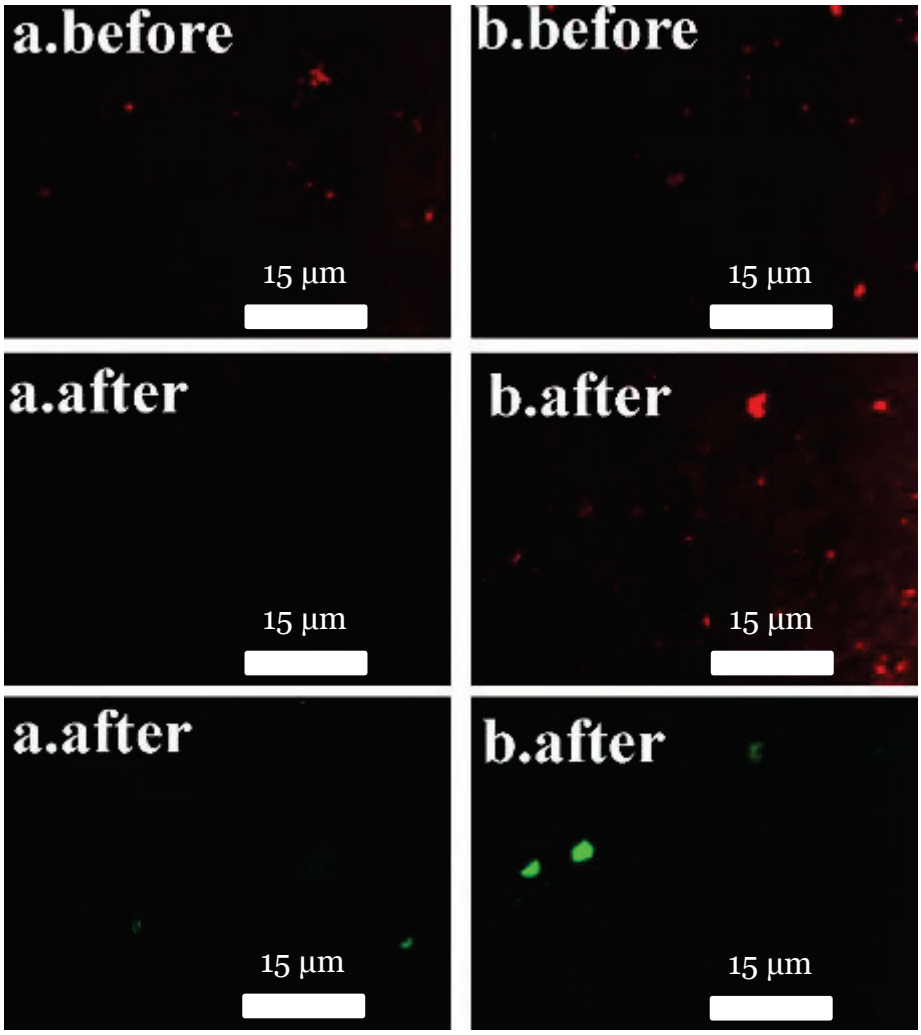


Figure 29. B4 Sensor. Cy5.5 (Near-IR) and GFP (Green) filter images after tap water exposure.

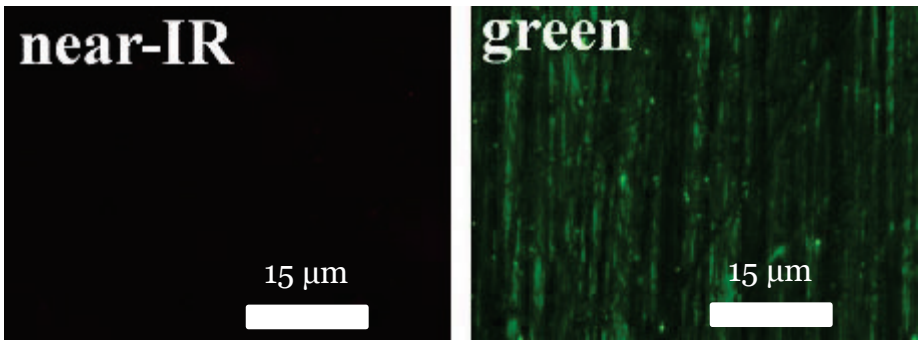


Figure 30. D1 Sensor. Cy5.5 (Near-IR) and GFP (Green) filter images after tap water exposure.

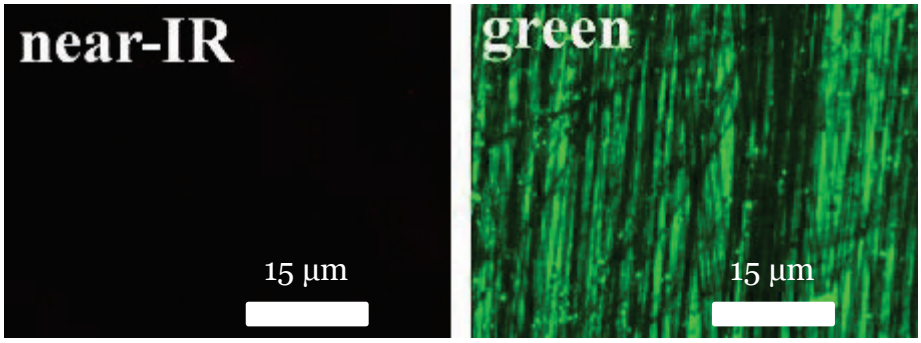


Figure 31. Coldero Dam (Luzon, Philippines), Enterococcus antibody, PicoGreen stained, 40x. Scale bar represents 26.6 µm, not the 40 µm shown.

