Evaluation of a New Rapid Method for Determining Recreational Water Quality

By

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BS, Purdue University 2008

THESIS

Submitted as partial fulfillment of the requirements
For the degree of Master of Science in Public Health
In the Graduate College of the
University of Illinois at Chicago, 2013

Chicago, Illinois

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LIST OF ABBREVIATIONS

ADV	hydrophobic adsorption
ATP	adenosine triphophate
BEACH	Beaches Environmental Assessment Closure and Health
CAWS	Chicago Area Water System
CDC	Centers for Disease Control
CFUs	Colony Forming Units
CHEERS	Chicago Health, Environmental Exposure, and Recreation Study
COV	Covalent Bonding
E.coli	*Escherichia Coli*
EPA	Environmental Protection Agency
IMS/ATP	Immunomagnetic Separation/Adenosine Triphosphate Bioluminescence
mL	milliliters
PBS	Phosphate Buffer Solution
qPCR	Quantatative Polymerase Chain Reaction
RLU	Relative Light Units
RPM	Revolutions per minute
UIC	University of Illlinois at Chicago
USGS	US Geological Survey

SUMMARY

For 25 years, the US Environmental Protection Agency (EPA) has relied on a standard test to determine the bacterial concentration in recreational waters. This test involves taking a water sample, filtering it, and incubating it for 24 hours in a Petri dish. This means that recreators and beach managers do not know the actual bacteria concentration of the water until the next day, resulting in overexposure to harmful bacteria. The EPA has allocated funding to stimulate research into developing a rapid method to determine the bacterial water concentration. One of the new methods being evaluated is called Immunomagnetic Separation, Adenosine Tri-phosphate Bioluminescence, or IMS/ATP for short. This method uses antibodies to select for specific bacteria, small iron beads to bind to these antibodies, and magnets to separate out the beads from the rest of the water. Once separated, the concentration of bacteria is estimated through the measurement of luminescence. This method can return results of bacterial concentration in water within two hours of sampling.

For this study, this method was evaluated in the Chicago area waterways in the summer of 2009. The IMS/ATP analysis ran concurrent to the Chicago Health, Environmental Exposure, and Recreation Study, or CHEERS, which involved the collection of water samples and their analysis for bacteria and other microbes from 2007–2009. Researchers from CHEERS were trained by US Geological Survey (USGS) employees on how to run the IMS/ATP method, and results from IMS/ATP can be compared to the EPA standard methods, that were already being performed as part of CHEERS.

SUMMARY (continued)

This report can be viewed as an experimental method evaluation, evaluating accuracy, precision, and repeatability of a new method. The data show that the method performed well on repeatability and precision, but performed poorly on accuracy. Overall, the new IMS/ATP method did a poor job of predicting bacterial concentrations found by the EPA standard methods.

I. BACKGROUND AND LITERATURE REVIEW

Waterborne illness has long been recognized as one of the important problems that may impact the health of the public. Due to dependence on water, ensuring the quality of the water has been a goal of public health departments for several decades. Millions of Americans every year are exposed to water that contains harmful bacteria while recreating in surface waters, such as lakes and streams. These waters do not undergo the rigorous tests of the nation's drinking water, and therefore can be a possible source for harmful bacteria and other pathogens. This water can be harmful to several different organ systems in the body, but the primary concern is recreational waterborne gastrointestinal illness. Since 1971, the Centers for Disease Control (CDC) and the EPA have maintained a system for collecting and reporting on occurrences of waterborne-disease outbreaks (WBDOs). During the years 2005–2006, there was an occurrence of 78 WBDOs in 31 reporting states, with more than 4,400 people becoming ill. These cases resulted in 116 hospitalizations and five deaths (Yoder, 2008). The burden of over-exposure to polluted waters can have dire consequences to the public.

A. Fecal Contaminants

Fecal contaminants represent all types of bacteria, viruses, and protozoa that are typically found in human and animal feces and digestive tracts. High levels of fecal contaminants in water mean that exposure to the water may induce an infection or disease in humans. Due to the myriad of types of fecal contaminants that can be found in water, health departments typically monitor only two types of contaminants, coliforms and fecal streptococci. Typically monitoring agencies test total coliforms, fecal coliforms,

Escherichia coli (*E. coli*), fecal streptococci, and/or enterococci. (EPA, 2010) These bacteria are called indicator bacteria, because their presence indicates the presence of more harmful pathogenic bacteria, viruses, or protozoans. Studies conducted by the EPA have determined that the correlation between different bacterial indicators and digestive illness is strongest in freshwater with *E. coli* and enterococci. As a result, some states have dropped all other types of sampling and only sample for *E. coli* and enterococci, as they serve as the best indicators of potential digestive illness. (EPA, 2010) The *E.coli* is a species of fecal coliform bacteria that is specific to fecal material from humans and other warm-blooded animals. According to the EPA, *E. coli* is recommended as the best indicator of health risk in recreational waters (EPA, 1986). Enterococci are a subgroup of fecal Streptococcus bacteria but are typically more predictive of human-specific disease than other fecal Streptococcus bacteria. The EPA recommends enterococci as a useful indicator in freshwater (EPA, 2010). There are several potentially harmful bacteria, protozoans, and viruses that exist in surface water. While listing all of the potentially harmful contaminants would be lengthy, listing some of the more common pathogens from surface waters can be useful. Some protozoans that cause adverse effects to human health are *Cryptosporidium* and *Giardia intestinalis*. Some common pathogenic bacteria include *Shigella*, *Campylobacter,* and *Salmonella*. These can lead to the common gastrointestinal symptoms of diarrhea, cramps, and vomiting. Viruses found in water include enterovirus, hepatitis A, norovirus, and rotavirus. Some strains of enterovirus can cause viral meningitis, and norovirus and rotavirus can cause gastroenteritis (CDC, 2009). It is important to remember that although these pathogens are not directly tested in each water sample, the presence of the indicator bacteria *E. coli* and enterococci

correlates with the presence of one or more of these pathogenic contaminants (CDC, 2009).

The EPA recommended *E. coli* as an indicator test for recreational water quality in 1986 (EPA, 1986). The 30-day geometric mean standard for *E. coli* was set at 126 colony forming units (CFUs) per 100 milliliters (mL) of water. This microbe concentration was correlated with gastrointestinal illness rate of about eight individuals out of 1,000 swimmers. This standard was based upon a study in Lake Erie, Pennsylvania and Keystone Lake in Tulsa, Oklahoma. Swimmers were interviewed before swimming, and then a follow-up interview was conducted eight to ten days later to determine development of respiratory or gastrointestinal illness. Incidence of illness was then compared to microbe concentration. The strongest correlation existed between *E. coli* and enterococci and gastrointestinal illness incidence, with little to no relation existing between total fecal coliform concentration and gastrointestinal illness (Haack, 2007).

Understanding that high levels of microbes can lead to disease is important, but it is also important to know where the fecal microbes come from. Typical sources for fecal contaminants include waste water from sewage treatment plants and drainage from septic tanks. In municipalities with combined sewer systems, that is where storm water and wastewater use the same infrastructure, during periods of high rain, untreated sewage effluent may be released into waterways to ease the overall burden on the sewage system. Additionally, runoff from agriculture and food processing plants along with storm runoff which can carry animal feces, can all be sources of fecal contaminants into a water system. The concentration of fecal contaminants in the water is a direct function of distance from source of fecal contaminants. That is, as one travels further downstream from a sewage treatment plant, the fecal bacteria concentration is expected to decrease.

The rate of decline is a function of environmental conditions that may influence the survival of each contaminant (EPA, 1986).

The primary exposure route that has caused the most concern for water recreation is ingestion. Every activity that involves surface water has some risk of potential exposure to pathogenic material. Exposure to the water can be through dermal and respiratory routes as well. These routes have different disease outcomes, including skin rashes and acute respiratory infections; however, the indicator bacteria standards have been developed in order to prevent gastrointestinal illness, and as such, should be considered as the primary route of exposure.

B. Current Indicator Tests

Currently the EPA uses certain standard methods to determine concentrations of the fecal indicator bacteria *E. coli* and enterococci in surface waters. These standard procedures are published and reviewed under the Beaches Environmental Assessment Closure and Health (BEACH) program (BEACH, 2008). These methods are to be used for all waters that are under the BEACH program. As mentioned before, the EPA has recommended testing for *E. coli* and enterococci since 1986. The EPA published Method 1600 for enterococci (EPA, 2002a) and Method 1603 for *E. coli* (EPA, 2002b) originally in 1986, but has been slightly modified throughout the past 25 years. These methods have some similarities and some differences, and each will be discussed below.

Method 1600 requires clean sampling equipment; disposable or autoclaved plastic bottles work well. Collection methods that avoid personal contamination are required. Using a glove or pole to collect water samples are ways to stop personal contamination. The water is then transported at a temperature of 1–4°C to the laboratory and the procedure must be initiated within six hours of collection. At the laboratory, the water is

then shaken to ensure uniformity of water components. The water is then filtered and the used filter is placed on a membrane-*Enterococcus* indoxyl-ß-D-glucoside (mEI) agar plate. The sample is then allowed to incubate for 24 hours at 41°C. After incubation, the number of colonies of bacteria grown are counted and then divided by the proper dilution volume to determine colony forming units per 100mL (EPA, 2002a). Method 1603 uses the same collection and transportation rules as method 1600. Afterward, the water is filtered and placed on an mTEC agar plate. The plate is then allowed to incubate at 35°C for two hours, then transferred in to a bag, and allowed to incubate for another 22 hours at 44.5°C (EPA, 2002b). After incubation, the plate is then analyzed to see how many bacterial colonies have formed. The results are then recorded as CFUs per 100 mL of sample water (CFU/100 mL).

