Microbiological Water Safety Monitoring, Spring 2016

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Abstract

The ultimate goal for the microbiological water safety monitoring (MWSM) team is the development of a test that detects pathogens in water. The test must be of low cost (under ten dollars for each test), have a reduced incubation time from the standard 48 hours, and be able to be used in a low-resource setting such as Honduras. The team tested methods indicating the presence or absence of bacteria compared to quantitatively determining bacterial presence. Upon understanding the cost and efficiency of each method, it was possible to narrow down the methods that could be used as a model for microbial detection for AguaClara purposes.

Introduction

The goal of this team was to develop a test that could be used to determine the safety of the water treated by the AguaClara plants by ensuring that there are no pathogens in this water. A sample of water can only be certified to be safe if there are no microbes detected. Any presence of microbes would allow for reproduction and population increase, and would cause dietary health issues for widespread consumers. Thus only a presence/absence test for microbes was all that was required, but the team hoped to design a quantification device such that the data collected could be used by plant operators to adjust filtration accordingly. Though the most recent Honduras team tested the water treated by a newly installed plant and found it to be free of E. coli, a number of events could take place during the lifetime of a plant which could affect its ability to clean water. A leak or failure of one of the pipes or tanks, improper operation and cleaning of parts of the tank including the backwashing of filters, and increased precipitation during the rainy season all affect the plant's effectiveness and would require the E. coli content of the treated water to be re-evaluated. Typical microbiological water tests are often expensive and must be carried out in a lab. However, conditions at the plant sites made these kinds of tests impractical. Additionally, in order to be certain that AguaClara plants successfully eliminate pathogens from the water, microbiological tests on the treated water must be run once a day. The method previously used cost 10 dollars per test, which put a significant financial strain on the communities in which these plants operate. For these reasons, the MWSM team was created to develop a cheap, fast, and reliable test that can be used in a low-resource setting and to produce results which can be interpreted by plant operators. This new test would not only allow

the operators to easily and inexpensively confirm the safety of the treated water before it is sent out of the plant, but would also provide valuable information on which processes in the plant are eliminating microbial contamination.

Literature Review

Low Resource Setting Experimentation

Murcott et al. (2015) was a paper on the effectiveness of different detection methods in areas with low resources such as Ghana and India. The "effectiveness" was a culmination of the quantified records for accuracy, precision and speed. This paper's locations and its resources are parallel to those of the water treatment plants of AguaClara. The study was conducted by comparing the effectiveness of the detector using ground water of unknown bacterial concentration and bottled water of known bacterial concentration. Overall, the two different settings of India and Ghana were shown to have slightly differing results but nothing of significance. For some reason, some of the tests fared better in Ghana than in India based on Total Coliform Performance Ratings which was basically a method for comparing tests that used consumer reports as the main source of information. The methods tested were: Hydrogen Sulfide TARA Aquacheck, ORlab H2S, Lab made H2S test, IDEXX Quantitray 2000, 3M Petrifilm, EasyGel cards, Aquagenx compartment bags Murcott et al. (2015). The TARA Aquacheck and ORlab both determined the quantity of bacteria by picking up traces of hydrogen sulfide which were produced by the target bacteria. The Lab made H2S test was a more accurate version of TARA and ORlab, but requires a laboratory setting. The IDEXX Quantitray 2000 used a dish with many chambers to statistically approximate the quantity of microbes but requires a laboratory setting as well. The statistical method commonly used called the most probable number method compares the number of innoculated chambers against the number of total chambers to calculate the approximate original microbe concentration in the water. The 3M Petrifilm used a color changing chemical indicator and a small amount of sample water which lead to inaccurate quantification readings. The EasyGel cards was a scaled down version of the Quantitray that only required 1mL of sample but only provided inaccurate readings. The Aquagenx compartment bags used the same concept as the Quantitray but with different sized compartments of bags and without the requirement of a laboratory setting. These tests were a mix of quantifiable detection and presence-absence detection methods. The results took into consideration the cost, specificity, and sensitivity, thus addressing key problems in developing a detector: applicability, accuracy and precision. In terms of reliability and accuracy, the three top forms of detection were IDEXX Aquacheck, ORLab, and lab-made H2S tests , which all required high technology lab settings thus were determined to be infeasible for the situations in the AguaClara water plants.

When testing the methods, the researchers looked for low false positives and low false negatives which are both important in determining the true quality of the water. A false positive occurs when a test incorrectly indicates the presence of a condition, while a false negative occurs when a test incorrectly indicates the absence of a condition. In this system, a false positive would occur when

water that is pathogen-free is labeled as contaminated. This could lead to an increase in filtration mechanisms which would unnecessarily increase costs. On the other hand, false negatives are also harmful for treatment plants because that would mean that contaminated water would be passed off as safe water, thus putting the consumers at risk. False positives and false negatives must both be minimized to ensure the reliability of tests.

Considering the cost, sensitivity and specificity, the compartment bags proved to be most realistic. Although the compartment bags only indicate the presence or absence of bacteria, the water plants seem to only require that much, thus the compartment bags may be useful for AguaClara. However, the compartmental bags require too much incubation time to provide useful information for plant operators in the time required. In the case that this sort of delay is acceptable, the compartmental bag test seemed to be the most viable in low resource settings.Murcott et al. (2015).

Summary of Different Tests

A study was done by several institutions that summarized different microbial drinking water tests.Bain et al. (2012) This study identified 44 different tests, with 18 being presence/absence tests, and the other 26 quantitative tests. The goals of this study were similar to the goals of this subteam: they defined primary characteristics to be considered when selecting a test for drinking water and combined this information to find a suitable test given the resources available. Three different resource settings were tested: low resource, medium resource, and high resource. In a low resource setting, there is no laboratory or electricity by the water source. A medium resource setting is one with a basic laboratory and electricity within 24 hours of the laboratory. A high resource setting is one with a regular laboratory and running electricity. The laboratory in this setting is comparable to standard university testing labs Bain et al. (2012). The low resource setting is the most comparable setting to the target site in Honduras. The tests were then evaluated for different conditions. The conditions that determined how the tests were compiled included cost, detection of faecal indicator bacteria, development of the test, and whether there was at least 1 ml volume of the sample Bain et al. (2012). Most tests were relatively cheap, and as predicted: the tests that utilized E-coli as the indicator organism tended to be more prevalent in a low-resource setting.Bain et al. (2012). Tests using total coliforms and hydrogen sulfide rather than E-coli were also included. Bain et al. (2012). This study is valuable because it compiles every test and aids in the selection process. The table below shows the different presence/absence tests, most probable number tests, which are estimates of the amount of pathogens, and different enumeration tests. The figure evaluates price, incubation time, and other factors.