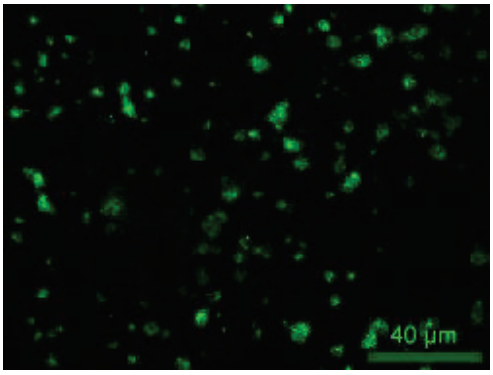


Figure 32. Palawan (near Puerto Princesa Bay, Philippines), Enterococcus antibody, PicoGreen stained, 40x. Scale bar represents 26.6 µm, not the 40 µm shown.

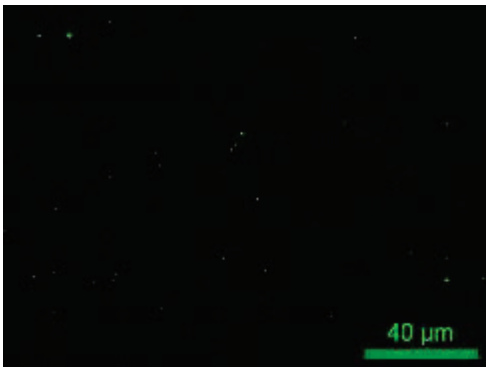


Figure 33. Coldero Dam, Coliform antibody, PicoGreen stained, 40x. Scale bar represents 26.6 µm, not the 40 µm shown.

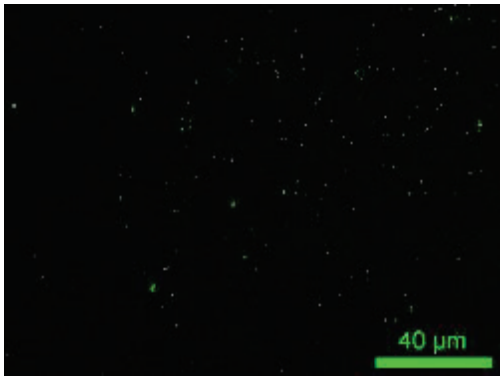
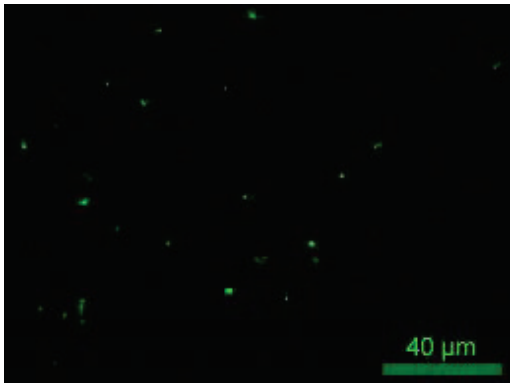


Figure 34. Palawan, Coliform antibody, PicoGreen stained, 40x. Scale bar represents 26.6 µm, not the 40 µm shown.



Looking at a micro-scale of the biosensor thin films, the study shows more information about what is occurring at the molecular surface of interaction. The use of tap water is known to degrade the biosensor active surface sensing elements. Tap water is typically treated with a number of chemicals in order to kill bacteria and other microorganisms. Additionally, it may contain other undesirable contaminants like toxic metal salts, hormones and pesticides, or it may become contaminated by chemicals or microbes within pipes (e.g. lead, bacteria, and protozoa) (Marciano-Cabral et al. 2010). This may be the reason for the sensor surface degradation when each is exposed to tap water. Testing in PBS is unrealistic in the field, but does prolong the sensor construct.

Normalization of sensor constructs is difficult as the pre-testing near-infra-red (IR) images showed, the background signal is inconsistent. Cartridges were shown to have striation of protein on the sensor surface, general background signal, or inconsistent surface decoration of the

supposed cell-antibody constructs. IR was needed for sensing the target as this construct was designed. In testing, it appears that an increase in near-IR signal (Figures 28-30) occurs or is concentrated after testing. This could be explained when the antibody originally attached to the surrogate's captured target. The live targets are the intended binding targets of the antibody used in the original sensor construct. The surrogate used was intended to only weakly bind to an antibody. The sensor was constructed so the surrogate and the labeled antibody would displace together, capturing the live target and reducing the fluorescent signal. The live target instead bound to the displaced surrogate's antibody, but concentrated the labeled antibody. This can be seen by green and red appearing in the same locations in Figures 34-36. The nature of the current sensor construction could be simplified greatly, reducing this effect, simply by using the surrogate itself as the fluorescent source. This could be accomplished by many means such as DNA dyes, cellular dyes, or covalent linkage of fluorophore directly to the surrogate.

This sensor design is not the most robust, but the principle of using a surrogate has potential. Improvements that reduce the complexity of the system would be of great benefit. Covalent attachment of antibodies to surfaces would remove background noise and improve system stability. Internally or permanently labeling the surrogate would be another way to reduce system complexity and increase stability. The next series of tests were completed on newly constructed thin films to replace the current AQUA PATH Biosensor thin films. When using covalent attachment of antibodies with a different model for antibody construct design, the thin film sensors showed improvement for sensor stability and sensitivity.

The stability of the cartridges was analyzed based on visual confirmation. The DI water runs show a decrease in the initial 700 nm near-infrared (n-IR) signal after exposure. The post PicoGreen stained surface reveal striations on the surface. This pattern was seen in the Cy5.5 filter on other sensor surfaces prior to testing. The negative controls and exposure to distilled water and PBS helps to first explain what would occur to the sensor thin film cartridges without any exposure to bacteria present in the samples. As stated previously, exposures for each sensor to target bacteria was a minimum of 30 min, pre- and post- as designated in the figures.

All images show high binding. The capture area was uniformly decorated with antibodies and bound 100-400 cells per image. Figures 21 and 24

show binding of cell clusters from the environment. This binding underestimates the bacterial concentration with a loss of signal, but provides very strong interactions between target and the antibody. Figures 22 and 23 are more characteristic of laboratory testing, where cells captured were in an individual state. The lack of background signal was evidenced by the dark black background of the image. This lack of signal was a benefit of using the amine conjugation instead of a protein layer for attachment. Further testing occurred on the serial dilutions, with natural pond water from the GMU S&T Campus Pond (Manassas, Virginia).