As can be seen, each sample takes 24 hour hours to incubate, with potentially six hours of transportation time. This could lead to an overall test time of up to 30 hours. That means if water is sampled on early on day one, then accurate results will not be available until early or midday on day two. Health departments' normal strategy is to issue a beach notification, such as a swim advisory or swim ban, on day two if the water from day one's test is above the standard concentration for microbes. This means that for a whole day, swimmers and other recreators are at risk for exposure to potentially dangerous waters. Additionally, if the water on day two is actually below the standard level, then the beach would have been closed unnecessarily. This can be burdensome to local businesses and bothersome to local residents. As a response to this problem, the EPA started the BEACH program, which, among other things, provided grants to support scientists in creating new rapid tests to determine water quality. The goal being to inform

the public as early as possible if there high levels of bacteria at the beach or waterways in use (BEACH, 2008).

C. Experimental Rapid Detection Methods

A new rapid test measures water quality for harmful contaminants. It is called quantitative polymerase chain reaction (qPCR) and analyzes the DNA of the contaminants in the water. Water is filtered and then the bacteria and other solids are extracted from the filter. The bacteria are then analyzed for their genus specific DNA (or RNA) chains using a special promoter to select for the DNA target sequence of interest, which is then duplicated repeatedly. The concentration of indicator bacteria in the water is estimated by the number of cycles of duplication required until the target sequences are detectable. This test has been shown to be promising in delivering accurate results in a timeframe of less than four hours. In a 2006 publication, enterococci measured by the qPCR method were found to be associated with the incidence of gastrointestinal illness at beaches on Lake Michigan and Lake Erie (Wade, 2006).

In addition to qPCR, other rapid methods have undergone evaluation. One such method quantifies the amount of adenosine triphosphate (ATP) in bacteria to get an overall picture of how many bacteria are in a sample of water but the overall method. This will be described in greater detail in the Methods section. A 2004 report by Lee and Deininger from the University of Michigan first described the use of (IMS/ATP) for use in recreational water quality assessment (Lee and Deininger, 2003). On two beaches on the Great Lakes and two inland beaches, 24 samples of water were pulled through filters and analyzed by standard methods at a Michigan health department lab for *E. coli*. Another sample of the same water was analyzed using *E. coli* antibodies as part of the new IMS/ATP method in the university lab. The results were given for the standard

method in CFU/100 mL and for the experimental method had units of relative light units (RLU) per 100 mL. The observed relationship between the standard culture method and the new IMS/ATP was:

LogCFU/100 mL = 0.91*logRLU/100 mL-0.503 n = 24 r^2 = 0.9287

The small sample size requires that more research be done, but the association between culture and IMS/ATP was remarkably strong. This study served as a preliminary proof of concept for the method (Lee and Deininger, 2003).

After the promising results from this study, several other studies have investigated the effectiveness of this method. In a study performed by scientists at the USGS from 2004–2006, the method was subject to an evaluation on a larger scale. Conducted in the Cuyahoga River, samples were analyzed for both enterococci and *E. coli* using EPA standard methods 1600 and 1603. Samples undergoing the IMS/ATP method were analyzed both with pre-filtering the water and without pre-filtering. Later analysis showed that pre-filtering the water was unnecessary and would actually filter out bacteria that would not be analyzed. A total of 228 samples were analyzed for *E. coli* and 35 samples were analyzed for enterococci using the unfiltered direct analysis. Sixty-seven percent of the *E. coli* and 91% of the enterococci samples analyzed under the standard method exceeded the EPA single sample maximum limits for moderate, full-body contact (Bushon, 2009). The regression between standard plating and unfiltered IMS/ATP for *E. coli* was:

LogCFU/100 mL = 0.8033*logRLU/100 mL-1.9364 n = 228 r^2 = 0.65 (Bushon, 2009)

And for enterococci the regression between standard plating and unfiltered IMS/ATP was:

LogCFU/100 mL = 0.9974*logRLU/100 mL-3.2337 n = 35 r^2 = 0.77 (Bushon, 2009)

This study included additional analysis indicating the differences between the results of the standard method and the IMS/ATP method. In other words, when the standard method results would lead to a beach closing, would the IMS/ATP come to the same conclusion? The decision rule for IMS/ATP was formulated from the study's regression equations, meaning a certain value in RLUs would equal a value in CFUs that would result in a beach closing. For *E. coli* the tests were concordant 67% of the time, with 25% false positives, meaning IMS/ATP would suggest a beach closing while EPA would not, and 8% false negatives, meaning IMS/ATP determined the water safe, when the EPA test concluded an elevated concentration of bacteria. The enterococci IMS/ATP and culture results were concordant 80% of the time with 14% false positives and 6% false negatives. Accounting for the somewhat high percentage in false positives, the researchers mentioned the inability for the method to select out injured and nonviable bacteria or a lack of specificity in the antibodies to select for only the bacteria of interest. There were only enough replicates to be properly analyzed for *E. coli*. Comparison of the replicates of the plating method and the IMS/ATP method indicate a similar average difference in the replicates for both methods, but a higher variance in IMS/ATP than plating (Bushon, 2009). Their regression correlation (r^2) was similar to that obtained by Richard Haugland in a qPCR analysis under similar conditions (Haugland, 2005). The researchers posit higher initial cost and cost per test as compared with IMS/ATP.

Researchers in southern California wanted to compare several methods for analyzing the same water samples, using standard culture methods as the gold standard. Each method tested samples with known levels of *E. coli* and enterococci (determined by culture). These samples included negative controls of phosphate buffer solution (PBS), seawater spiked with sewage, and water polluted with seagull guano. Each method was

evaluated under four sets of criteria. First was the ability of the tests to detect their target in spiked samples, and to detect nothing in control samples. Second was the ability to detect the target over a series of dilutions. Third was repeatability, measured using replicate samples. Finally, the ability of the test to differentiate between human and gull fecal material. Samples of clean and ambient water were split in two, and one of each was spiked with fecal material to see if the test could correctly identify the spiked and unspiked samples. The IMS/ATP was able to repeat results successfully and correctly identified 100% of sewage spiked-clean water and sewage spiked-ambient water. However, it also displayed a high rate (66%) of false positives, in that it indicated high values for *E. coli* and enterococci in waters that contained neither. The researchers attributed this to the use of antibodies that were not specific to the target bacteria (Griffith, 2009).

Recently, a new method has emerged to bind the antibody to the magnetic bead in the process. Historically, the bead/antibody compound attached via hydrophobic adsorption (ADV). The researchers wanted to see if a new method using covalent bonding (COV) provided a stronger bond. They tested the method with each binding strategy, and compared it to the standard plating method. They sampled both freshwater (typically higher bacteria concentrations) and saltwater samples. The samples were also analyzed onsite, with a mobile laboratory setup at each beach location. Their results showed that the complex retained 41.6% of the bead/antibody compound after normal pipetting and mixing, and 11% of the compounds after vortexing. The new COV method experienced low detectable loss (<1.1%) of compounds during pipetting and mixing or vortexing, in essence, showing that the bond between the COV is much stronger and more durable than the bond created in the ADV. Additionally, the COV-binding process

still retains a high correlation between standard and IMS/ATP methods for *E. coli* and enterococci. The regression equation for *E. coli* is:

Log RLU/100 mL = 0.66*logCFU/100 mL + 3.48 r^2 = 0.87

And for enterococci the regression equation is:

Log RLU/100 mL = 0.69*logCFU/100 mL + 3.93 r^2 = 0.94

Perhaps more remarkably, this new method had a higher rate of correct test classification, with the *E. coli* test concordant on 92% of the tests, and the enterococci concurrent on 94% of tests. The study also found a decreased ATP signal when the sample was held on ice rather than analyzed immediately. A drop in 26% of the signal was found after 15 minutes of hold time, a drop of 30% after two hours and 38% drop after six hours. This was true for both ADS and COV binding methods. The ability of the IMS/ATP method to successfully be done in the field allows for maximum ATP signal and reporting of beach closings to the public (Lee, 2010).

The next study evaluated the performance of IMS/ATP in different waste water systems. Water samples were analyzed in five municipalities with a large population range in three states (Bushon, 2009): Delaware, Ohio; Morehead City, North Carolina; Chapel Hill, North Carolina; Avalon, California; and Orange County, California. A similar comparison was made between EPA standard methods and IMS/ATP methods. The study found a strong positive correlation to the IMS/ATP data and the culture-plated CFUs in four of the five municipalities. There was poor correlation in Orange County; the reason for this is believed to be due to a contaminant in the water that would affect the bacteria/antibody bond. In previous studies that analyzed IMS/ATP samples in Orange County wastewater, the procedure consistently underestimated the levels of enterococci in the water samples that had been spiked in the lab, indicating that

something in the water might be inhibiting proper binding. The conclusion is that IMS/ATP needs to be evaluated in various water systems to determine if proper binding complexes can be created.

II. STUDY OBJECTIVES

This purpose of this project is to evaluate the performance of the IMS/ATP in the Chicago water system. The IMS/ATP needs more evaluation before it can be considered a viable alternative to the current EPA standards. This study hopes to add additional data to the relatively small pool that already exists. This project will determine IMS/ATP's ability to predict the bacterial water concentration by comparing the IMS/ATP results to the EPA standard results. This will be the first study in which non-USGS personnel run IMS/ATP in the field. Blank analysis, 1:10 dilutions, and spot checks by USGS staff will ensure that the analysis and procedures are being run according to the specifications. The USGS also checked results and provided guidance to the University of Illinois, Chicago (UIC) staff constantly throughout the sample collection and analysis.

III. METHODS AND MATERIALS

A. Sampling Locations

The water for this study was taken from 19 locations throughout the Chicago region, comprising five location groups (Table I), and included Lake Michigan, the Chicago River, and other inland rivers and lakes. A concerted effort was made to collect water upstream and downstream the water reclamation plants (WRPs) in the Chicago River. The sampling locations were chosen to ensure large variability in microbial pollution.