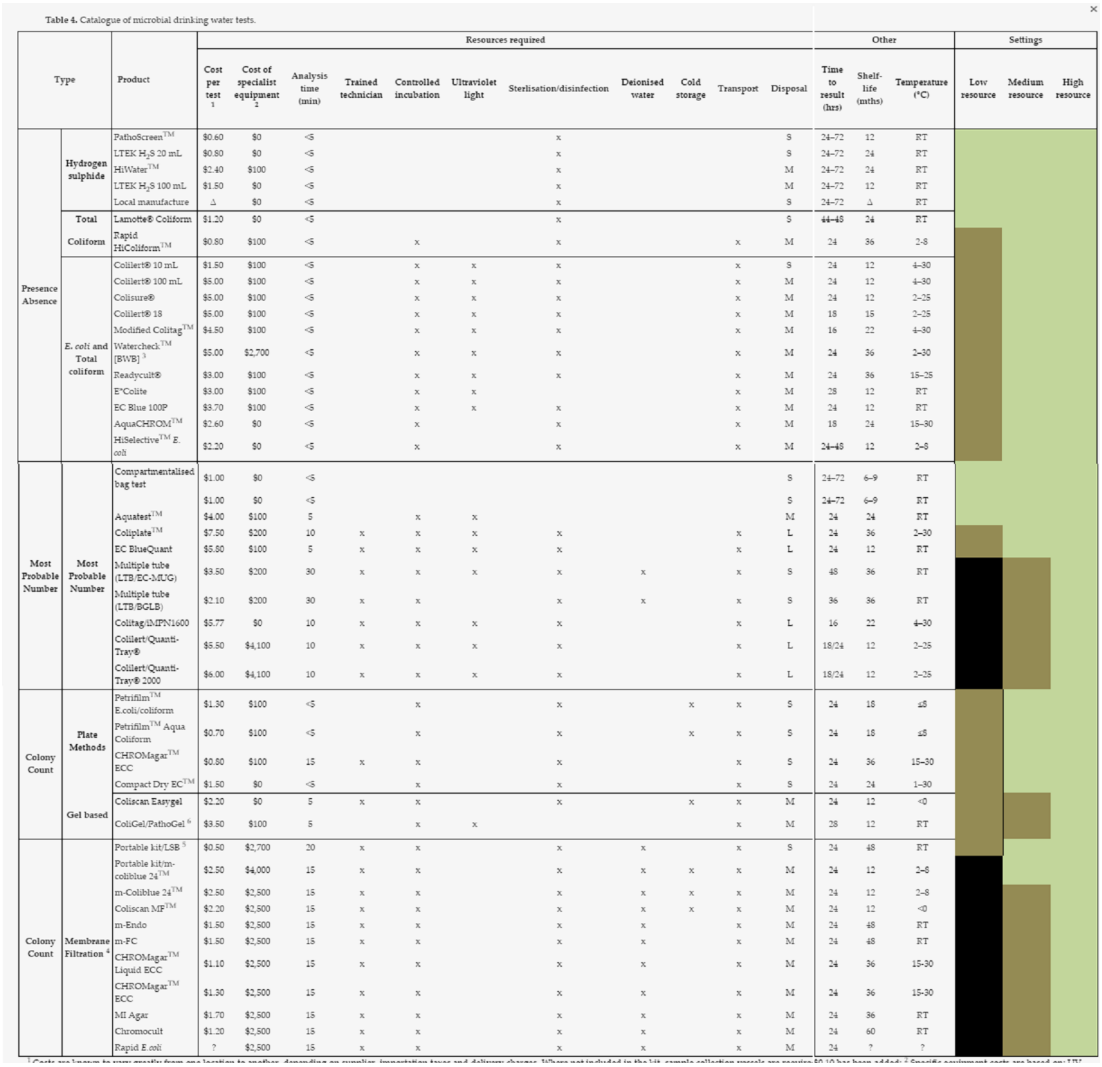

Table 4. Catalogue of microbial drinking water tests.

Type		Product	Resources required										Other			Settings		
			Cost per test [1]	Cost of specialist equipment [2]	Analysis time (min)	Trained technician	Controlled incubation	Ultraviolet light	Sterlisation/disinfection	Deionised water	Cold storage	Transport	Disposal	Time to result (hrs)	Shelf-life (mths)	Temperature (°C)	Low resource	Medium resource	High resource
Presence Absence	Hydrogen sulphide	PathoScreen™	$0.60	$0	<5				x				S	24–72	12	RT			
		LTEK H_2S 20 mL	$0.80	$0	<5				x				S	24–72	24	RT			
		HiWater™	$2.40	$100	<5				x				M	24–72	24	RT			
		LTEK H_2S 100 mL	$1.50	$0	<5				x				M	24–72	12	RT			
		Local manufacture	Δ	$0	<5				x				S	24–72	Δ	RT			
	Total Coliform	Lamotte® Coliform	$1.20	$0	<5				x				S	~~44–48~~	~~24~~	RT			
		Rapid HiColiform™	$0.80	$100	<5		x		x			x	M	24	36	2-8			
	E. coli and Total coliform	Colilert® 10 mL	$1.80	$100	<5		x	x	x			x	S	24	12	4–30			
		Colilert® 100 mL	$5.00	$100	<5		x	x	x			x	M	24	12	4–30			
		Colisure®	$5.00	$100	<5		x	x	x			x	M	24	12	2–25			
		Colilert® 18	$5.00	$100	<5		x	x	x			x	M	18	18	2–25			
		Modified Colitag™	$4.80	$100	<5		x	x	x			x	M	16	22	4–30			
		Watercheck™ [BWB] [3]	$5.00	$2,700	<5		x	x	x			x	M	24	36	2–30			
		Readycult®	$3.00	$100	<5		x	x	x			x	M	24	36	15–25			
		E*Colite	$3.00	$100	<5		x	x				x	M	28	12	RT			
		EC Blue 100P	$3.70	$100	<5		x	x	x			x	M	24	12	RT			
		AquaCHROM™	$2.60	$0	<5		x		x			x	M	18	24	15–30			
		HiSelective™ *E. coli*	$2.20	$0	<5		x		x			x	M	~~24–48~~	12	2–8			
Most Probable Number	Most Probable Number	Compartmentalised bag test	$1.00	$0	<5								S	24–72	6–9	RT			
			$1.00	$0	<5								S	~~24~~–72	6–9	RT			
		Aquatest™	$4.00	$100	5		x	x					M	~~24~~	~~24~~	RT			
		Coliplate™	$7.50	$200	10	x	x	x	x			x	L	~~24~~	36	2–30			
		EC BlueQuant	$5.80	$100	5	x	x	x	x			x	L	~~24~~	12	RT			
		Multiple tube (LTB/EC-MUG)	$3.80	$200	30	x	x	x	x	x		x	S	48	36	RT			
		Multiple tube (LTB/BGLB)	$2.10	$200	30	x	x		x	x		x	S	36	36	RT			
		Colitag/iMPN1600	$5.77	$0	10	x	x	x	x			x	L	16	22	~~4~~–30			
		Colilert/Quanti-Tray®	$5.50	$4,100	10	x	x	x	x			x	L	18/24	12	2–25			
		Colilert/Quanti-Tray® 2000	$6.00	$4,100	10	x	x	x	x			x	L	18/24	12	2–25			
Colony Count	Plate Methods	Petrifilm™ E.coli/coliform	$1.30	$100	<5		x		x		x	x	S	24	18	≤8			
		Petrifilm™ Aqua Coliform	$0.70	$100	<5		x		x		x	x	S	24	18	≤8			
		CHROMagar™ ECC	$0.80	$100	15	x	x		x			x	S	24	36	15–30			
		Compact Dry EC™	$1.50	$0	<5		x		x			x	S	24	24	1–30			
	Gel based	Coliscan Easygel	$2.20	$0	5	x	x		x		x	x	M	24	12	<0			
		ColiGel/PathoGel [6]	$3.80	$100	5		x	x				x	M	28	12	RT			
Colony Count	Membrane Filtration [4]	Portable kit/LSB [5]	$0.80	$2,700	20	x	x		x	x		x	S	24	48	RT			
		Portable kit/m-coliblue 24™	$2.50	$4,000	15	x	x		x	x	x	x	M	24	12	2–8			
		m-Colibblue 24™	$2.50	$2,500	15	x	x		x	x	x	x	M	24	12	2–8			
		Coliscan MF™	$2.20	$2,500	15	x	x		x	x	x	x	M	24	12	<0			
		m-Endo	$1.50	$2,500	15	x	x		x	x		x	M	24	48	RT			
		m-FC	$1.50	$2,500	15	x	x		x	x		x	M	24	48	RT			
		CHROMagar™ Liquid ECC	$1.10	$2,500	15	x	x		x	x		x	M	24	36	15-30			
		CHROMagar™ ECC	$1.30	$2,500	15	x	x		x	x		x	M	24	36	15-30			
		MI Agar	$1.70	$2,500	15	x	x		x	x		x	M	24	36	RT			
		Chromocult	$1.20	$2,500	15	x	x		x	x		x	M	24	60	RT			
		Rapid *E.coli*	?	$2,500	15	x	x		x	x		x	M	24	?	?			