A decrease in signal intensity is seen when comparing Figures 21-26 in all post images when no target is present in all buoys when tested. A more pronounced decrease is seen when distilled water is used v. 1x PBS. The PBS likely retained more signal for many factors that may include common generation conditions as the sensor, lack of dissolved metals, chlorine (if tap water is used), sulfur, etc. Further research and development was then conducted towards stabilizing the biosensor design to maintain the sensor apparatus. Unfortunately, this sensor design was not as fruitful as first thought. Some experiments were conducted with the remaining cartridge designs to see the effects when exposed to serial dilutions of the waterborne pathogen indicator, *E. coli*, and some field source waters along with some newly designed thin films (Figures 31-34). This research and development led to the collaboration with Nanosonic, Inc. and new thin films for the biosensor for the AQUA PATH Biosensing Buoy System.

The response to spiked *E. coli* was tested in PBS to give optimum conditions for stability and selectivity of the antibodies on the sensor. Dose response was tested at 100, 1,000, and 10,000 CFU/mL.

At all concentrations, there were significant unique PicoGreen cells in the GFP filtered images. The general trend over time with exposure of the sensor to tap water, PBS, or challenge targets, is the loss of the near-IR signal. This appears to be the result of the labeled antibody being removed from the surface through diffusion or chemical attack. The tap water is chlorinated, which may quench the fluorphore itself, or lend towards the degradation of the surrogate cells holding the sensor sandwich together. The testing with PBS showed the same trending of loss in signal with time, making a stronger case for a loss of signal resulting from detaching fluorophore complexes. To check whether the antibody-fluorophore

complex or the surrogate was being removed, the post-run tap water samples were dyed with PicoGreen to locate surrogates. The dots and strands/streaks of sensor antibody-fluorophore complex are shown in (Figures 29 and 30).

In both cases, very little red signal is seen, but the red signal does overlap with dyed green surrogates remaining on the surface. These post green dyed images show significant signal resulting from the DNA staining by the PicoGreen. The resulting cells stained on the surface suggest the whole surrogate complex is being removed from the surface. If only the antibody were being removed, then the density of cells would be similar, Figures 29 and 30, green as they are in Figures 21 and 22 pre-inoculation.

The co-localization of the red and green signals in the PBS runs present an interesting scenario of function. The antibodies are not developed to attach to heat inactivated dried cell surfaces, but rather live cells. It is likely the weak binding antibodies attaching the surrogates to the sensor surface are causing binding to the live *E. coli* more tightly. The displaced labeled antibody-fluorophore complexes are concentrating on the bound live cells. This would explain why only some green signals have red signals when overlaid and why some green signals do not. The expected result would be for the surrogate-antibody-fluorophore complex that was removed completely, be exchanged for a green live cell.

The sensors constructs do not appear to be constructed to withstand normal water conditions (e.g. relatively pure water). A number of construction elements may contribute to this weak design and are included as determined from the third-party assessment. These are, but not limited to, poor antibody choice and instability of heat inactivated-surrogate. Optimization was done in buffered solution with high ionic content and/or sensor surface delamination. Antibodies are naturally formed inside host organisms, and the closer the reaction solution is to those conditions (i.e. blood), the better the antibodies will bind. The majority of antibodies used in research are generated in rabbits, goats, sheep, mice, and cows. Matching the binding environment in water samples to the original animal's blood conditions is difficult and will always present a level of difficulty for environmental sensors using them.

In 2012, the team took the AQUA PATH Biosensing Buoy Systems to an invited event to participate within the U.S. Pacific Command (PACOM)

Marine Forces Pacific (MARFORPAC) Experimentation Center [MEC], which were interested partners in water quality bacterial sampling at selected sites as part of Balikatan ’12 (Luzon Island and Palawan Island, Philippines). The team was able to evaluate the biosensors via third-party following the exercise with HP Environmental, Inc. All samples demonstrated a diminished fluorescent signal or positive detection at various levels of detection of *Enterococcus* and Coliforms, which is how the biosensor was set up to determine (a reduction in fluorescent signal) upon detection (Figures 21-30). Data from the Coldero Dam (Luzon, Philippines), Palawan (near Puerto Princesa Bay, Philippines) and samples are provided in Figures 31-34. The biosensors did detect bacteria, mainly Sphingmonoas, a Coliform via non-specific binding, which is associated with environmental bacterial Coliforms. Some non-specific binding to the *E. coli* sensor was seen and this is under investigation for the improvements on the thin film. The DNA fingerprint, data not shown, run in-house at the George Mason University shared ERDC-GRL laboratory, did show the presence of *E. coli*. This was found on the biosensors brought back from the Thailand field water testing.

Sample water was taken (~50 mL) from Palawan, Philippines and at the Coldero Damn, Luzon, Philippines when the glass thin film capture surfaces were submerged. The MDE cartridges were stored for four months before testing. Upon analysis, each capture surface was stained with 5 µL of 1x PicoGreen DNA stain for 5 min. The surfaces were rinsed and then imaged using a GFP fluorescent filter set at 20x magnification with a Leica fluorescent microscope and Olympus digital camera for image capture.

4 Network Communications of AQUA PATH Biosensor Buoy System

Sensor data using the Sporian Microsystem gateway was taken. Sampling was set to run the pump of the system for 9 sec of flow, every 60 sec. With data being recorded every 60 sec for the AQUA PATH Biosensing Buoy System's optical biosensor, the Sporian Microsystem gateway system was allowed to record the data of the sensors in distilled water for five min to confirm communications and function of the data and gateway. Resulting data collected included information such as temperature related internal to the buoy, global positioning system (GPS), and the biosensor. Data was recorded in laboratory tests within 10 ft of the AQUA PATH Biosensing Buoy Systems and field tests ranged from 10 ft to over 1,000 ft. Further research for establishing satellite radio communications would allow for global communication with the system.