TABLE I

LIST OF SAMPLING LOCATIONS GROUPED BY WATER SOURCE

Location Group Heading	Samples Analyzed (n)	Sampling Locations
CAWS-N	38	Clark Park, North Avenue Marina, Skokie Rowing Center
CAWS-S	16	Worth, Alsip
CAWS-O	38	Chicago River—Main Stem, Ping Tom Park, Canal Origins, Lawrence Avenue Fisheries
Lake Michigan	49	Montrose Beach, Leone Beach, Jackson Park
Inland Lake	26	Skokie Lagoons, Mastodon Lake, Tampier Lake, Beaubien Woods, Lake Arlington

The water was collected and analyzed by CHEERS water samplers and was run concurrent with other analyses by the research study. A mix of all possible water types from various locations was collected and analyzed. Ninety-two samples were analyzed from the Chicago Area Water System (CAWS). Overall, 38 samples were taken from the north side of CAWS, 16 samples from the south side of CAWS, and 38 samples from other locations in CAWS. Forty-nine samples were from inland lakes, 26 from Lake Michigan, and eight from other inland rivers.

B. **Collection Methods**

The water was collected by using a 2 L bottle at the end of an 8-foot pole to obtain a grab sample away from the water shore. The one water sample was then placed into two equal sized disposable plastic containers for replicate comparison. The samples were placed in a cooler and delivered to the UIC Environmental and Occupational Health Science's Water Quality Lab at 2121 W. Taylor Street, Chicago, Illinois. The water was then placed in a refrigerator and stored for a few hours until the researchers could begin running the procedure.

C. **Analysis Materials**

Each water sample was analyzed for three antibodies which correspond to the indicator bacteria. Antibodies A and B were used to analyze *E. coli* while Antibody C was used for enterococci. All reagents and equipment were provided by the USGS, Columbus, Ohio. Antibody A was monoclonal mouse anti-*E. Coli* antibody, B was polyclonal rabbit anti-*E. Coli*, and C was polyclonal rabbit anti-enterococci antibody. In total, each water sample was analyzed twice for each antibody, and twice again after a

1/10 dilution with sterile Phosphate Buffer Solution (PBS, pH = 7.4) for each antibody, meaning one water sample yielded 12 test results. The sterile iron beads were coated with goat anti-mouse IgG antibody for Antibody A solutions and goat anti-rabbit IgG antibody beads for Antibodies B and C. The final step required the addition of a compound called *luciferin luciferase* to the solution. This compound is designed to glow in the presence of ATP. The machine used for analysis was called a luminometer, which measures light intensity. This Modulus™ Single Tub Multimode Reader Luminometer was provided by the USGS and features a touch screen interface and easy-to-use procedure. It was calibrated regularly by plugging in a calibration hardware device and running a preprogrammed calibration procedure.

D. **Analysis Methods**

A 25 mL water sample was transferred to a 50 mL conical vial by pipette, and antibody was added to the sample. The amount of antibody was 4 µL, 40 µL, and 20 µL for EC monoclonal, EC polyclonal, and EN, respectively. Upon addition of the antibody to the sample, the samples turned in a tube turner at 18 revolutions per minute (rpm) for 15 minutes. Next, 200 µL of bead solution was added and are turned at 18 rpm for 45 minutes. The spinning induces mixing, which allows the bacteria, antibody, and bead to bind together and form a complex. The tubes were then inserted into a magnetic holder that attracted the complex against the back wall of the tube. The tube was rocked gently and the excess water was drained, leaving only the bacteria-antibody-bead complex. The PBS was then used to wash the complex and the solution was transferred to smaller 1 mL tubes. The wash step was repeated two times in conjunction with magnetic separations to remove any non-target bacteria stuck to the bead. Finally the bead antibody bacteria bond

was broken, leaving only the bacteria. The bacteria were then lysed with a cell-releasing agent, freeing the intracellular ATP into solution. The sample was placed in the luminometer and *luciferin luciferase* was added, and generated light was measured by the luminometer and recorded. The recorded value was then compared to the sample water that was collected at the same time and was analyzed according to EPA standard methods 1600 and 1603 performed by Microbac Laboratory, Merrilville, Indiana.

E. **Quality Assurance**

Before an experimental method performance can be analyzed, internal validity must first be ensured. To accomplish this, split dilution and blank samples were analyzed. These are the basis for determining whether or not the method is repeatable or if analysis of the same water samples will yield the same results. The sampling schedule was set up to achieve sufficient internal validity to be confident in the results. After a week of sampling, a schedule established that each water sample would be analyzed in replicate, with one sample of water being analyzed in 1:10 dilution per day. Additionally, a laboratory blank sample would be analyzed every day as well. Overall, 69 samples were taken and analyzed for antibody A, with 65 done in replicate and 28 done in replicate 1:10 dilutions. For antibody B, 63 samples were analyzed, with 59 done in replicate and 26 done in replicate 1:10 dilutions. For antibody C, 49 samples were analyzed with all 49 done in replicate, and 18 done in replicate 1:10 dilutions. In total, 76 blank samples were analyzed across all antibodies.

IV. RESULTS

A. Distributions

As discussed in previous sections, the IMS/ATP analysis output is RLUs. This is an arbitrary measurement compared to a set of standards, and indicates the relative luminescence. Each antibody returned a unique set of results (even though antibodies A and B both measured *E. coli* concentrations). In order to understand the most appropriate way to statistically analyze the data, it must first be determined if the data are normally distributed. Figure 1 below show the distribution of log10-transformed RLUs for each antibody. Data for each antibody, when run through the Shapiro-Wilk test for normality, show that the data are not distributed normally (Shapiro-Wilk $p = 0.02$). Therefore the data cannot be considered a normal distribution, and must be evaluated using non-parametric statistics.

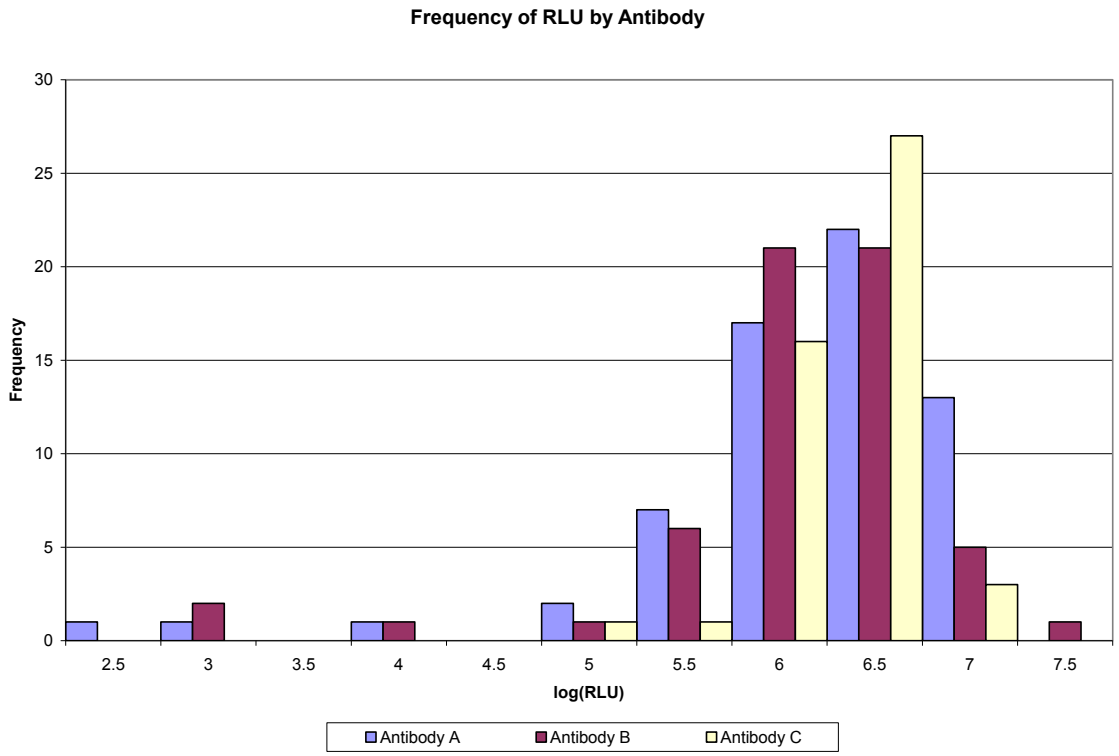

Figure 1. Frequency of RLU by antibody.

Basic statistics outlining the distributions can be seen in Table II.

TABLE II

STATISTICAL DISTRIBUTION OF ANTIBODY HISTOGRAM

	Antibody A (E. coli, monoclonal)	**Antibody B (E. coli, polyclonal)**	**Antibody C (Enterococci, polyclonal)**
N	65	59	49
Mean	5.87	5.79	6.01
Median	6.04	5.87	6.16
Std Deviation	0.81	0.86	0.39
Skewness	-2.15	-1.81	-0.70
Coeff Variation	13.77	14.91	6.35

Figure 2 shows that the data for the blank analysis of antibodies A, B, and C have similar distributions.