Figure 1: Table of Tests

Compartmentalized Bag Test

A study was performed specifically on the compartmentalized bag test, which is one of the more developed and well-known tests, that tested its accuracy by comparing it to a standard lab filtration test using agar.Stauber et al. (2014). The filtration test used a selective medium containing chromogenic and fluorogenic Beta-glucuronide and Beta-galactoside substrates used for the detection and quantification of E. coli and coliforms. Chromogenic substrates react with proteolytic enzymes, or enzymes that break down proteins, to create the color change that identifies E-coli and coliforms. Fluorogenic substrates, however, react with enzymes to produce a fluorescent compound, which also identifies E-coli and coliformsStauber et al. (2014). The bag test, however, was based on the metabolism of glucuronides to the enzyme Beta-glucuronidase. This reac-

tion caused the color change to blue, as shown in the figure below. This blue color indicated the presence of E-coliStauber et al. (2014). The two different tests agreed on results 70, 71.4, 73.5, and 92.8 percent of the time for four E. coli concentrations of <1, 1–10,11–100, and >100 MPN/100 ml respectively Stauber et al. (2014). The higher concentrations of E-coli returned the more accurate results. There were some problems found with the compartmentalized bag test, including false negative readings. When the test indicated the presence of pathogens, it was 95 percent accurate, but if it returned a negative there was a chance that the water was still contaminated, which is an example of the previously defined false negative result to a test. Out of 263 samples tested with both the compartmentalized bag test and the membrane filtration test, 12 were found to be negative for pathogen presence with the bag test but positive for pathogen presence with the membrane filtration test. Therefore, there was a 4.6 percent chance that a false negative test occurred in this experiment Stauber et al. (2014). Overall, however, the compartmentalized bag test was found to be generally reliable and an efficient test in that it can be used when other microbial water testing resources are not available.

Figure 2: Color change reflects presence of E-coli

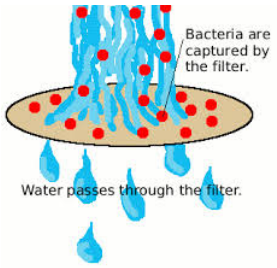

Figure 3: The standard membrane filtration test

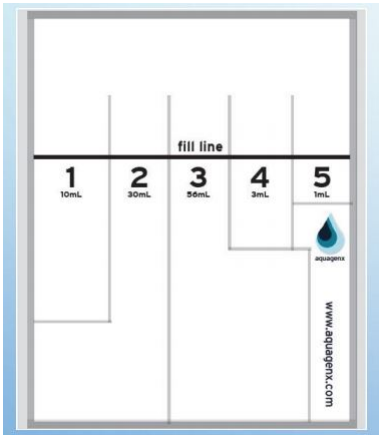

Figure 4: The different size compartments are shown in this figure.The specified volume of each compartment is what is put into each.

MBS Method

In an effort to develop a test for contaminants in drinking water which did not require a laboratory, expensive equipment, or trained technicians, researchers developed the Microbiological Survey Method. This method, which makes use of total coliforms as an indicator of contamination, uses disposable reaction vials that "measure the catalytic activity of redox enzymes of the main metabolic pathways of bacteria" and can be read by a change in color of the contents of the vial Arienzo (2015). To test this method, sixty-one wells were made around Douala, Cameroon. The majority of these wells were dug wells between 1.5m and 6m in depth, while others were drilled wells deeper than 20m. Water samples from each well were collected in each of three distinct seasons: once in the rainy season characterized by heavy, frequent rainfall and temperatures around 30-38 degrees Celsius, once in the dry season with no rainfall and high temperatures around 40-45 degrees Celsius, and once in the beginning of the rainy season, which has both high temperatures and heavy precipitation. These water samples came from climates similar to those of Honduras though from differing sources (well water from Douala versus surface water in Honduras), and water from both Honduras and Douala were often contaminated with faecal bacteria. The rainy season facilitated the growth of more microbes and the drilled wells tended to have more contaminated samples. The difference in water sources between the Honduran and Cameroon water would have been the cause for incomparable results since surface water and well water are two different environments. For this method, the quantity of CFUs (Colony Forming Units) were used to standardize and compare the results of each experiment. Researchers commonly refer to colonies as CFUs as a way of assuming that one colony grew from only one cell for the sake of simplicity.

To develop a timeline for the color change of the vial content, water samples with known amounts of contaminant were tested in the lab. The water samples from Cameroon were treated with thiosulfate to inhibit the antimicrobial effects of the chlorine that was being used to treat the water. The results of the test are interpreted through the change in color of the vial content over

time: color change after 14 hours indicates a level of total coliforms above 100 CFU/mL, color change only after 24 hours indicated total coliforms lower than 100 CFU/mL, and no color change indicates the absence of any coliforms. A visual of the color change can be seen in figure 5

Figure 5: The color change used for the microbiologicla survey method.

The MBS method, when tested on the water samples from Cameroon, was found to underestimate the bacterial concentration by 0.5 log units, or units defined on a logarithmic scale, whereas the most probable number technique has a given uncertainty of about 1 log unit. The most probable number technique works by identifying the most likely number of pathogens in a sample. However, this only applies when the test is performed in the presence of a coliform concentration of 100 CFU/mL or higher. When the bacterial concentration (expressed as the log of CFU/mL) was plotted against water sample color change time, a high level of correlation (0.98) was found in the relationship between the MBS method and the bacterial concentration. The correlation factor between the CFU/mL obtained via traditional methods and the MBS method was 0.94 for total coliforms Arienzo (2015). Although the water sources of Douala and Honduras are different, and this method may not be completely applicable to the MWSM team, the team was made aware of this method and the logic of this method could contribute to the development of AguaClara's microbe detector.

This test may give a rough estimate of coliform concentration, but it can be regarded essentially as a highly accurate presence/absence test as the 100 CFU/mL concentration level is far above what is considered the maximum level for safe drinking water. However, this means that the test is only reliable for relatively high concentrations of bacteria and provides false negatives for concentrations lower than 100 CFU/mL which makes this test impractical for AguaClara use.

Motility of E-coli

The team looked into the idea of a sliding colony counter, or a tube in which a water sample and media is placed in. The coliform and E-coli colonies would change color under UV light, which would make these colonies easier to identify. A sliding colony counter, or an electronic device used to quantify colonies, would then slowly slide down the tube, counting colonies. To look into the possibility of this method, information was required on the swimming speed of E-coli. E-coli swims when exposed to chemicals it are attracted to, including amino acids and sugars. This process is called chemotaxis Mittal et al. (2003). A study

done at Harvard University found that an E-coli cell swam at an average speed of 27 μm (micrometers/s) Mittal et al. (2003). This study grew E-coli in agar and placed the mixtures on glass slides to track the speed of E-coli. This speed was consistent to E-coli swimming 1-2 body lengths per second. The speed was converted to 2.33 m/day. This experiment showed the high speed of E-coli and the difficulty of identifying colonies in a tube due to this rate. Since E-coli swims at a high speed, it would be difficult to isolate individual colonies and count them. For AguaClara purposes, this method of using a sliding colony counter would not be ideal unless the motility of E-coli was reduced.

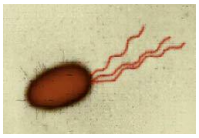

Figure 6: E-coli

Lactose Fermentation as Indicator of Total Coliforms

One of the most commonly used indicator media for the identification of pathogenic E. coli (E. coli O157:H7) is the Sorbitol MacConkey Agar supplemented with Cefixime and potassium-tellurite (SMAC). This uses sorbitol instead of the lactose used by traditional MacConkey agar to identify bacteria that can ferment sorbitol and those that cannot. Because non-pathogenic E. coli can ferment sorbitol and O157:H7 cannot, the acidic environment created by the fermentation of sorbitol and the basic environment created by the use of peptones as a food source change the color of the included pH indicator neutral red. SMAC agar Cefixime and potassium-tellurite allow for the selective growth of E. coli O157:H7. This prevents false positives from other non-sorbitol fermenting bacteria but also inhibits the growth of other strains of E. coli. (Muller 2005). There is no evidence that the response of pathogenic strains of E. coli to water treatment processes is different from the responses of other strains of E. coli. Therefore the WHO has determined that conventional testing , such as using MacConkey agar, is sufficient for indicating the pathogen content of drinking water World Health Organization (2011). A single SMAC plate costs $17.87 and must be stored at a temperature between 2 and 8 degrees Celsius, and therefore this media would not be suitable for the purposes of this test. The idea behind the traditional MacConkey agar – that lactose fermenting bacterial colonies create an acidic environment and therefore can be identified with a pH indicator – is not completely unusable. Most total coliforms have the ability to ferment lactose at high temperatures around 30-35 C and therefore could be identified using this technique. World Health Organization (2011)

Colitag

Another method that has been used extensively for water safety testing is Colitag. The way results are obtained through Colitag is similar to the compartmentalized bag test. The components of the test are added to a 100 ml sample, and after an incubation time, the sample changes to a yellow color indicating

the presence of coliforms. Then, an ultraviolet light is used to check for fluorescence, which indicates the presence of E-coli. The incubation time for Colitag is reported to be 16-22 hours, which is shorter than other existing tests, and the incubation temperature was reported as 35 degrees C Hach (2016). This temperature is higher than that of Honduras, but Colitag is simple to use in a low resource setting and has a short incubation time. The test is cheap as well, but can only be bought in bulk. Therefore, Colitag is an inexpensive option that fits the majority of requirements for an optimal test.
The figure below shows the results of a Colitag test, where the yellow color indicates the presence coliforms and the fluorescence indicates the presence of E-coli.