5 Conclusion

Before this project started there were few, if any, systems developed for the use of in-the-field biosensing systems in source waters. Although this project was not as comprehensive and in depth towards the scientific analytics, it still demonstrated the feasibility of such a biosensing system is in the realm of the possibility to use in the field for pathogenic bacterial detection. This project began the scientific work that in the end demonstrated the presence/absence of pathogenic bacterial indicators for polluted source waters. It did provide new methods in thin film development, biosensor development, and implementation for use in the source water environment. What started as an idea became an in laboratory "on the bench" prototype to an in the field prototype. The new system demonstrated the capability and has driven small business to develop the systems further for greater study and use in the commercial market. The novelty of the AQUA PATH Biosensing Buoy System is a signal conditioning circuits and sensor control board comprised of firmware/hardware that provides a unique ability to allow the biosensor to act autonomously reporting (less than 30 min) for levels of pathogenic bacteria in source waters. This distinctive system design provides a new measuring technique using novel thin films, advanced electronic hardware and software to capture improved and rapid data collection (less than 48 hr) within the water quality community for total bacterial detection. This type of water resource data collection technique or method has yet to be demonstrated anywhere commercially to the best of our knowledge. Additionally, the AQUA PATH Biosensing Buoy System, test set ups, and exercise participation are shown in the Appendix. The development of the prototype to show feasibility of this is an achievement in the field of environmental sensing related to presence/absence of bacterial loads for monitoring/reporting within source waters. Further analysis should be conducted to evaluate the system against the EPA approved standard developed by IDEXX Inc., who manufactures and distributes the Colisure® 16 hr presence/absence test method for water quality analysts.

The development of this system moves the Army one step closer to being able to provide the Soldier with a new tool to provide immediate notification globally regarding the presence/absence of pathogenic bacterial indicators (< 30 min) of threats or conditions to decision makers of source water supplies and for the homeland or civil works community recreational use water sources and supplies. (Currently, the system

demonstrated a < 30 minute time of detection of high levels of bacterial loading in a source waters. These preliminary estimates were around 1,000 CFU/100 mL). These levels, though not ideal, show promise of rapid reporting and advancement in detection technology located in the field versus laboratory sampling and measurement and would be better for use in a comparative analysis to the EPA approved standard Colisure® 16 hr presence/absence test method. In the future, improved technological advancement in the thin film design for bacterial detection could provide an improved stabilized biosensor that can handle harsh environmental conditions and provide closer to 1 CFU/100 mL or less reporting values which would are more desirable in the water quality bacterial biosensor community. EPA regulations currently hold at 100 CFU/100 mL for the standard in assessing water quality bacterial loads and are measured in a laboratory setting [The standard is a joint publication of the American Public Health Association (APHA), the American Water Works Association (AWWA); and the Water Environment Federation (WEF); APHA 1985; Federal Register 1975; TB-MED-577 2005)].

Innovative advancements in smart sensor-based sensing systems over the past few decades have provided new technologies the ability to closely monitor and map natural environmental occurrences. The ability to fully implement this technology reliably has yet to be further explored within the water quality community. Water quality monitoring typically involves sample collection, and analyses are then performed in a laboratory setting. The results are, at best, presented after an 18-48 hr period. Pretreatment of source waters in advanced countries, including the United States (U.S.), still face adverse effects of unclean source waters from introduction of uncontrolled contaminants, as previously mentioned. These contaminants serve as health risks to recreational water use and drinking water that result in undocumented effects on population exposures. Additionally, there are concerns regarding contaminated drinking water effecting social and economic impacts especially in countries without pretreatment facilities. As research advances for innovative solutions in the developed world, such as this study, placing focus on deploying automated digital sensing systems for reservoirs, recreational area, and other source water locations can be useful for remote monitoring and reporting to reduce the risk of exposure.

The AQUA PATH Buoy System Biosensor's established prototyped design and construction addresses each of these challenges and demonstrates the

potential for future technology to provide a cost savings based on the use of the biosensor system. This was intended to extend the use of the ultra-filtration filters and delay replacement either at a treatment facility system or detached at an offsite location of a treatment system. The buoys were intended to demonstrate a cost savings to the civil community for initial analysis of water quality data collection for recreational beach use or for disaster relief efforts when in-house water treatment facility test equipment is not functional or evaluation at remote locations is necessary. The geospatially-enabled wireless network was intended to be used and applied through the addition of various sensor types and for detection of multiple pathogenic indicating bacteria. The technology for rapid modular construction of bio-chemical capture surfaces is necessary for environmentally detached or remote sensing applications. Reliance on proprietary third-party manufacturing reduces the ability for rapid response, limits the number of detection targets, and ensures high costs for vital monitoring and sensing needs. Collaboration with small businesses led this effort to evolve into a lower cost breakout with innovative prototyped technology. Attempts are still being made to further reduce costs for the highest impact for the use of these systems in monitoring water quality globally. With commercial applications increasing demand, costs could be reduced when the product becomes available and ready for this use.

The test and evaluation of the AQUA PATH Biosensing Buoy System demonstrated some technological advancement towards the state-of-the-art of water quality technology. The results provided in this technical report were useful in formulating an informative outlook for additional research and development of the technology that still needs to be addressed. Evaluations presented in this document summarize the culmination of over a decade of investigations into new developmental milestones in the field of water quality technology assessment for use in civil works and military domains. This research developed better insights towards sensor phenomenology and use in the field of how bacteria bound to targets of interest will aid in the building of such as a biosensor mechanism. The team learned how to increase the survivability and reuse rate of the biosensor thin films in harsh environmental settings. The initial assessment of response to the biosensor was demonstrated. Future analysis will need to involve developing calibration curves of known bacterial concentrations exposed to the biosensor components of the AQUA PATH Biosensing Buoy System. This demonstration serves as an

initial baseline and a measurement of the percent drop in signal strength versus time in minutes as shown in the results of this study.