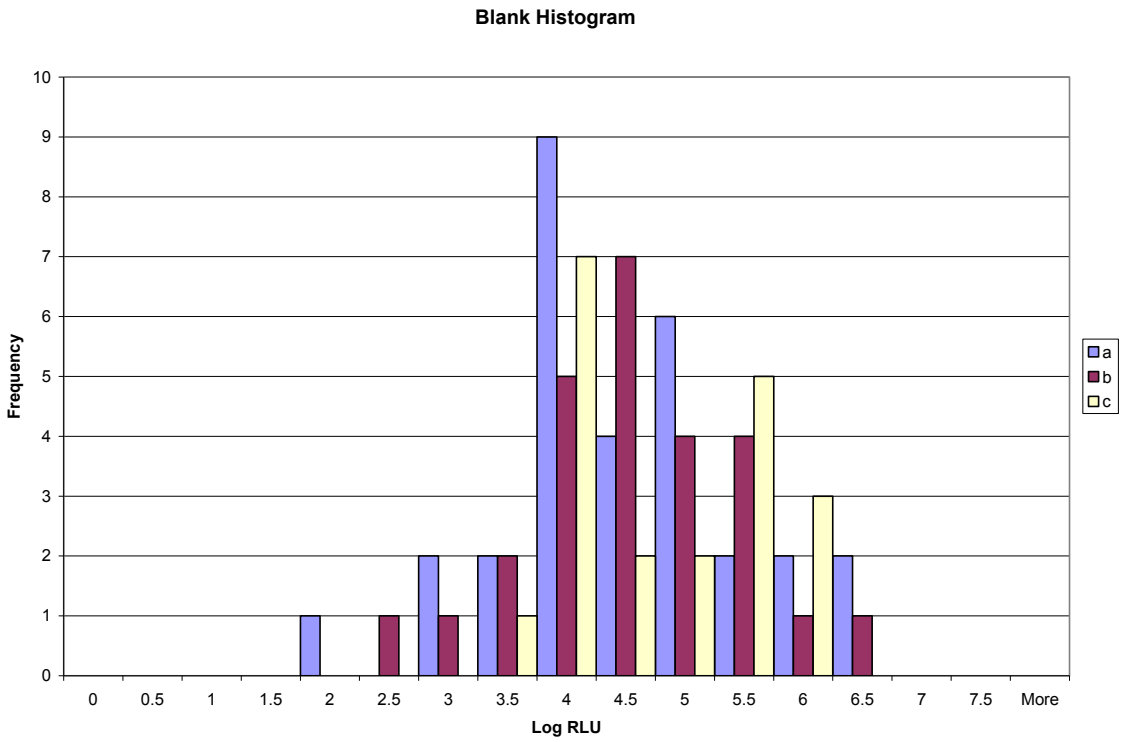

Figure 2. Blank histogram.

B. **Blank by Date**

Figures 3–5 below show the results for blank by date of sampling. Blank results are presented on a logarithmic scale.

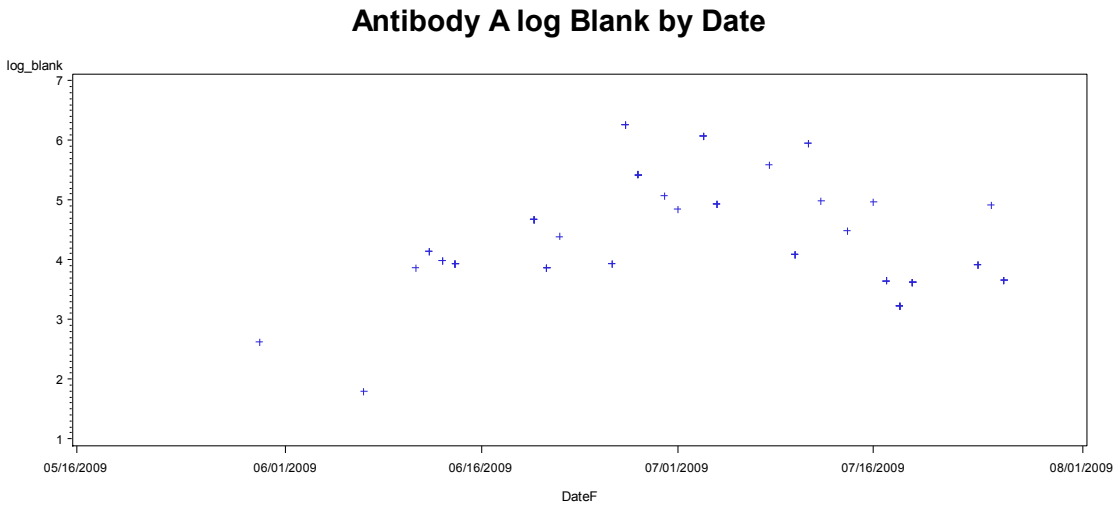

Figure 3. Antibody A log blank by date.

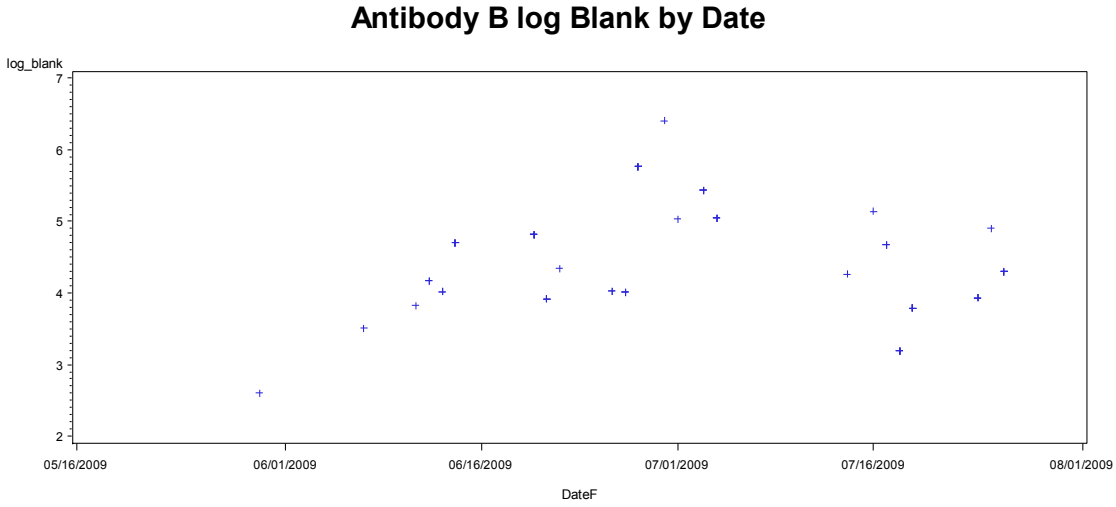

Figure 4. Antibody B log blank by date.

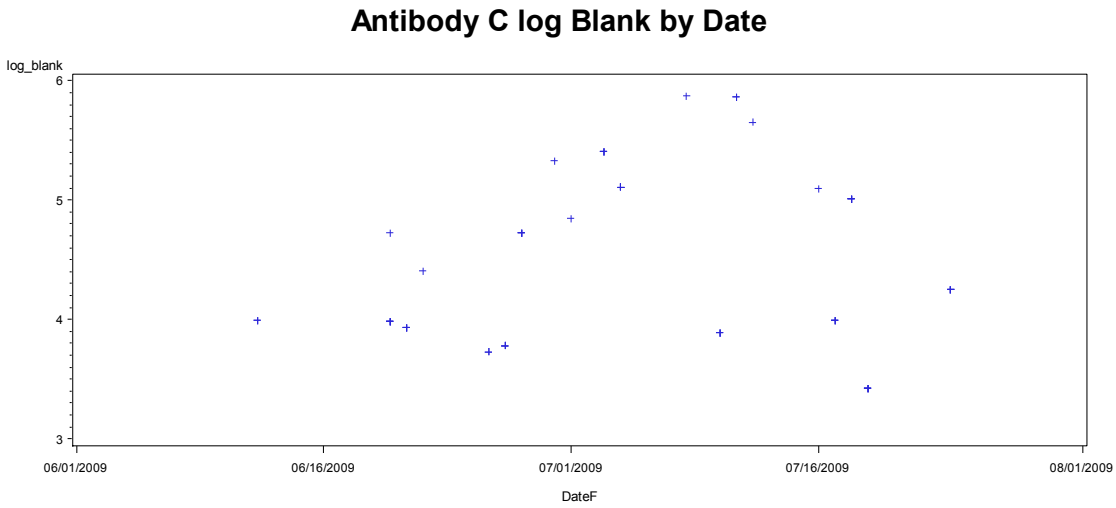

Figure 5. Antibody C log blank by date.

As can be seen, the blank results have an upward trend throughout the beginning of the sampling and then peak around the first or second week of July 2009. The reason for this spike is unknown. To investigate the effect of the spike on the overall dataset, figures 6–8 show the results of a simple adjustment of the sample results. This was accomplished by subtracting the log of the blank results from the log of the sample results.

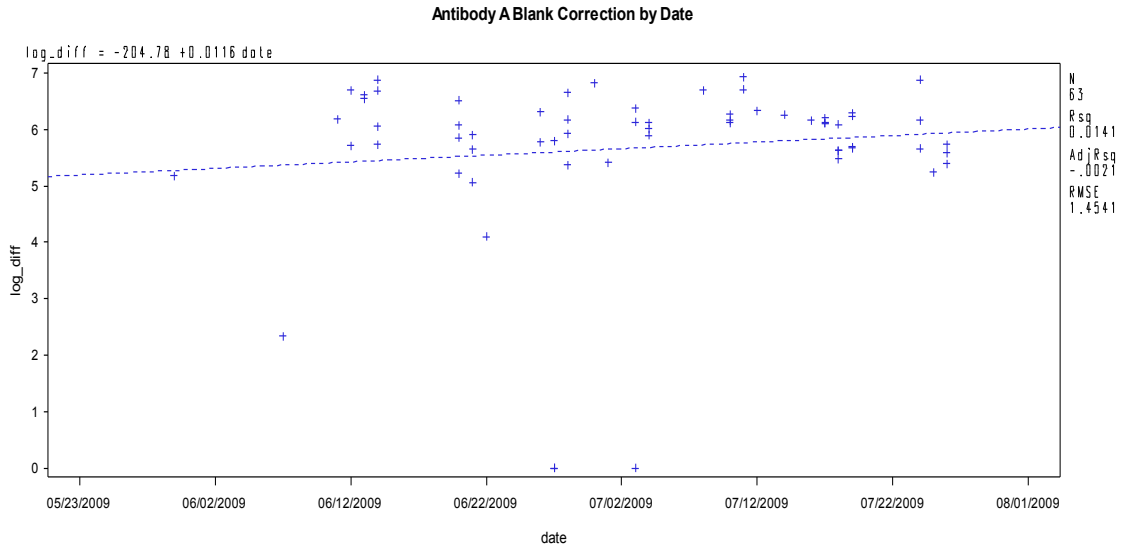

Figure 6. Antibody A blank correction by date.