Figure 7: Colitag Test

Loop-mediated Isothermal Amplification Method

The loop-mediated isothermal amplification, also known as the LAMP method is a method for amplifying bacterial DNA without the controlled fluctuation of temperature. Typical polymerase chain reactions used to replicate DNA require heating and cooling to allow denaturation, elongation and annealing of DNA by enzymes called polymerases and ligases. This kind of temperature control requires a laboratory grade machine and would be too costly for AguaClara plants. However, Kaneko et. al. have found that the LAMP method is just as effective in amplification as PCR. The elimination of the temperature fluctuations would reduce costs by also eliminating the need for a temperature controlling machine. The DNA could just be incubated at one temperature and there would be no side effects to the cloning. This method would also eliminate the danger of killing bacteria through heating and thus denaturing. On top of this benefit, the LAMP method also loops multiple copies of the DNA to reduce the need for primers which are typically required at the starting ends of the DNA to begin the replication process. This LAMP method in conjunction with EvaGreen dye which attaches to DNA and fluoresces could potentially be another form of inexpensive coliform indication since the only requirements seem to be the enzymes and the EvaGreen dye.

Methods and Discussion

First Iteration

Initial Research

The purpose of this initial step was to use the literature research done prior in order to design the goal product and also design experiments to test the viability of said design. This required the preparation of the proper media and other materials.

Research Application Design

Upon gaining an understanding of microbial detection in water, the team went into a brainstorming phase of hatching ideas for potentially viable forms of microbe detection. The overarching goal of a tube form incubator and colony counter led the team to further research on E. coli motility and growth. Figure 8 is the rough design of the sliding colony counter. The tube would have thickened Colitag media and the coliform colonies would turn bright green and the E. Coli colonies would turn blue under UV light. The sliding colony counter, which works by counting individual bacterial colonies, would start on one side of the tube and slowly slide down the tube to count how many colonies of each microbe are present throughout the tube. Research was done on types of fluid media in which cultures could grow without stunted growth or cell death; hence, a media thick enough to prevent cells from swimming too far from the origin point and allows for segregation of colonies but thin enough to allow unrestricted colony growth in the media became the goal. The media used for the Colitag indicator turns bright green in the presence of fecal coliforms and glows blue under ultraviolet light in the presence of E. Coli. A combination of a semi-gel-like matrix and this color changing media remains the goal to allow for visible and distinguishable coliform and E. Coli colonies.

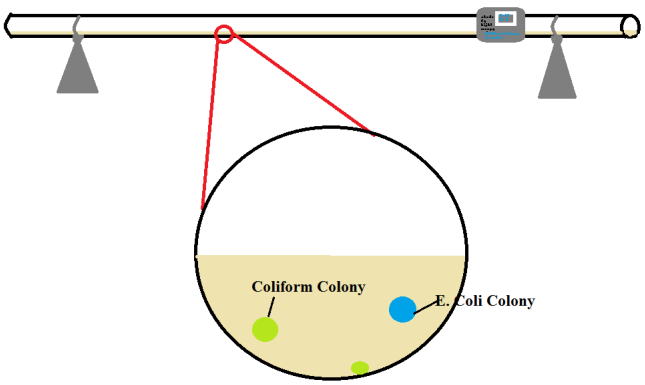

Figure 8: Sliding Colony Counter Design

Second Iteration

Description

The goal of this iteration of our experiment was to test how different quantities of gelatin affected the viscosity of media.

Materials

- 4 g of NaCl
- 4 g of Tryptone
- 2 g of Yeast Extract
- 4 Paragon Jars
- 1 Hot Plate
- Water sample from Beebe Lake
- Styrofoam Box Incubator
- 8.5 g of gelatin

Viscosity Experimentation Procedure

The goal of the sliding colony counter required the development of a viscous media to incubate the water sample with its potential microbes. However, this method had to be tested for its viability to determine whether or not the survival rate of cells would be affected by the heat of the melted gelatin, which was used for thickening the liquid. Dead bacteria would not be able to grow and thus there would be nothing to detect. The gelatinous samples were tested against a sample in liquid Luria Bertrani broth which is a basic media that fosters cell growth. Each 100ml sample of water had 1 g tryptone, 0.5 g yeast extract, and 1 g NaCl added to provide the bacteria with necessary nutrients to grow in the LB broth. The viscous media had the same amounts of chemicals, however there was melted gelatin added and mixed thoroughly to obtain even thickening. Three different amounts of gelatin (1g, 2.5g, 5g) were added to the samples and each of these amounts of gelatin were melted in 5ml, 5ml and 10ml of heated tap water respectively. The gelatin was melted prior to mixing with the contaminated water samples and broth. The water samples from Beebe lake were assumed to be contaminated such that it would contain cells. The samples were then incubated for 16-20 hours in a styrofoam box.

Results

As expected, the control grew colonies, verifying that the water sample had bacteria. The colonies were mostly floating on the surface and along the walls. The 1 g gelatin sample had a thick layer of gelatin on the bottom and it seemed that the gelatin was holding the colonies at the bottom. The 2.5 g gelatin sample had a ball of gelatin at the bottom of the bottle. The ball of gelatin had many air bubbles and the turbidity of the sample around the gelatin seemed to indicate the presence of many but indistinguishable colonies. The 5 g gelatin sample

had a layer 1.25 cm thick of gelatin on the bottom with many air bubbles. The entire surface seemed to have colonies growing. This indicated that there was too much gelatin in the mixture, or that the gelatin was not mixed well with the water sample and media. The gelatin was found in clumps because it was at a high temperature when mixed with the water samples and media, which were at a low temperature. This experiment was repeated to attempt to replicate or better our results. For the next iteration, the gelatin was mixed more uniformly with the media to create a thickened media.

Another issue that arose was that an extra 5 ml of water was added to the 5 g gelatin sample to ensure that the gelatin completely dissolved. Thus, the volume of each sample was not the same. This resulted in a lack of continuity between samples in the experiment, and may have resulted in a different result for the 5 g gelatin mixture. The experiment will be attempted again to solely test the amount of gelatin in the mixture rather than the volume of the mixture.

Table 1: Second Iteration

Jar	Observations/Results
Control	Microbes grew on sides of bottle.
1g gelatin	Microbes on the sides of bottle as well the bottom
2.5g gelatin	Semi-thick layer of gelatin and microbes at bottom or jar
5.0g gelatin	1.27 cm thick layer of gelatin and microbes on bottom of jar