This technology has helped push the envelope of water quality analyses and aims to move data collection from the analog stage to a digitized stage of collection and data management for rapid decision making. To the best of our knowledge, this is the first time that geospatially enabling networked components were developed to provide near real-time detection of pathogenic indicator bacterial values or levels (presence/absence) in the field. Although the systems were not planned to go beyond commercialization from the SBIR Phase III, they are available from Sporian Microsystems, Inc., Boulder, CO. Additional partnerships with industry and academia should be sought out to provide a greater impact to the systems operational capability towards its optical biosensor analyses related to rapid in-field bacterial detection in source waters. Future experimentation will need to be conducted to aid towards a 1 CFU/mL sensitivity and selectivity in detection of water quality related bacterial indicators, such as coliforms (Total Coliforms), *E. coli* and *Enterococcus*. Experimental development within the laboratory and field is highly recommended and should be sought out by civil works and military interests. Water is and always will be vital to operations.

5.1 Laboratory test discussion

In-house preparation of a laboratory-based test and evaluation were a success showing the various dilution limits of detection with known concentrations of select bacteria. Limitations of the biosensors exposed to tap water, for example, were informative, showing that MDE glass thin film has restraints based on thin film design and prompted additional research into more structurally-durable thin film construction. A third-party state certified laboratory ran water quality analyses that were completed during the duration of the experiments on the biosensors and the water. Tests performed were the Colilert and membrane filtration. The results of these tests are provided within this report. The additional work, shown in Anderson et al. (2012), provides input towards biosensor thin film stability and reusability in the harsh field environment. The collaboration conducted with Nanosonic, Inc. showed promise and paves the way towards future use and reuse of this technology in the field, reducing cost, and providing rapid water quality data as part of this advancement.

5.2 Field test discussion

The military exercises were marked opportunities for field analyses of the systems. Participation within these venues were invaluable to the research and development of the AQUA PATH Biosensing Buoy System. The Cobra Gold (Thailand) systems demonstration was successful because the network communications worked. Cobra Gold was also a learning experience for global shipping logistics, which prepared the team well for the Balikatan Exercise (Philippines). Due to experiences at Cobra Gold, preparation for the Balikatan Exercise was better planned regarding the biosensor logistics and testing.

The AQUA PATH Buoy System Biosensors did detect bacteria in a field environment from source waters, mainly *Sphingmonoas*, (a Coliform via non-specific binding), which is associated with environmental bacterial Coliforms. Some non-specific binding to the *E. coli* sensor, such as *Sphingmonoas*, was detected and because of this, it is recommended that further investigation to improve the thin film be done, pending further funding. The DNA fingerprint ran in-house did show presence of *E. coli* as well on the biosensors brought back from the Cobra Gold field water testing (data not shown).

The Balikatan Exercise was a successful exercise for the AQUA PATH Biosensing Buoy System with the system in its prototyped form. However, there was a large learning curve concerning how to perform the testing with the systems on a global scale. Success was measured by determining if the systems and the biosensors were functional during most of the exercise to capture enough data to determine a research and development value for effective participation in the exercise and detecting bacteria in source waters. Multiple samples were collected on-site and stored for further evaluation within the Philippines and brought back to the U.S. for further water quality analyses. Along with those successes, the prototypes did reveal some areas for improvement. A few systems did not respond well electronically to full days of exposure to the increased temperature fluctuations causing their reporting ability to be diminished. The tropical rainforest environment (i.e., high humidity 90-100% and high temperatures 90-100°F) showed new limits of temperature exposure for the internal buoy construction for the electronics housing.

The test and evaluation process at both exercises were impacted the most from the sheer scale of testing at the global versus the local scale. Global

transport of the temperature sensitive biosensors and the actual systems were addressed at this exercise. Many logistics lessons were learned from the Cobra Gold Exercise and this knowledge was passed on to the following Balikatan Exercise that occurred a few months after. This involved cold transport and shipment of the antibodies closer to the time of the testing, and involved personal transport of the biosensors rather than shipment via commercial carrier. Detection of bacteria occurred at both fresh and salt water locations for testing and evaluation. The bacterial concentrations for water quality analysis were also compared to standard EPA and military-specific testing such as the Colilert-18 hour test that was completed by a Navy preventative medicine unit. The biosensors were brought back for further third-party evaluation by a state certified laboratory that ran Colilert and membrane filtration tests to identify bacteria that were bound to the biosensor chips.

5.3 Project outcomes and recommendations for follow-on work

The mission of the world water community is to advance water quality improvement in order to sustain human life within a healthy ecological system. In the future, water quality techniques, specifically real-time reporting, may be innovative enough to support rapid monitoring and reporting. These innovative techniques could provide automated remote monitoring and reporting rather than the current method of on-site monitoring and reporting by individuals. The AQUA PATH Biosensing Buoy System establishes a new effort to provide field data digitally rather than by collection and using analog laboratory data collection techniques to aid in providing a rapid assessment of source waters bacterial loads at near shore inner coastal locations and via a remote wireless network for digitally derived data dissemination. The prototyped AQUA PATH Biosensing Buoy System did show promise of a functioning bacterial load detection capability to detect the presence/absence of harmful levels of bacterial loads in source waters (EPA standard: 100 CFU/mL), which was geoenabled as well. The system did show promise in development of a unique signal conditioning circuit and sensor control firmware/hardware that allow the sensor to act autonomously, reporting in less than 30 min for levels of pathogenic bacteria in source waters, which included geolocation of where it was sampling from with date and time stamped information. This unique system within the water quality community and type of water resource data collection method has yet to be demonstrated anywhere commercially that the team is aware of. In addition, it is also important to mention that this project did not cover a more in depth