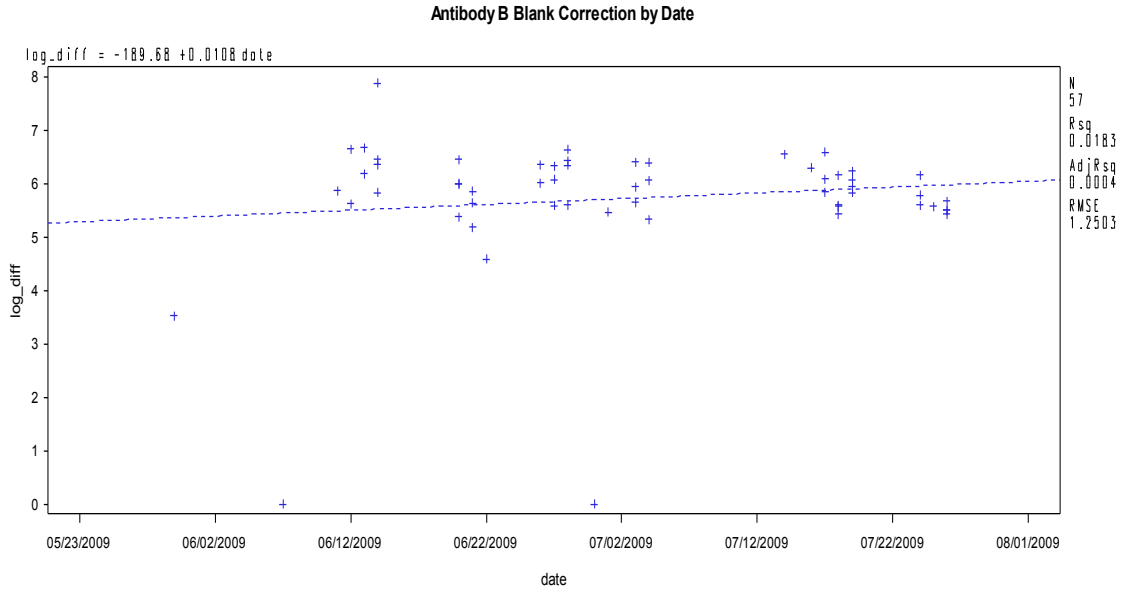

Figure 7. Antibody B blank correction by date.

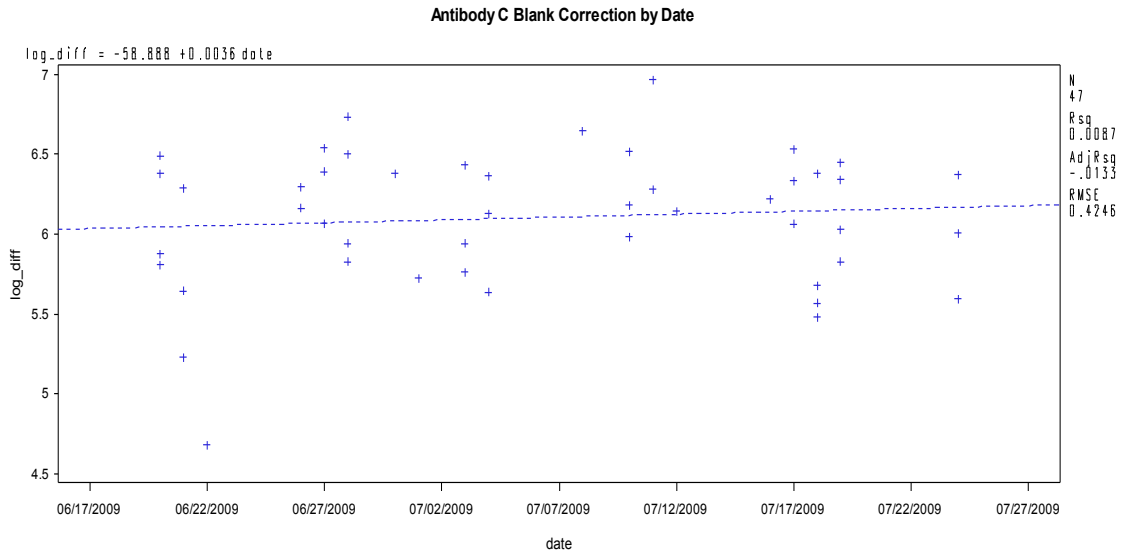

Figure 8. Antibody C blank correction by date.

So the spike in the blanks was also accompanied by an increase in the sample results, although to a smaller degree. Most of the adjusted data points remained positive, with a few points becoming negative (meaning the blank values were higher than the sample values). The data will therefore undergo a simple adjustment of the blanks without any further modification.

C. **Replicate Sample Analysis**

During sampling, every sample was split and then analyzed for each antibody twice, meaning that one sample of water was analyzed six times (three antibodies, twice per antibody). Therefore, each antibody can be compared for repeatability, if each test of the same water yields the same result. Below, figures 9–11 show the distribution of the

replicate samples. One of the replicates is on the x axis, and the other is on the y axis. The line indicates a 1:1 relationship.

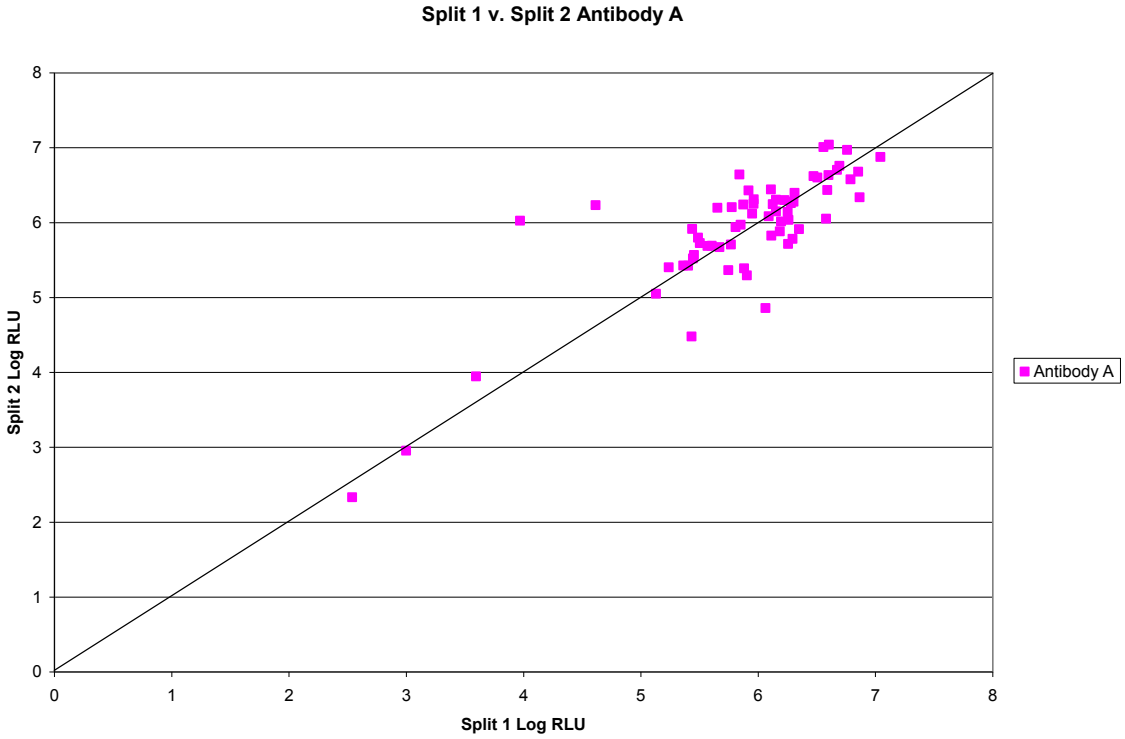

Figure 9. Split 1 versus split 2 antibody A.

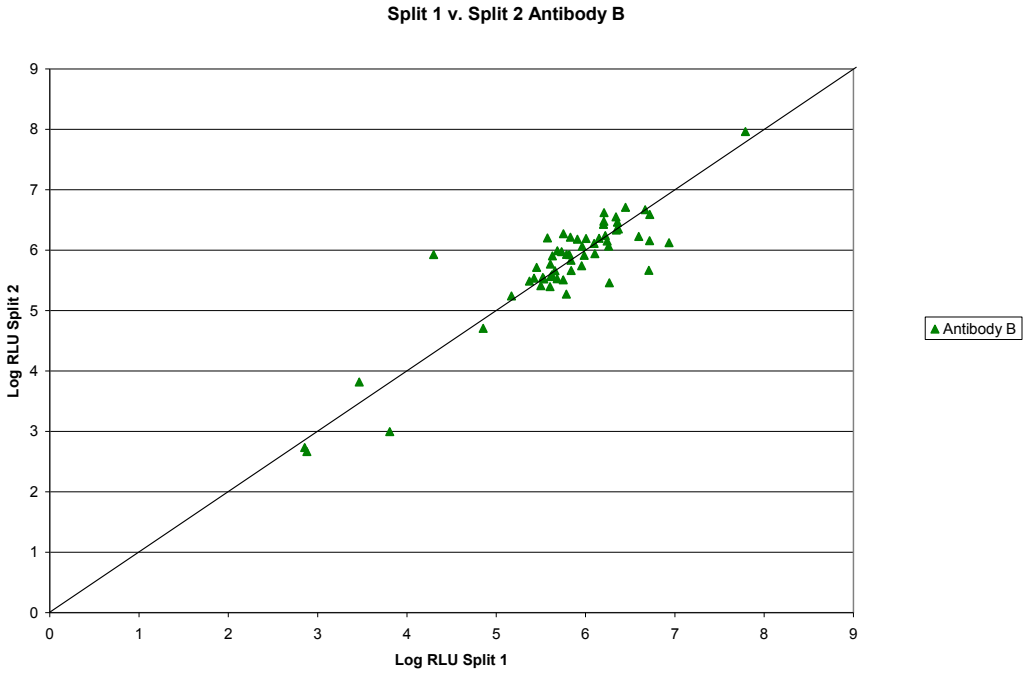

Figure 10. Split 1 versus split 2 antibody B.