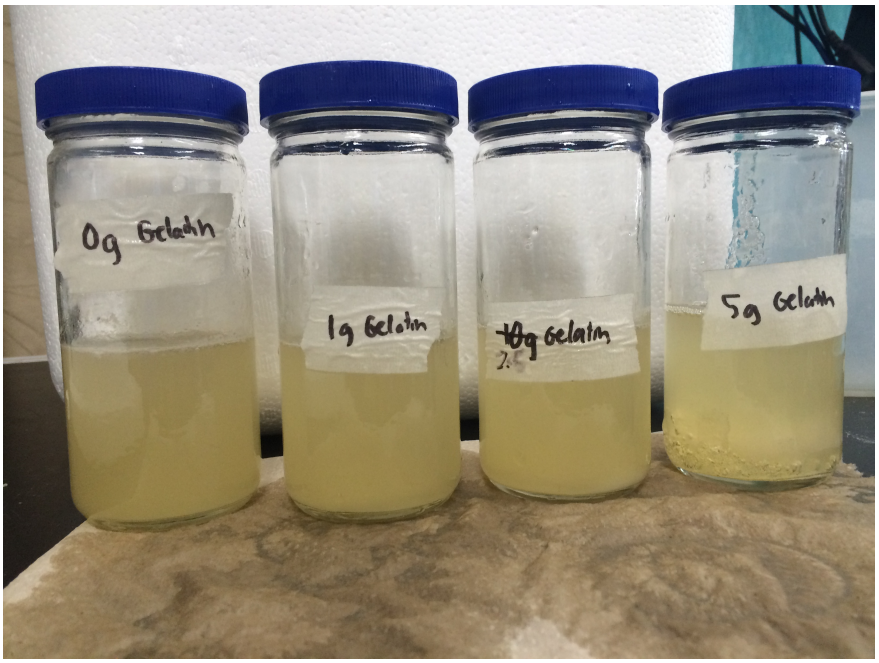

Figure 9: All viscosity experiment samples after incubation

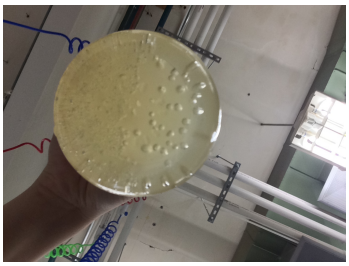

Figure 10: Bottom layer of sample with 5 g of gelatin

Figure 11: Bottom layer of sample with 2.5 g of gelatin

Figure 12: Bottom layer of sample with 1 g of gelatin

Figure 13: Bottom layer of control sample

Third Iteration

Description

For this iteration, the media containing the water samples was heated up before the gelatin was added. The goal of this technique was to ensure a more uniform mixture of gelatin and media. Results were also taken after both 24 and 48 hours to determine an ideal incubation time.

Procedure

This procedure followed almost the exact procedure as in the second iteration. No new materials were required for this iteration. The contents were mixed once more in four paragon jars. One jar was left with only the broth as a control, and placed in the styrofoam box incubator. The control jar was heated up in future iterations. In the other three jars, the thickened media was set up. However, for this iteration, the media was heated up to 38 C and then mixed with 1 g, 2.5 g, and 5 g of gelatin, respectively. This allowed the gelatin to melt while mixing with the media. The maximum tolerable temperature for E. Coli was found to be 45 C thus the temperature of the media was measured constantly to ensure that this critical temperature was never exceeded during the heating process. Then after mixing in the gelatin, the samples were heated up again to let the gelatin dissolve better and which removed some gelatin from the walls to prevent bacterial growth on the walls as was the case in the previous iteration. The goal of this was to ensure a more uniform mixture of gelatin and broth.

Results

The results of this iteration was very informative in that it indicated that the 5 g gelatin after only 24 hours worked the best in achieving the goal of isolating colonies as seen in Figure 15. Although some of the colonies combined together to form large groups of CFUs, there were visible chunks of bacteria at the bottom of the bottle. However, most of the liquid was in a sense wasted since it was not thickened in any way. Only a thin (1.27 cm) layer resting on the bottom of the bottle was thickened and the rest of the liquid media was simply sitting on top. The same observations were made for the other samples as well with thinner bottom gelatinous layers. The 1 g bottle at fist glance looked nearly identical to the control, but the bottom of the bottle seemed to have more bacteria observed through general turbidity. However, there were no defined colonies. Another difference between the 1 g and the control was that when the 1g bottle was tipped over a few degrees, the bottom layer seemed to stay together as opposed to the control where the bacteria would just scatter through the liquid. the 2.5 g had a bottom layer similar to the 1 g except that the layer itself was slightly thicker. Figure 14 is a side by side comparison of the samples after 24 hours of incubation wherein the layers of gelatin are visible on the bottom of the bottles as described earlier.
After 48 hours of incubation, the 5 g bottle no longer had distinguishable colonies for the proliferation of the bacteria grew to be too much to the point of complete amalgamation of all colonies. The control bottle had more of an overall turbid look after 48 hours rather than a thin layer on the bottom. On the other hand, the 1 g bottle still maintained its layer of bacteria and gelatin on the bottom.

The layer did not grow any thicker but rather more turbid.
Possibly due to the difference in the method for mixing gelatin, there were almost no bacteria growing on the walls of the control nor the 1 g gelatin bottles. The previous method did not have a second heating and swirling cycle, and did not allow the gelatin stuck on the walls to melt off thus facilitated bacterial growth on the walls.

Table 2: Third Iteration

Jar	24 hours	48 hours
Control	Microbes throughout bottle	No change
1g gelatin	Thin bottom layer of microbes	Maintained layer
2.5g gelatin	Semi-thick bottom layer	Maintained layer
5.0g gelatin	Thick bottom layer	Layer lost thickness

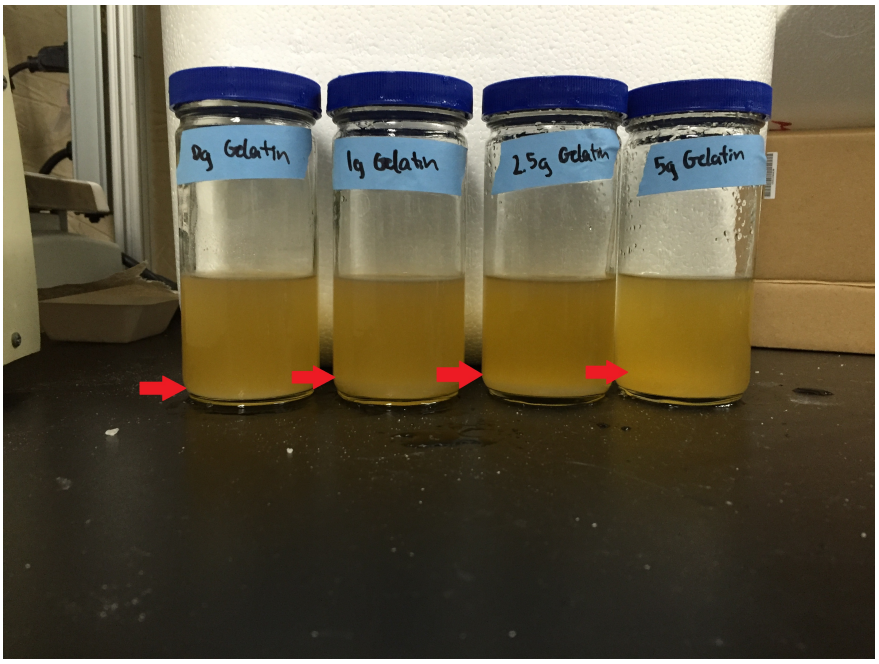

Figure 14: Profile view of all bottles after 24 hours of incubation. Note the gelatin layers on the bottom of the jars for 1g, 2.5, and 5g gelatin.

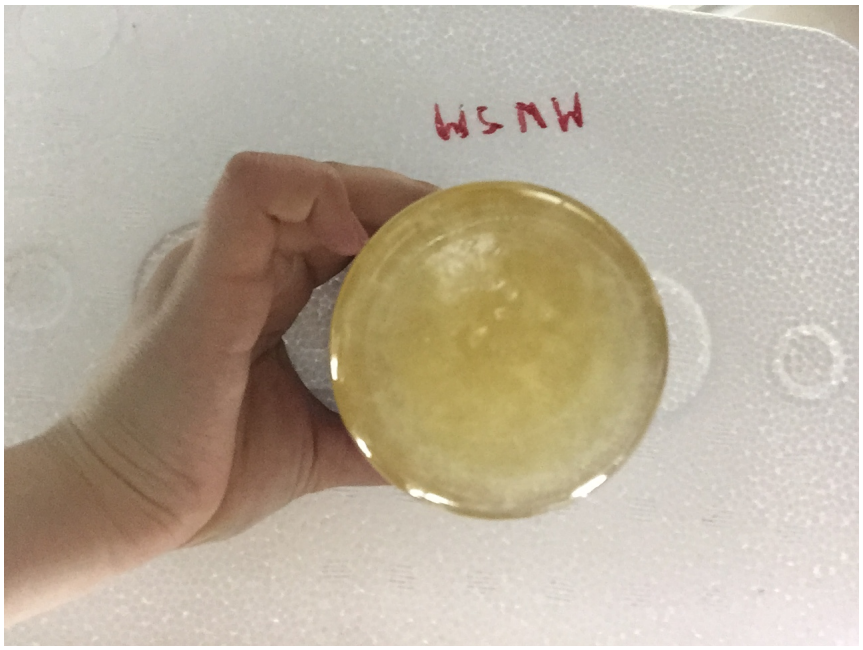

Figure 15: Bottom view of the 5g jar after 24 hours incubation. There are visible flocs of colonies dispersed all around the bottom.The white dots are bacterial growths.