exploration of the replication of testing of the biosensor components, full array of interferences from the environment, and the full remote monitoring/reporting capabilities that the project investigators would have liked to achieve. The systems analysis, at best, is estimated in the test and evaluations to be around 1,000 (CFU/mL) (EPA standard: 100 CFU/mL), but further testing would be necessary to verify these numbers. At a minimum, the AQUA PATH Biosensing Buoy System could distinguish the presence/absence of these harmful bacteria in less than 30 min. Therefore, the system is more comparative to the EPA presence/absence testing rather than the numerical specific comparative testing. There would need to be further research and development in the future for numerical specific comparative testing. At a minimum, these initial tests demonstrate the current prototyped AQUA PATH Biosensing Buoy System could be used as a test to replace the 16 hr IDEXX, Inc. Colisure® test. It was recommended that the new system go through a rigorous test and evaluation procedure to be able to have it listed as a test available to the military via the Army Test and Evaluation Command (ATEC) and civil via the EPA water and standards communities pending funding to support such an effort. The IDEXX, Inc. Colisure® test is the classical test it was compared against. Discussions occurred with the U.S. Army Public Health Command, GVSC (TARDEC), the Quartermaster, G4, and ATEC for the possibility of this to be considered in the future. The system, in its current configuration, can produce an absence/presence confirmation in under 30 min, which is significantly faster for presence/absence testing and makes a leap forward. It is important to note the biosensor thin film research and development that occurred during this project as well. In the future, improved technological advancement in the thin film design for bacterial detection could provide an improved stabilized biosensor that can handle harsh environmental conditions and provide closer to 1 CFU/100 mL or less reporting values, which are more desirable in the water quality bacterial biosensor community. The collaboration conducted with Nanosonic, Inc. showed promise and paves the way towards future use and reuse of this technology in the field, reducing cost, and providing rapid water quality data as part of this advancement.

Many innovative advancements in smart sensor-based sensing systems over the past decade have provided new technological advancements that aim to provide the ability to closely monitor and map natural environmental occurrences via small business investments. The ability to fully implement

this technology reliably has yet to be successful within the water quality community. Current water quality monitoring typically occurs when samples are collected, analyses are performed within a laboratory setting, and results are at best presented at after an 18 to 24 hr period or more likely 48 hr following the Standard Methods for the Examination of Water and Wastewater [The standard is a joint publication of the American Public Health Association (APHA 1985), the American Water Works Association (AWWA 2016), and the Water Environment Federation (WEF 2016)]. Currently, the USGS and the NOAA have mastered and shown the way within the scientific community for broad area mapping and monitoring sensing systems, such as measuring earthquake activity and oceanic Tsunami alerts to river water levels on a National and global scale. As research advances in the developed world, there will be more innovation towards deploying a number of automated and digital sensing systems that the scientific community can one day utilize to monitor water quality parameters within a reservoir or water resource to provide a way to monitor the risk for contamination without taking samples to a laboratory and monitoring directly in the field. The goal for this research and development effort was to push this technology towards a new technique or method of monitoring and reporting, which would allow for USACE-ERDC to collaboratively aid other federal agencies, such as the USGS and the USDA in monitoring the U.S. and global waterways for such water quality parameters. Advancements, such as the AQUA PATH Biosensing Buoy System, can provide faster opening and closures of recreational waters and determine critical threats to the source waters due to non-point source occurrences related to contamination or pollution. Potential application areas for this autonomous system for both civil works and military use can be seen at municipal water and wastewater testing, tactical field use for the military, homeland security, agriculture, transportation industry, and food production.

Further interest and funding was culminated into additional integration of the geospatial wireless mesh network from AQUA PATH Biosensing Buoy System's conceptual design to show the technology can move from analog to a digital tool kit. It was also being tested to determine feasibility into the CCDC GVSC. This work would serve as a basis for additional verification tests and evaluation, however would rely on further funded interest of the CCDC GVSC (WQAS-P program of record) or other civil works and/or military programs related to water quality research and development efforts.

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Appendix A: AQUA PATH Biosensor Buoy System

SBIR Topic written and submitted based on the need for a rapid (Gap/Needs statement of a system capable of less than 5 min detection of biological/bacterial material in the environment with a rapid reporting capability) water quality biosensing system for the Army and the Defense Threat Reduction Agency.

Phase I 2004

Concept of Operations rendering for the AQUA PATH Biosensor Buoy System.

Phase II SBIR Prototype
2007

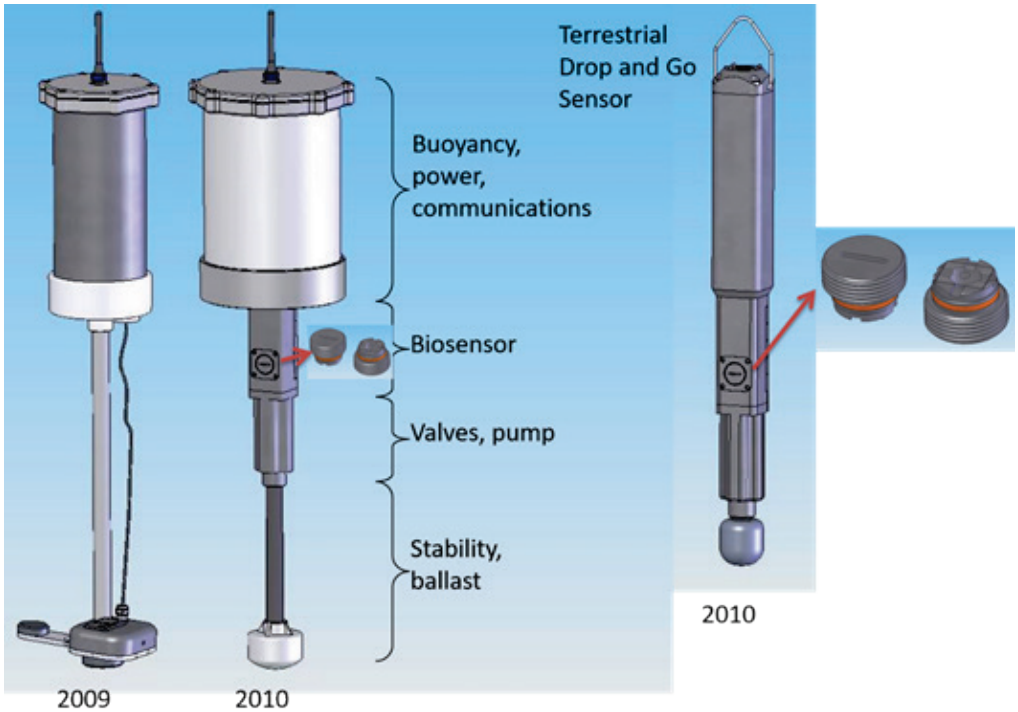
Phase III SBIR Prototype 2
2009

Phase III SBIR Prototype 3
2009
(First Field Ready System for
Test and Evaluation)