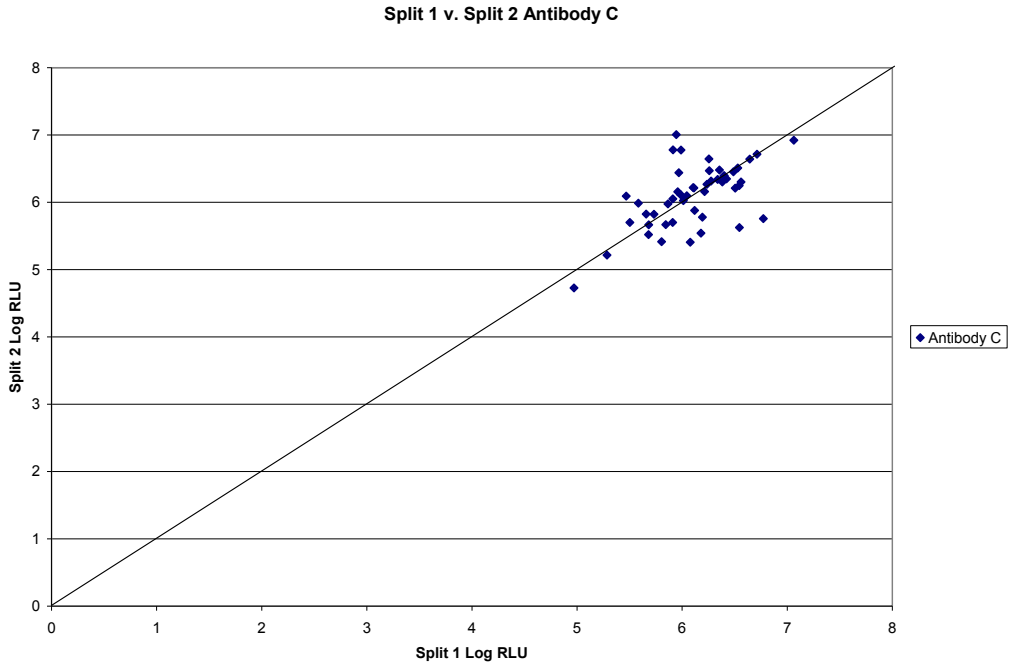

Figure 11. Split 1 versus split 2 antibody C.

D. **Dilution Replicates**

Each day of sampling, a 1:10 consisting of 2.5 mL of sample water and 22.5 mL of PBS buffer was analyzed to check internal validity of the day's samples. The purpose of this was to check the method to see if the results from the dilution sample would return to be about one tenth the value of the actual sample. Additionally, these dilutions were done in replicate. Figures 12–14 show the results for each antibodies performance with the 1:10 dilution quality assurance test. The line on the graph indicates a 1:10 relationship.

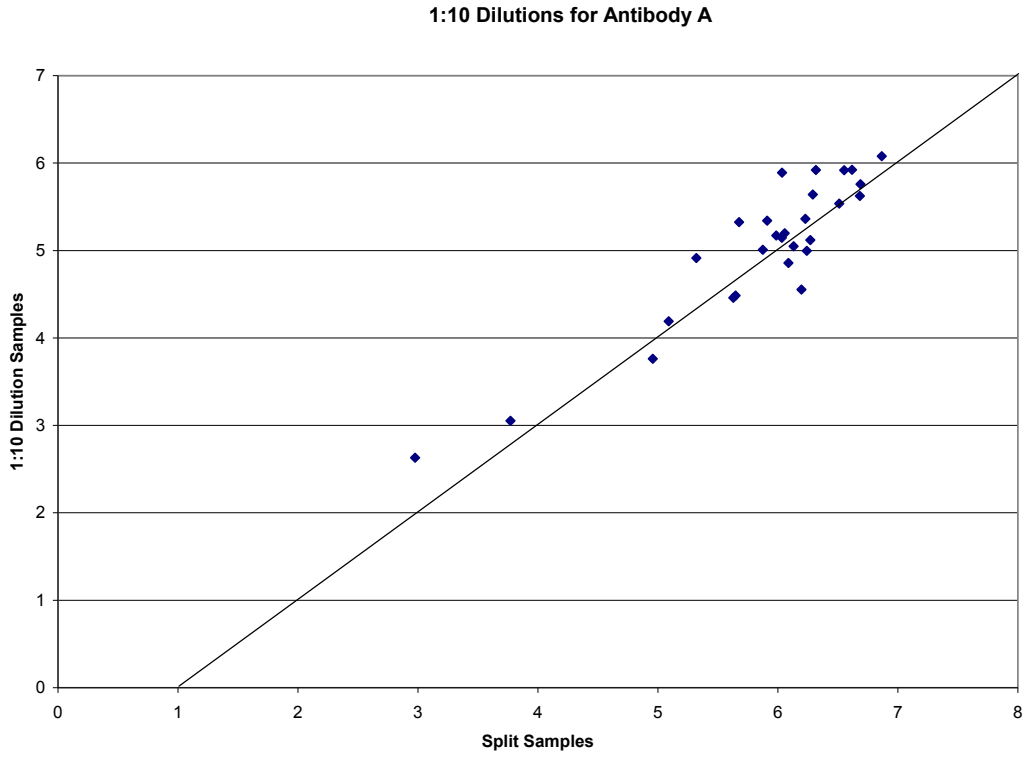

Figure 12. 1:10 dilutions for antibody A.

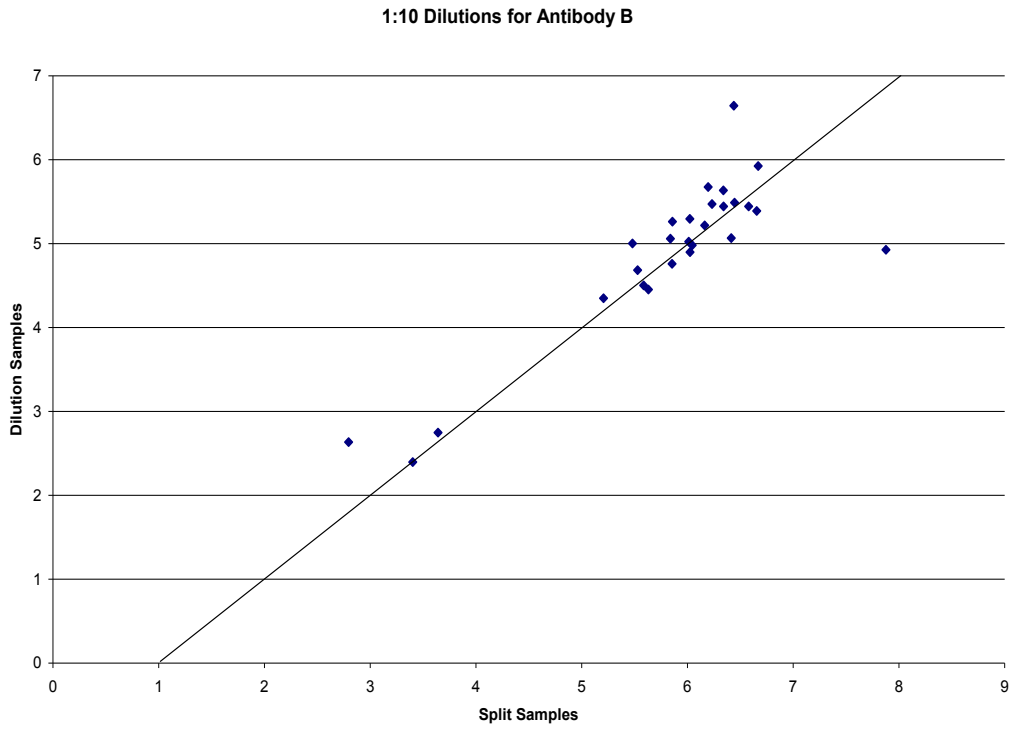

Figure 13. 1:10 dilutions for antibody B.