Fourth Iteration

Description

This iteration focused on testing smaller quantities to observe whether results remained consistent. For this iteration, petri dishes were used to mix media and gelatin. The goal of this experiment was to create a thick media throughout the petri dish. On top of that, the control jar was heated along side the other samples in order to maintain consistency throughout all samples. The previous iteration did not take into account that the heating of the samples could kill off some bacteria, thus the unheated control bottle would not have been an accurate representation of the number of cells that would have grown without gelatin. Measures were taken to ensure the uniformity of the experiment.

New Materials

- 2 Petri Dishes

Procedure

The protocol for the 95 ml samples was nearly identical to that of the third iteration. The only difference was that the control 0 g gelatin jar was also heated as a precautionary measure for consistency across the board. As for the two petri dishes, 1 g of gelatin was added to a total of 16 ml of water. 15 ml of water was used for dissolving the gelatin and 1 ml of sample was used for the pour plate method. The pour plate method allows for the plating of liquid

samples with colonies growing within the gelatinous media. Since the 15 ml of tap water was the only liquid heated, the 1 ml sample containing E. Coli was not heated, thus lowered the chances of microbial death. Once the 1 g of gelatin was melted in the 15 ml of water, the 1 ml sample was poured onto the plate, followed by the melted gelatin. Following this swift step, the lid of the petri dish was placed back on the dish and was gently swirled clockwise and counterclockwise before the gelatin hardened. The petri dishes were sealed with tape around the perimeter to ensure minimal contamination. Both the petri dishes and jars were incubated for 24 hours and 48 hours. Although the pour plate method would not have provided the thickness desired, it would have allowed for the isolation of visible colonies, as desired.

Results

The results of this iteration was very similar to that of the third iteration in the way that the gelatinous layers formed on the bottom of the jars. The results for the 5 g bottle were consistent with those in the third iteration as one could observe by comparing Figure 14 and Figure 17. The bottles in Figure 17 are much more turbid overall although the gelatin layers are still visible. The petri dishes did not solidify in the way that was expected. Since the gelatin to media ratio was greater than that of the jars, the petri dishes were expected to solidify completely. The contents of the petri dishes were still very fluid and there did not even seem to be a gelatin layer on the bottom as with the bottles. Instead, there were large films of bacterial growth that developed in the media as observable in Figure 19.
In an attempt to obtain only the bottom gelatin layer of the 5 g bottle (figure 17), the team poured out the top media, but was unsuccessful since even the gelatin layer could not handle being tipped over above the horizontal position and the gelatin layer was flushed out along with the rest of the media. From these results, the team attempted another iteration to test only the thickening of water without media or sample water with bacteria.

Table 3: Fourth Iteration

Jar	24 hours	48 hours
Control	Microbes on walls	No change
1g gelatin	Thin bottom layer of microbes	Maintained layer
2.5g gelatin	Semi-thick bottom layer	Layer was more motile
5.0g gelatin	Thick layer with visible colonies	Layer lost thickness
Petri Dish 1	Visible colonies in thick layer	Less thick
Petri Dish 2	Heavy condensation on lid	no solidification

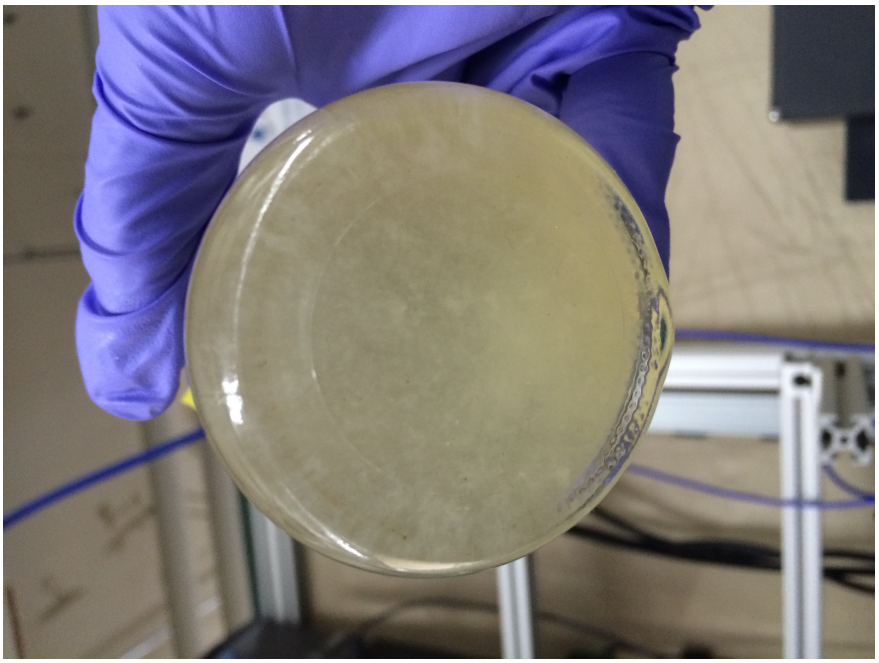

Figure 16: Bottom view of the 5g bottle after 24 hours of incubation. Again, there are visible colonies formed on the bottom of the jar, much like those of the 5g jar of the third iteration.

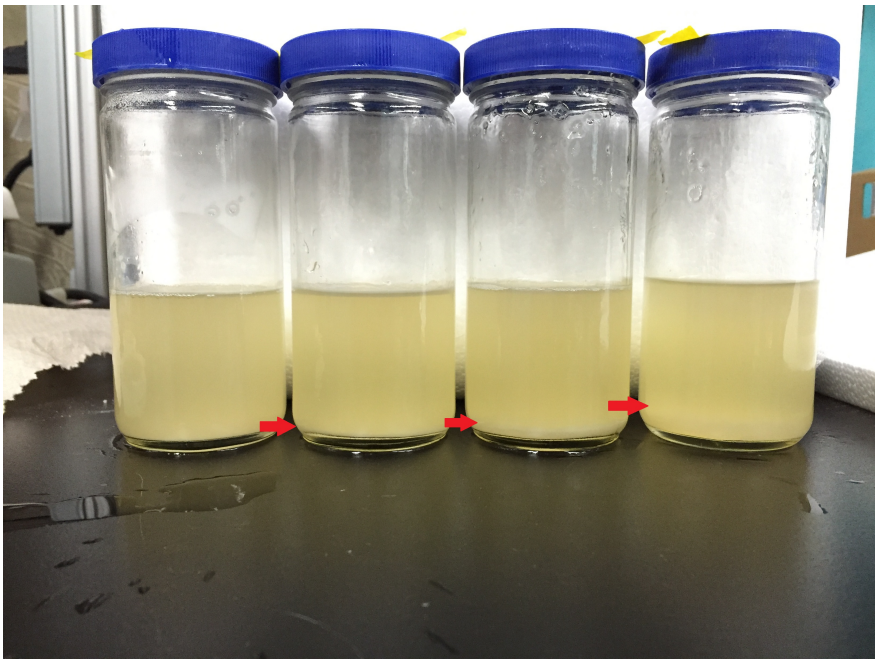

Figure 17: Profile view of all bottles. From left to right: Control, 1g gelatin, 2.5g gelatin, 5g gelatin. Note the layers on the bottom of the jars for 1g, 2.5, and 5g gelatin and the overall turbidity of the control jar.