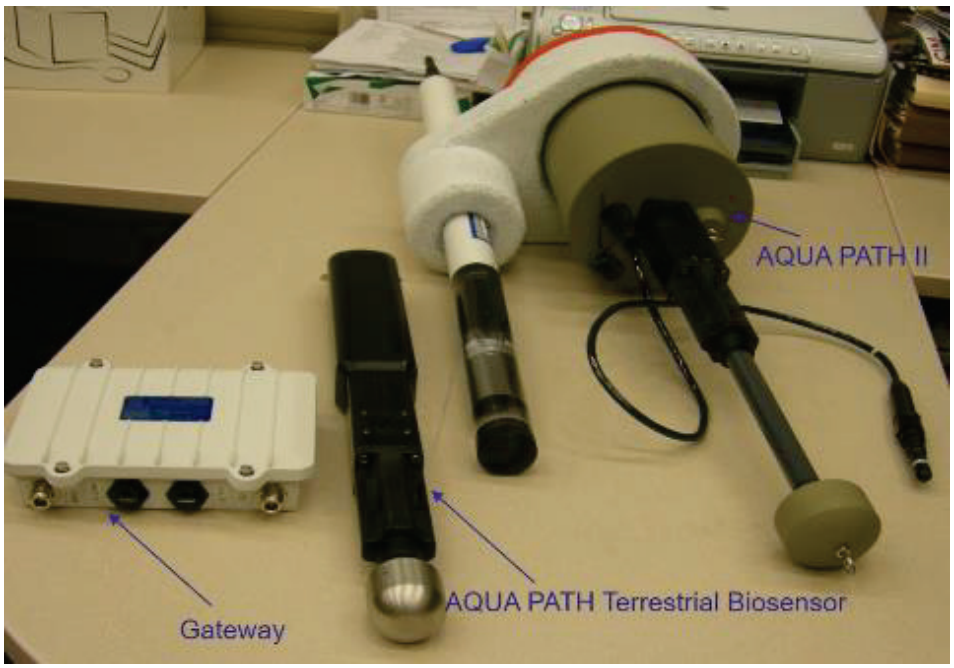
Original prototyped design of the AQUA PATH I Biosensor Buoy System: Biosensor, MDE Cartridge replacement with thin film comprised of fluorescent antibody based bacterial detection material. Storage in Phosphate Buffered Saline (PBS) at 4°C.

AQUA PATH I Biosensor Buoy systems deployed during initial field test and evaluation. Selfridge Air National Guard Base (SANGB), Lake St. Clair, Michigan, 2009. Inset: Left: AQUA PATH Communications Gateway: Right: Buoys floating with foam support and without.

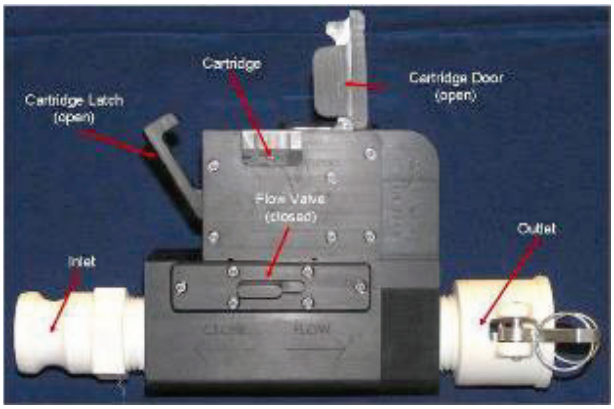
From left to right: AQUA PATH I Biosensor Buoy System, AQUA PATH II Biosensor Buoy System, and the AQUA PATH II Terrestrial Biosensor Well Analysis Unit. Includes updated biosensor module and cartridge designs. (2009-2010).

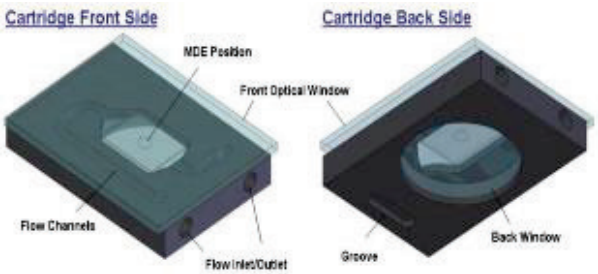


From left to right: AQUA PATH Network Gateway Box. AQUA PATH II Biosensor Well Analysis Unit. AQUA PATH II Biosensor Buoy System with Hach Hydrolab MS5 Sonde Attachment (Sonde measures: pH, Conductivity (Salinity), Dissolved Oxygen, and Turbidity, 2010-2011).



AQUA PATH In-Line Biosensing System and cartridge inserts (2011).





AQUA PATH II Biosensor Buoy System with Hach Sonde Attachment, 2012. Assessment of communications and biosensor Snake River, Idaho. Department of Energy, Idaho National Laboratory. (Cover Photo).

Balikatan Philippines: AQUA PATH II Static Display Opening Day Ceremony.
Left to Right: Alex Ly, Drs. Clint Smith, Andmorgan Fisher, and Michael Anderson.

Balikatan AQUA PATH II Demo in Palawan, Philippines Palawan Public Health Department Palawan State University.

Aiding the MEC to Engage within the Pacific Realm providing Humanitarian Assistance and Disaster Relief (HA/DR) and Education to prevent Insurgency of the Future: Cobra Gold Thailand & Balikatan Philippines 2012.

Cobra Gold Thailand AQUA PATH II Demo with ERDC researchers, the MEC, Royal Thai Navy and the Defense Science & Technology Office February 2012

ERDC researchers: Drs. Clint Smith and Michael Anderson prepare to deploy AQUA PATH II at Ft Magsaysay, Luzon, Philippines Balikatan April 2012

Logo designed for marking the participation in the 2012 military exercises within PACOM with the AQUA PATH II Biosensing Buoy System
Cobra Gold February 2012 and Balikatan April 2012.