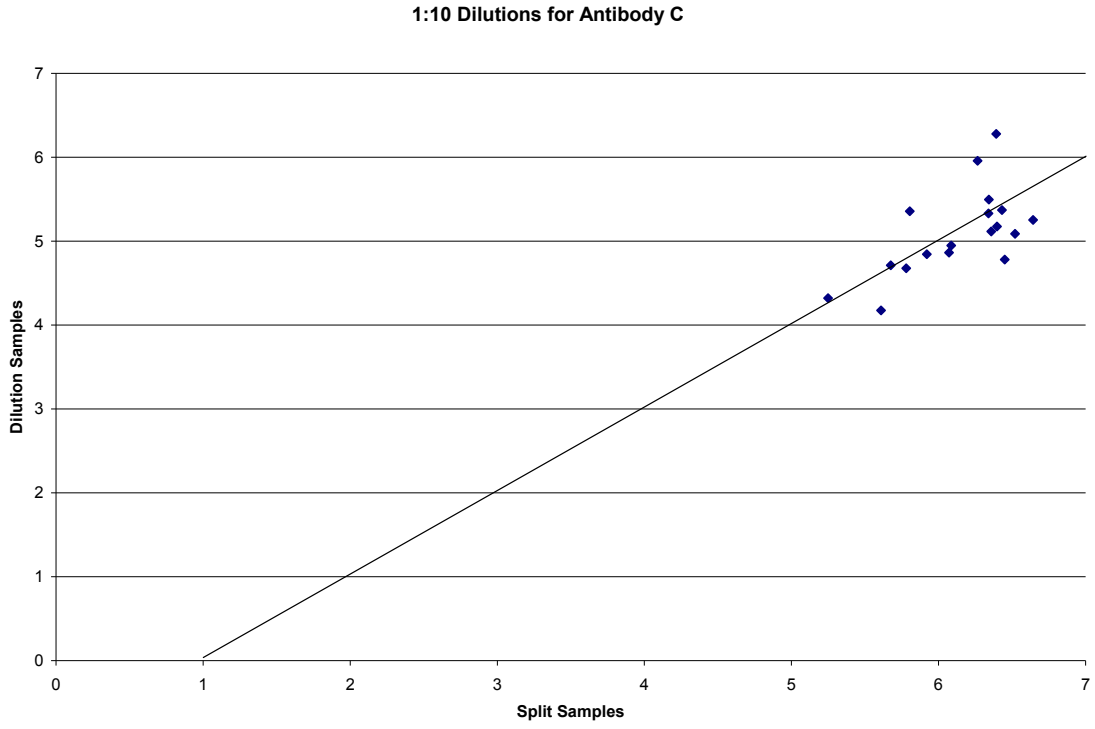

Figure 14. 1:10 dilutions for antibody C

As can be seen, all 3 antibodies performed well in the 1:10 test. The split samples were, on average, about 9 times higher than the dilution samples.

E. Comparison to Standard Culture Method

As already discussed, antibodies A and B detect *E. coli* and antibody C detects enterococci. Thus the comparison of A and B to 1603 results and C to 1600 results will determine if IMS/ATP results are a good predictor of EPA standard results. Log transforming the standard results is necessary for direct comparison. So this results in a Log (RLU) to Log (CFU) relationship. Figure 15 shows the correlation between antibody A and method 1603, Figure 16 shows the correlation between antibody B and method 1603, and Figure 17 shows the correlation between antibody C and method 1600. Below, each graph shows the description of the Spearman correlation between the two variables. Spearman correlation is used because of the non-normality of the distribution.

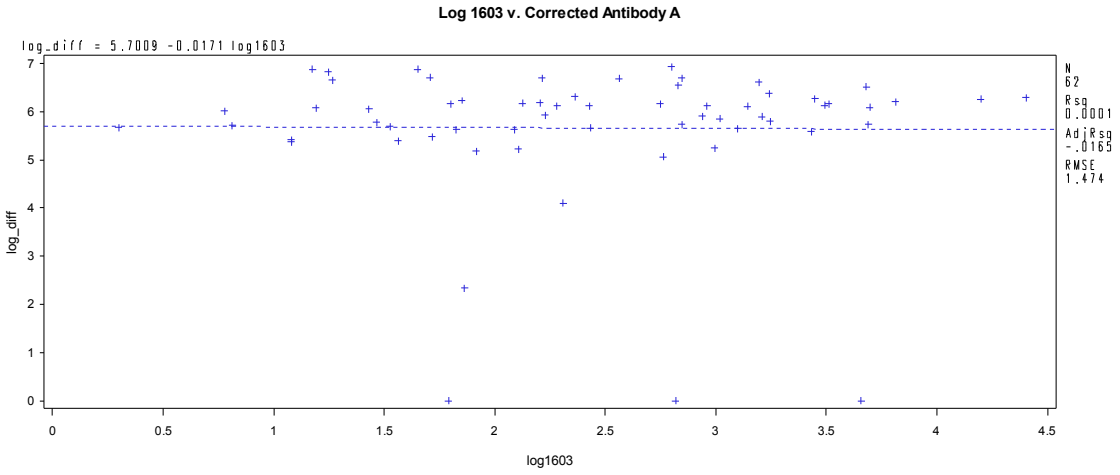

Figure 15. Log 1603 versus corrected antibody A.

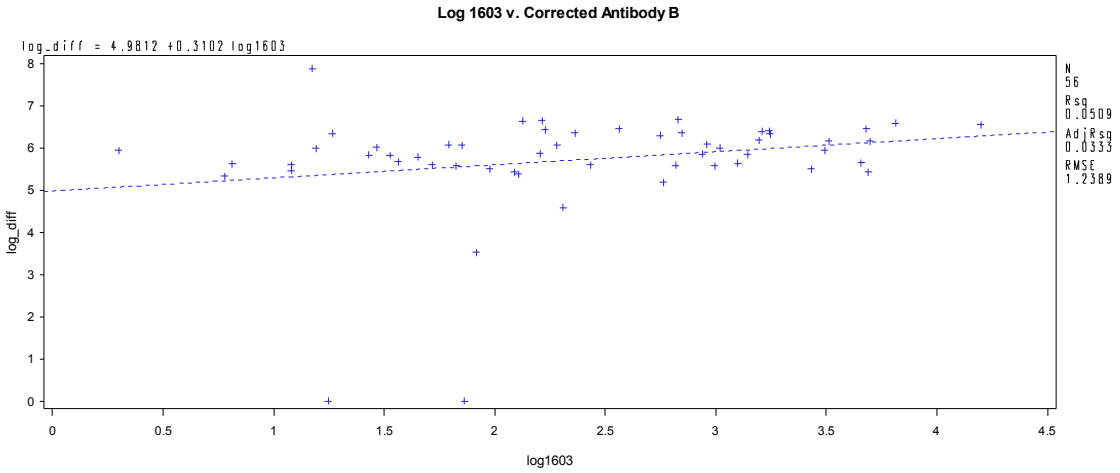

Figure 16. 1:10 dilutions for antibody B.

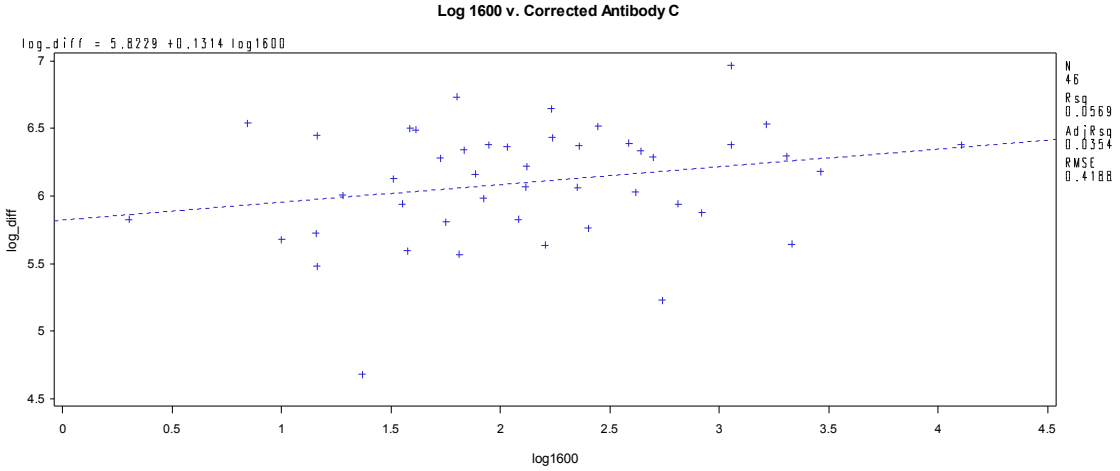

Figure 17. 1:10 dilutions for antibody C.

In Table III, the Spearman correlation is described with regards to each antibody group. Antibody C performed the best, but still did a poor job of predicting the outcomes of the standardized EPA tests.

TABLE III

SUMMARY OF SPEARMAN CORRELATION BY ANTIBODY

Antibody	Spearman Correlation (p-value)
A	0.0403 (0.75)
B	0.1286 (0.34)
C	0.19280 (0.19)

In order to get a better understanding of the data, the results have been broken down, highlighting the difference in performance based upon the location group of the water. Table IV show the performance for each antibody from each location group.

TABLE IV

SPEARMAN CORRELATION BY ANTIBODY AT EACH LOCATION TYPE

Antibody	CAWS-N	CAWS-S	CAWS-O	Lake Michigan	Inland Lake
A	0.15	0.21	0.18	0.11	0.10
B	0.12	0.09	0.18	0.19	0.20
C	0.15	0.17	0.22	0.20	0.17

The values correspond to the Spearman Correlation of each of the antibodies at each of the location groups. As can be seen, none of the correlations are very strong, and this indicates that the antibodies did a poor job of predicting EPA standard method results. This table, however, goes on to show that the antibodies performed equally poorly across all location groups. This is important because the water composition in the CAWS system is very different than Lake Michigan and Inland Lakes. In other words, pollution and bacterial concentrations differ location to location, and if pollution is interfering with IMS/ATP performance, the correlations should be stronger or weaker across the different location groups.

As can be seen, the distribution of the difference of log-transformed RLUs of samples and same-day blanks is not always ideal. A lot depends on the bacterial concentration at the location sampled. On some samples for antibodies A and B, the logarithm of the blank result was higher than the sample result, as can be seen in the negative results in the plots on figures 15–17. This seemed to happen during a few-day span of sampling and resolved itself near the end of sampling. There was no relation between location group, location, antibody, or recent rainfall to explain the result of the high-blank values for the few dates of sampling as can be seen in Table III. Overall, the negative difference value had no effect on the comparison of the experimental sample results to the standard sample results. As can be seen, IMS/ATP has done a poor job of predicting bacterial water concentrations in Chicago area recreational water. Across all antibodies, the predictive capacity for IMS/ATP is poor and does not appear much different than random noise.