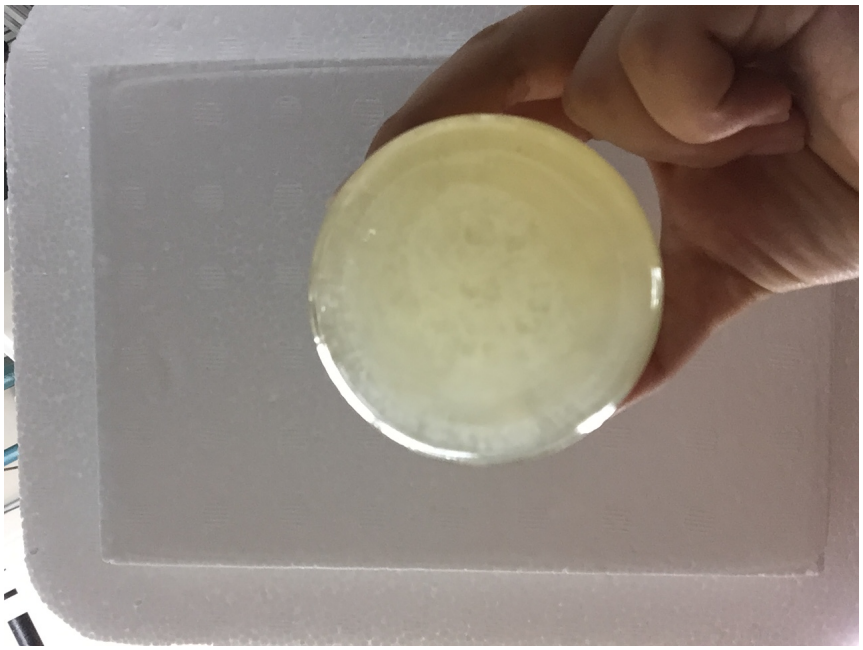

Figure 18: Bottom view of the 5g gelatin bottle after 48 hours of incubation. The bottom of the jar is more turbid overall and the individual colonies that were able to be seen before are no longer distinguishable.

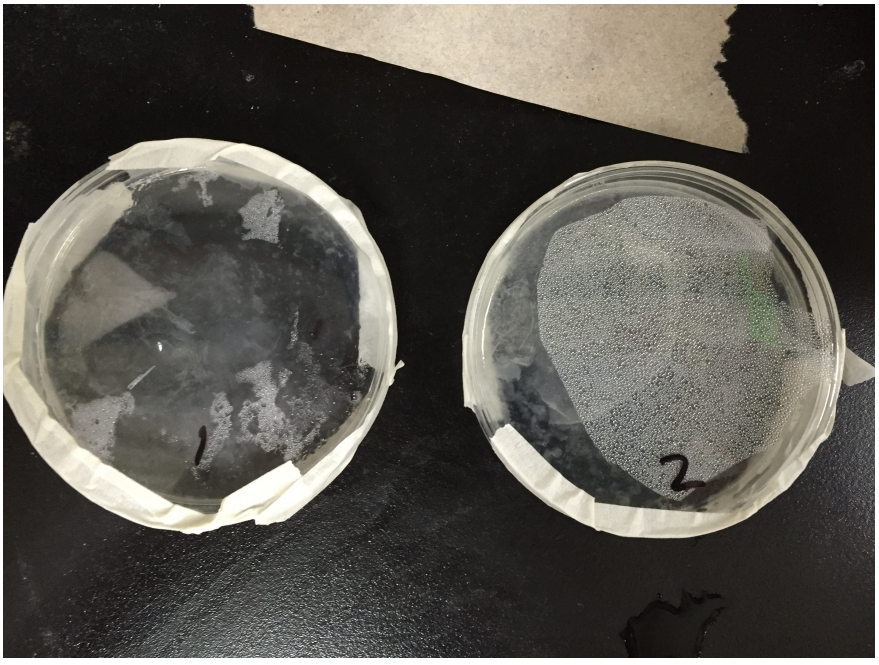

Figure 19: Top view of the petri dishes. There is a lot of condensation visible on dish 2 and the white bacterial films are visible in dish 1.

Fifth Iteration

Description

After experimenting with growing bacteria in media with different concentrations of gelatin, the team took a step back to focus on just gelatin. While the gelatin thickened media at the bottom of the jars in the previous iterations, the mixture was not uniformly thickened. This iteration focused on determining the necessary gelatin concentration to completely thicken a liquid.

Materials

- ProCoDA thermometer
- Tap Water
- Paragon Jars
- Styrofoam box

Experimenting with only Gelatin Procedure

This procedure required just tap water, gelatin, and the paragon jars used in the previous procedures. To test how effective different amounts of gelatin were for liquid, smaller quantities of liquid were used. 20 ml of water were added to each of four paragon jars. 20 ml were used as opposed to 100 ml to use smaller quantities of gelatin. The ratio of gelatin to water would be the same for 100 ml of water, however. For example, 1 g of gelatin in 20 ml of water would be equivalent to 5 g of gelatin in 100 ml of water. The amounts of gelatin used in each bottle were 1 g, 2.5 g, 3.75 g, and 5 g, respectively. The jars were heated to 38.5C, while ProCoDA was used to track the temperature over time. At this temperature, the gelatin was mixed with the water and allowed to melt. Once the gelatin had melted, the jars were placed in the styrofoam box incubator to cool for 24 hours.

Results

The results of the fifth iteration showed the viability of gelatin as a thickener. All four jars had a solid mixture. Therefore, all four quantities of gelatin worked well at thickening liquid. One result was surprising, however, as the 1 g of gelatin thickened 20 ml of water, but 5 g of gelatin could not completely thicken 100 ml of media in the previous iterations. This leads to a question on whether gelatin thickens water and media differently. This question leads to the next iteration.

Table 4: Fifth Iteration

Jar	24 hour Results
1g gelatin	Thick layer of gelatin
2.5g gelatin	Thick layer of gelatin
3.75g gelatin	Thick layer of gelatin
5.0g gelatin	Layer was very thick: difficult to remove from jar

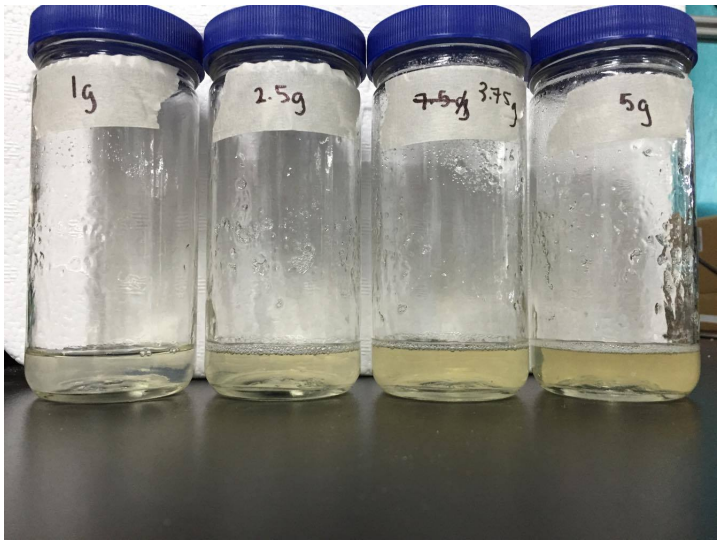

Figure 20: Side-by-side comparison of the bottles. Increasing gelatin concentration increases the darkness of the gel while thickening the sample more as well.

Sixth Iteration

Description

This purpose of the sixth iteration was to test whether gelatin thickens water and media differently. In the previous iteration, 1 g of gelatin was enough to thicken 20 ml of water. This iteration replicated the previous procedure, but with 20 ml of LB broth.

New Materials

- Petri Dish
- Three Paragon Jars
- Ingredients for LB Broth

Experimenting with Gelatin in Media Procedure

This procedure was very similar to the previous procedure. For this iteration, only two quantities of gelatin were tested. From the previous iteration, it was determined that 3.75 g and 5 g of gelatin were higher quantities of gelatin that necessary for thickening 20 ml of liquid. Therefore, only 1 g and 2.5 g were tested. A petri dish was also included in this experiment to observe the thickened media in a plate. The same procedure as the second, third, and fourth iterations was used to create the LB Broth. 20 ml of broth and tap water were then added to three jars. One of these jars was used for the petri dish, as a petri dish cannot be heated using a hot plate. The three jars were heated to approximately 38.5 C, and then 1 g, 2.5 g and 1 g of gelatin were added to each, respectively. The temperature was once again measured using ProCoDA.

After the gelatin melted in the broth, the contents of the third jar were poured into the petri dish. The two other jars and petri dish were then placed in the styrofoam box to cool for 24 hours.