Appendix B: AQUA PATH Biosensor Buoy System

The AQUA PATH technology introduces a new biosensor capability to remotely monitor along with physical water quality parameters, digitizing data flow, and collection of data for further monitoring and reporting. The AQUA PATH technology evolved from monitoring in situ bacterial presence/absence to a physical water quality analysis unit from 2009 to 2014 to form what is now the Water Diagnostics Operations Gear (WaterDOG). The last prototype developed was in 2017.

To explain this further, the AQUA PATH II Biosensing Buoy was used in conjunction with a HACH® mini-sonde multi-parameter to take additional measurements such as temperature, conductivity, pH, and turbidity. This system had a focus on biosensing and physical water parameter data collection. The USG team branched out to internally investigate developing a physical water quality analytics system that would operate like the AQUA PATH system, but did not use the biosensor component. WaterDOG, the new system, placed focus on taking measurements of just the physical water quality parameters of water quality monitoring and reporting the data digitally and remotely.

As stated, the WaterDOG focused on physical water quality measurement in conjunction with small, 10 gallon per hour (gph) and 3,000 (gph), large capacity water purification equipment, respectively. Traditionally, remote operations and testing of physical water quality parameters have been done by analog based toolkits. Colorimetric test strips and aqueous solutions that are mixed on site and recorded by pencil to paper for record and pushed to others in the chain of command by mail. The WaterDOG utilizes a calibrated sensor network in an in-line bypass format to monitor and record measurements of the physical water quality in a flow through environment at a treatment facility or in the field with other specialized water purification equipment. This allowed for either on-board storage of the digitally collected data, linkage to wireless technology, or other forms of remote communication for the data to be transmitted and provide a decision maker the ability to make assessments rapidly.

Moving from analog to digitally-based data collection was not available to the Quality Assessment System-Purification (QAS-P) program of record

system used by the Army. This lead to the development of the WaterDOG in-house at the US Army ERDC, which enabled a rapid way to share the knowledge for the physical water quality analytics digitally versus a manual process. The WaterDOG technology is briefly discussed to show advancement in the technology that occurred during 2013-2017, but the focus of this report is on the AQUA PATH Biosensing Systems research, development, test and evaluation.

Unit Conversion Factors

Multiply	By	To Obtain
degrees Fahrenheit	(F-32)/1.8	degrees Celsius
feet	0.3048	meters
gallons (US liquid)	3.785412 E-03	cubic meters
inches	0.0254	meters
microinches	0.0254	micrometers
microns	1.0 E-06	meters
mils	0.0254	millimeters
pints (US liquid)	0.473176	liters
pounds (force) per square foot	47.88026	pascals
pounds (force) per square inch	6.894757	kilopascals
pounds (mass)	0.45359237	kilograms

Acronyms and Abbreviations

APTES	3-Aminopropyl triethoxysilane
APHA	American Public Health Association
AQUA PATH	Autonomous QUerying And PATHogen threat agent sensor
ATEC	Army Test and Evaluation Command
AWWA	American Water Works Association
CCDC-GVSC	Combat Capabilities Development Command - Ground Vehicle Systems Center
CFU	Colony Forming Units
DI	deionized
DNA	Deoxyribonucleic Acid
DoD	Department of Defense
DSAB	Data Signatures Analysis Branch
EL	Environmental Laboratory
ERDC	Engineer Research Development Center
FIF	Future Innovation Fund
G4	G4 Logistics - 7th Army Training Command
GFP	Green Fluorescent Protein
GPS	Global Positioning System
gph	gallon per hour
GRL	Geospatial Research Laboratory
GMU S&T	George Mason University Science and Technology
IR	Infra-red
HQUSACE	Headquarters, U.S. Army Corps of Engineers
MARFORPAC	Marine Forces Pacific
MDE	Molecular Detection Element
MF	Membrane Filter
MIP	Molecular Imprinted Polymers
MPN	Most Probable Number
MTF	Multiple Tube Fermentation
n-IR	near-infrared
NOAA	National Oceanic Atmospheric Administration
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PdM PAWS	Product Manager Petroleum and Water Systems

PEO CS&CSS	Program Executive Office Combat Support and Combat Service Support
PM FP	Project Manager Force Projection
RTC	Real Time Clock
SBIR	Small Business Innovative Research
TARDEC: Now CCDC GVSC	Tank Automotive Research Development and Engineering Center: Now CCDC-GVSC Combat Capabilities Development Command - Ground Vehicle Systems Center (CCDC GVSC)
TRIS	Tris (hydroxymethyl) aminomethane
TS	Tryptic Soy
TSB	Tryptic Soy Broth
U.N.	United Nations
U.S.	United States
USACE	U.S. Army Corps of Engineers
USG	United States Government
USGS	United States Geological Survey
USPACOM	Pacific Command
WaterDOG	Water Diagnostics Operations Gear
WEF	Water Environment Federation
WQAS-P	Water Quality Assessment Set-Purification

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14. ABSTRACT

Contaminants serve as health risks to recreational water, potable water, and marine life that result in undocumented effects on population exposure. In many areas of the world, the concern lies in contaminated drinking water, which would immediately effect social and economic order. As research advances for innovative solutions, the deployment of automated systems for source water monitoring could reduce the risk of exposure.

Water quality monitoring typically involves sample collection and analyses that are performed in a laboratory setting. These results are normally presented after an 18–48 hr period. This report details the prototyped Autonomous QUerying And PATHogen threat agent sensor (AQUA PATH) geoenabled system that is able to detect the presence/absence of pathogenic bacteria indicators in source waters and report these values in the field, in less than 30 minutes. The AQUA PATH system establishes rapid field data collection and reports assessment of source waters bacterial loads at near shore inner coastal locations, which makes a leap forward compared to current presence/absence tests standards established by the EPA.

15. SUBJECT TERMS

Water – pollution
Water quality – measurement – automation
Remote sensing
Biosensors
Pathogenic bacteria

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