V. DISCUSSION

Samples of water were analyzed across several different types of water in the Chicago area for fecal indicator bacteria using EPA standard culture methods and an experimental test called IMS/ATP. Developing a method to determine water quality in a rapid timeframe is essential to avoid unnecessary exposures to polluted water. The lack of rapid method is leading to overexposures to bacteria and other pathogens. Griffith sought to consolidate and evaluate reliability of new methods in his report (Griffith, 2009). New methods directly measuring viruses and protozoa appeared to be promising, but more tests across several different water matrices and municipalities are required to determine if methods can deliver reliable results. With the advent of qPCR as a viable option to deliver bacterial water concentrations in a matter of hours, recreators can now have a reliable source of information on bacterial water concentration. Evaluation of other tests is a necessity to determine the best method for delivering the most reliable results for the least cost. The IMS/ATP has presented itself as an alternative to qPCR method, and seeks to be a cheaper alternative to groups who cannot afford the startup cost of a qPCR program. However, reliability of method performance is the most important aspect of any water sampling protocol, and a cheaper method must still perform reliably to be considered a viable option. The data did not perform as well as hoped, and returned results that did a poor job in predicting the bacterial concentration in the water. Looking at some key facts of the results may help shed light on what happened during the sampling and analysis.

Laboratory blanks, run with sterile PBS instead of sample water, are used to identify sample contamination and evaluate the output of the luminometer in the absence

of ATP. If the laboratory blanks return high and inconsistent results (especially higher than the results from the sample water) then conclusions about the predictive ability of IMS/ATP will be effectively meaningless. As can be seen in figures 3–5, across all antibodies, laboratory blanks yielded mixed results. As sampling progressed, something seemed to cause the blank results to spike up for a few weeks, only to come back down to normal for the final weeks of sampling. This result is disconcerting, perhaps signifying that results from one time period could be less reliable than the other time periods. Another way to look at the data, however, shows a direct comparison of the blank results to the sample results. When Figures 6–8 are analyzed, it shows that although the spike-up in results of the blank samples had an effect, the effect was not as drastic as simply looking at blank results alone. The time period corresponding to the spike in blank results also corresponded to an increase in sample results. Although the increase in sample results is less drastic than the increase in blanks, the data determined that simply subtracting the blank results from the sample results for each day was appropriate, and was used to account for the steady increase in results among blanks. Looking at more quality control tools will help better determine the predictive ability of the test.

The best way to determine if a new method is repeatable is to take one sample, split it in two, and then run the same test for each split sample and compare the results. The data for split analysis can be found in Figures 9–11. As can be seen, across all antibodies, the results are fairly consistent along the 1:1 line. These data show that the method is repeatable, and analyzing two samples of the same water will yield similar results. The other quality-control procedure to assess repeatability and the method's predictive ability is to analyze a sample of water, and then a sample of 10% water and 90% PBS. If results of these two samples have a close to a 1:10 relationship, it shows that

the test is repeatable, and the method can show a difference in magnitude and can help shed light on the method's sensitivity. As can be seen on Figures 12–14, the results adhere closely to the 1:10 relationship line, indicating, as in the split analysis, that the method is repeatable and that the method can discern between higher and lower concentration results. As an interesting aside, the dilution samples contained 90% PBS. With that in mind, spikes in dilution results should have been observed, similar to that observed in the blank sample analysis. As can be seen in Figures 12–14, the data did not experience the same spike, as most of the results were around the 1:10 line, adding further mystery to the blank results. It has been established that despite issues in blank analysis, this method is repeatable and can distinguish between 1:10 differences in bacterial concentration. Therefore, the data will now be examined to understand the predictive ability of IMS/ATP in determining bacterial concentration in water as compared to EPA methods 1600 and 1603.

As can be seen in Figures 15–17, the IMS/ATP results were not associated with the corresponding culture results. The data show that blank adjusted IMS/ATP results do not perform better than simple random noise in determining bacterial concentrations in water. Furthermore, analyzing method performance with relation to location group of the water, as seen in Table III, show that the method poorly predicted bacterial concentrations in water regardless of the water source in Chicago.

A. Immunomagnetic Separation/Adeonsine Triphosphate Performance Compared to Literature

The results showed no correlation between IMS/ATP results and culture results, while prior studies did. The current data show a correlation of 0.04 and 0.16 for *E. coli*

antibodies, and 0.19 for enterococci. Bushon et al. in 2009 found r^2 of 0.65 for *E. coli* and 0.71 for enterococci using similar methods to the CHEERS study. Using the new covalently binding beads, Griffith et al. found r^2 of 0.87 for *E. coli* and 0.94 for enterococci. Clearly, something in the CHEERS study went differently than the Bushon and Griffith studies, yielding results that poorly predicted bacterial concentration. In order to understand the reasons for variation, a systematic evaluation of the method must be undertaken. An evaluation of the method's performance with an investigation to possible sources of error will help determine if this test is still a viable option as a rapid recreational water quality evaluation method.

Evaluating a new method requires an analysis into the method's repeatability, accuracy, precision, ease of use, and cost. As just shown above, the method performed poorly in the Chicago water system when compared to the EPA standard method. The data obtained yielded a much different and less reliable result than several of the papers published on IMS/ATP. This difference could be due to a number of factors. These factors could be in the sample water (some pollutant interfering with the reagents), the sampling methods, the laboratory personnel, the laboratory equipment, or the contract laboratory. These factors will be discussed below.

B. **The Sample Water**

The water matrices that compose each of the water systems that were analyzed are very different. Lake Michigan water is largely free of pollutants and biological material when compared to the treated wastewater of the CAWS system. Not only bacterial contaminants, but other pollutants including heavy metals, pesticides, salts, as well as general turbidity are much higher in CAWS than Lake Michigan and inland lakes. Table

III shows the lack of difference in IMS/ATP performance over the different water systems. As can be seen, the method's performance remained poor over all water systems. Thus, either some interfering pollutant is present in all water systems, or the sample water is not the issue. It is far more likely that the water is not the issue, as the likelihood for a uniform contaminant to be present in various water systems is unlikely.

C. **The Sampling Methods**

In grabbing the water sample from the river, transporting it, and holding it in the lab before analysis, there may be a possibility of contamination of sample water. Grab samples were performed by all samplers and placed in resealable plastic cups, placed in a cooler, and returned to the laboratory at UIC. Analyzers performed the test by mixing up the water and then taking a small sample and placing it in the conical vial to begin the experiment. However, since the samples were taken with the same methods and same vessels as the rest of the CHEERS project, suspect data would have been seen across all tests, including field blank and samples that were spiked with known bacterial concentrations. Since the quality assurance program for the CHEERS project performed very well, the methods by which IMS/ATP samples were collected should be considered reliable. It should be noted here that in a previous study, method recovery greatly improved with decreased time on coolers (Lee, 2010). Therefore there has been some data to suggest that performing the method on-site may improve recovery greatly. By placing samples awaiting analysis in coolers and refrigerators, this may have altered the sample in a way that could reduce recovery and method performance.

D. Laboratory Workers

Laboratory workers are a potential source for error in this procedure. Not using aseptic technique or wrongly carrying out the procedure could lead to unreliable results. However, this would lead to errors in all of the results, and would manifest itself as random noise across all sample results. Figures 9–11 show a positive relationship between replicate samples. This relationship would not exist if the laboratory workers were performing the method poorly, as the replicate sample results would be highly variable, and these graphs would appear more as random noise, than with a distinct correlation.

E. Laboratory Materials and Equipment

All of the equipment to analyze the water samples was supplied by the USGS. This included all the vials, pipette tips, test tube turners, magnetic holders, luminometer, and reagents. Plastic vials and pipette tips were used once and then discarded. Reagents were delivered on an as-needed basis, and were kept in the refrigerator when not in use, as per USGS recommendations. The luminometer was calibrated on a regular basis, although no record of actual calibration exists. During the last few weeks of sampling, calibration occurred every day, just before the procedure began. This calibration procedure consisted of attaching a piece of hardware to the luminometer and running a pre-programmed calibration procedure. However, unlike the other potential sources of error, this one is harder to disprove with our obtained data. It is possible that the reagents lost their effectiveness in the refrigerator or that the reagents needed to be brought to a certain temperature before use.

F. **Contract Laboratory**

The EPA standard water samples were sent to Microbac Laboratory in Merrilville, Indiana, which was responsible for determining the 1600 and 1603 results. It is possible that the samples run at Microbac contained a lot of statistical noise, which would have diminished the relationship between IMS/ATP and the standard methods. This possibility, however, is unlikely, as CHEERS sampling procedures included laboratory blinded blank, split, and spiked samples, and Microbac returned accurate results.

VI. CONCLUSION

Examining possible sources of error and limitations of the study, there appears to be no glaring source of error that would have caused the method to return results with so much variability, especially after the better performances of the method in other settings. During data collection, a USGS representative observed UIC laboratory personnel to evaluate their performance of the method. This representative found everything to be functioning properly, that laboratory personnel were following the method as prescribed, and no sources of error could be determined. Perhaps adopting the COV method described by Lee will increase the predictive value of the test by increasing the binding capabilities of the antibody-bead complex. This would ensure greater adherence of the complex, through buffer washings, meaning more bacteria would remain in solution at the time of lysing. The results from this trial do not disqualify IMS/ATP as a viable option for rapid detection of bacterial concentration. Like many experimental methods, this method has to be optimized to work in all water environments. Further trials and comparisons to other methods will help to ultimately decide the likelihood of using IMS/ATP as a broad-scale standard method. The literature, consisting of multiple well-designed studies, suggests good reliability of results, and one poor performance should not discredit the method as a whole. Overall, more evaluation is needed before officials rely on IMS/ATP to definitively determine bacterial water concentration.

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