Results

The results of this iteration were highly informative. Both jars and the petri dish had solid media, which indicated that the gelatin worked regardless of whether being mixed with water or media. Both 1 g and 2.5 g of gelatin thickened 20 ml of media. The 1 g of gelatin also thickened the media in the petri dish. One result that was not expected was the growth of bacterial colonies. As shown in Figure 21, there are many white dots on the bottom of the jar. These dots represent colony forming units, (CFUs), and where easy to distinguish from one another. Therefore, in 20 ml of media that used tap water instead of contaminated water, 1 g of gelatin was able to isolate bacterial colonies. The 2.5 g of gelatin also thickened the media, and created, and as shown in Figure 22, a darker color than the 1 g of gelatin. Only a few bacterial colonies grew in this thickened media. A few bacterial colonies grew in the petri dish. However, these colonies were unexpected since the water used for this experiment were thought to have been sterile. The colonies could very well have been due to contamination of the water or the equipment used. Another iteration could be done in the future to determine the true cause.

Table 5: Sixth Iteration

Jar	16 hour Results
1g gelatin	Solid layer with lots of CFUs
2.5g gelatin	Solid layer with air bubble cluster
Petri Dish	Solid, distinguishable CFUs

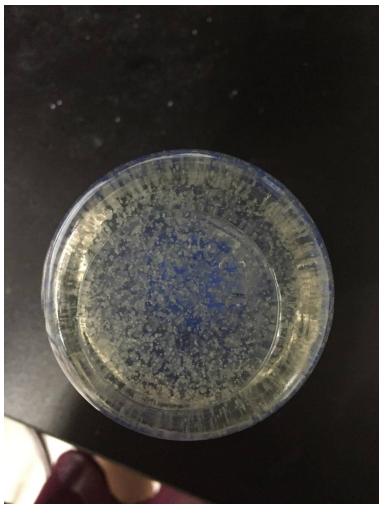

Figure 21: Bottom view of the 1g gelatin bottle with media.The many white dots are colonies that grew throughout the media.

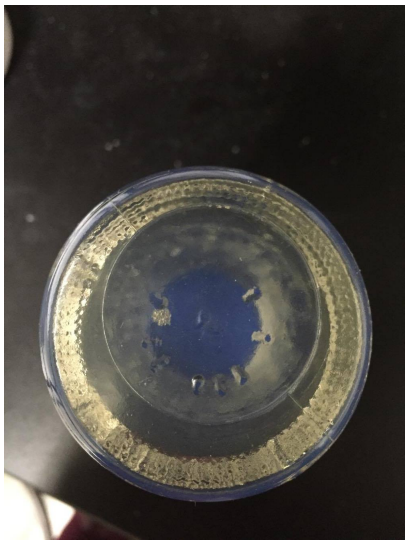

Figure 22: Bottom view of the 2.5g gelatin bottle with media.No colonies observed. The disturbance in the middle area is a cluster of air bubbles.

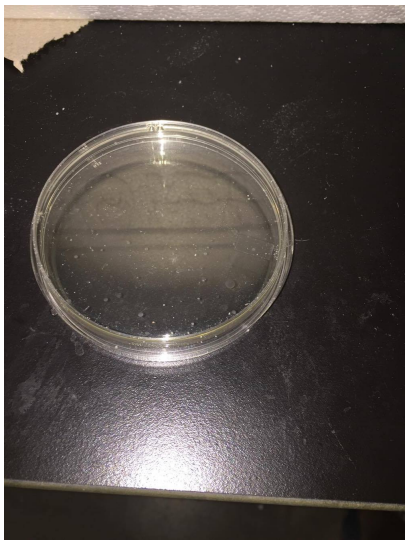

Figure 23: Top view of the 1g gelatin media petri dish. The visible white dots are bacterial colonies.

Conclusion

While keeping the ultimate goal of this team in mind, a few key points in carrying out the bacterial incubation and growth stood out. The optimal incubation time for visible and distinguishable CFUs was 24 hours. 48 hours showed to be too long and allowed multiple CFUs to combine together into larger conglomerates which defeats the team's purpose of quantifying the colonies. The

final iteration showed that spreading the media out on greater area such as a petri dish as opposed to allowing thick volumes over a small area as with the jars allowed for less colonial overlap thus CFUs were able to be distinguished from other CFUs. With testing only the gelatin, the team found that 1g of gelatin per 20ml of water was enough and possibly even more than the thickness desired. Any greater gelatin concentration would disrupt the visibility of the CFUs because the color of the media itself became darker. On top of the visibility disadvantages of excess gelatin, the media became more solid and some of the greater gelatin concentrations appeared to be too thick to allow bacterial growth. Iteration six allowed the liquid to thicken even with media. Considering the unexpected bacterial growth in the sixth iteration, petri dishes continued to prove themselves as the better option for a container because petri dishes are sterilized as opposed to the glass jars which are washed by the team members. The contamination of the jars could possibly be avoided by the implementation of an autoclave step in the cleaning process. Autoclaving involves highly pressurized saturated steam to sterilize laboratory equipment. The team was not able to test any color indicators this semester but found a few potential indicators for future semesters.Overall it seems that the team made significant progress in laying out the foundation for future the future of this subteam such that more specific and refined experiments could be carried out in the future.

Future Work

For the future, the primary goal of this subteam will be to move towards the development of the sliding colony counter itself. This semester, the subteam was able to thicken media completely with gelatin. This was one of the goals for this semester, and will lead into next semester's work. The implementation of the colony counting device will be the goal for next semester. Now that gelatin's ability to thicken media as well as the necessary amount of gelatin are known, the next goal would be to use this thickened media to actually count colonies. This would be done using an indicator to make the colonies more visible. One of the first steps for next semester would be to find the proper indicator. Once a suitable indicator is found, the team can move towards the goal of an actual sliding colony counter. If not, different methods will be researched as this subteam is important to ensuring that AguaClara is safe after plant treatment. The top options as of now are the EvaGreen dye and Colitag since they may possibly be affordable. Communication with the sellers will be sought to find out the pricing details. The contamination discovered in the last iteration must be resolved before any experimentation in the future, otherwise future experiments cannot be deemed accurate nor valid. The bacteria is thought to have been from either the tap water itself or residual bacteria left from previous iterations due to insufficient cleaning. Investigating this will most likely be a priority for next semester.

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Semester Schedule

Task Map

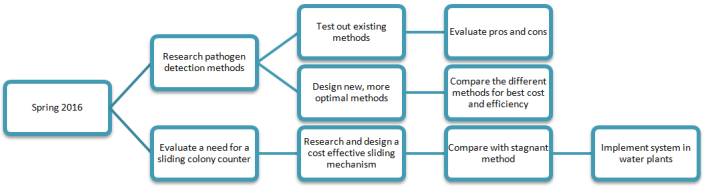

Figure 24: Task Map

Task List

1. Research methods for detecting pathogens using indicator species in drinking water that have been established and accepted by the EPA and WHO. Use resources from the EPA website, the WHO's "Guidelines for Safe Drinking Water" 4th ed., published papers on microbiological water testing.
   Target Date: 2/18/16 by Janak -Completed.

2. Determine what resources in Honduras are available to use for testing. The tests that can be done experimentally in a lab must be able to be replicated there.
   Target Date:2/18/16 Jacqueline -Completed.

3. Determine what the purpose of this project is as well as the limitations of the tests that can be run.
   Target Date: 2/22/16 by Emily -Completed.

4. Look into the possibility of using a liquid culture colony.The possibility of this method is something to discuss with Monroe.
   Target Date: 2/26/16 by Janak -Completed.

5. Compile a list of viable testing methods to research further. This list will be made through comparing indicators, prices, and incubation times of the different tests.
   Target Date: 3/1/2016 by Janak - Completed.

6. Once the list of testing methods has been analyzed completely, begin testing different methods to determine which are the most effective and efficient for the purposes of this project.
   Target Date: 3/17/2016 by Jacqueline - Completed.

7. Determine which techniques would be the most effective in meeting the requirements for our test: cheap (less than five dollars per test), functional under a wide range of environmental conditions, and easy to operate and interpret.
   Target Date: 4/7/2016 by Jacqueline - Completed.

8. Begin designing our own test based by modifying existing tests, or if the liquid culture colony approach showed promise, develop a new type of test based on this technique.
   Target Date: End of semester by Janak- Completed.

9. Use water with known levels of contamination to test this new method.
   Target Date: End of semester by Jacqueline

Report Proofreader: Jacqueline Dokko