

Monitoring of *Escherichia coli* in Jones Falls, MD: a comparison of methods – Research Paper

April 23, 2012

INTRODUCTION

Water is very essential to us especially when it is used for drinking or recreational purposes such as swimming or kayaking. It has long been established that there exists a link between fecal contamination and water-borne diseases such as cholera, viruses, harmful bacteria, parasites, and protozoa. Fecal pollution of water is a public health concern especially for those people utilizing the water for drinking or recreational purposes (Stepenuck et al., 2011). Detecting all “potential enteric” environmental pathogens in water can be expensive and difficult to isolate these pathogens and grow them in the laboratory. Therefore, using the presence of fecal indicator bacteria (FIB) like *Escherichia coli* (*E. coli*) – a common bacterium found in the gut of warm-blooded organisms, we can monitor water samples for fecal contamination. It is a coliform and contains both genes – the coliform *lacZ* gene the *uidA* gene unique only to *E. coli*. *E. coli* can be easily detected in water samples and in the environment. They are usually less risky to collect since many strains are not pathogenic, and they are easier and cheaper to grow than most pathogens. According to the US Environmental Protection Agency (US EPA), water is considered unsafe for drinking or recreational use if *E. coli* counts exceed 126 cfu/100 ml of water.

There are several *E. coli* monitoring methods like mTEC, Colilert, Coliscan® Easygel® Agar Plates from Micrology Laboratories (www.micrologylabs.com) and 3-M™ Petrifilm *E. coli*/Coliform Count (EC) Plates (www.3m.com). Both the mTEC and Colilert methods are EPA-approved for recreational waters, but are very expensive and require specialized equipments. Even though both the Coliscan® Easygel® and 3-M Petrifilm methods are not EPA-approved, these methods are used extensively by the non-profit organizations for the routine monitoring and enumeration of coliforms and *E. coli* in water samples. The focus of this project will be on comparing both these methods.

Both methods are colorimetric and have chromogenic substrates that produce different colored pigments if digested by the enzymes: β -galactosidase and β -glucuronidase. Coliform bacteria produce a pink pigment if β -galactosidase is digested and a blue pigment if β -glucuronidase is used. Since *E. coli* digest both these enzymes, it grows as dark blue or purple (red+blue) colonies in the Coliscan medium. The 3-M Petrifilm plates, in addition to indicator dyes in the medium, contain lactose. By definition, coliforms are able to ferment lactose or breakdown sugar. All coliforms have the *lacZ* gene. During fermentation, carbon dioxide is produced. Therefore, according to the manufacturer's instructions, coliform colonies appear pink with the production of gas bubbles (CO₂) and *E. coli* colonies appear blue with gas bubbles. Pink or blue colonies that do not produce gas bubbles are not supposed to be coliform & *E. coli* (www.3m.com).

The purpose of this study is to compare both the Coliscan® Easygel® and 3-M™ Petrifilm *E. coli*/Coliform methods with a focus on: accuracy in detecting true *E. coli* and total coliforms and *E. coli* counts. Proposed hypotheses are:

1. Hypothesis #1 – Both methods are identically accurate in detecting *E. coli*.
2. Hypothesis #2 – Both methods will give the same number of *E. coli* colonies.

The study is part of a larger research effort by the University of Baltimore to monitor and source track fecal contamination in the Lower Jones Falls and other tributaries of the Inner Harbor.

MATERIALS AND METHODS

Sampling: A volume of water was collected from the storm drain at Street Car Museum (SCO) on October 24, 2011 and put on ice. The samples were then taken to the laboratory as soon as possible, or within 6 hours of commencing sampling.

Processing: Samples were diluted 10-fold with sterile, distilled water, and mixed thoroughly.

Media/Plating: All plating was done in triplicates:

April 23, 2012

Coliscan Easy Gel: 1 mL of the 1/10 dilution was added to the already thawed media bottle. After addition, the media bottle was inverted gently ~25 times for thorough mixing and then the media was poured into the Easy gel plates. The plates were then swirled gently and set aside so that the media can solidify.

3-M Petrifilm *E. coli*/coliform: 1 mL of 1/10 diluted sample was added to the media. After addition, the plastic lid was rolled onto the sample to distribute the sample evenly within the circle.

Incubation: Plates were incubated at 35°C for ~24 – 48 hours.

Counting: After incubation, the plates were removed, inspected, and enumerated.

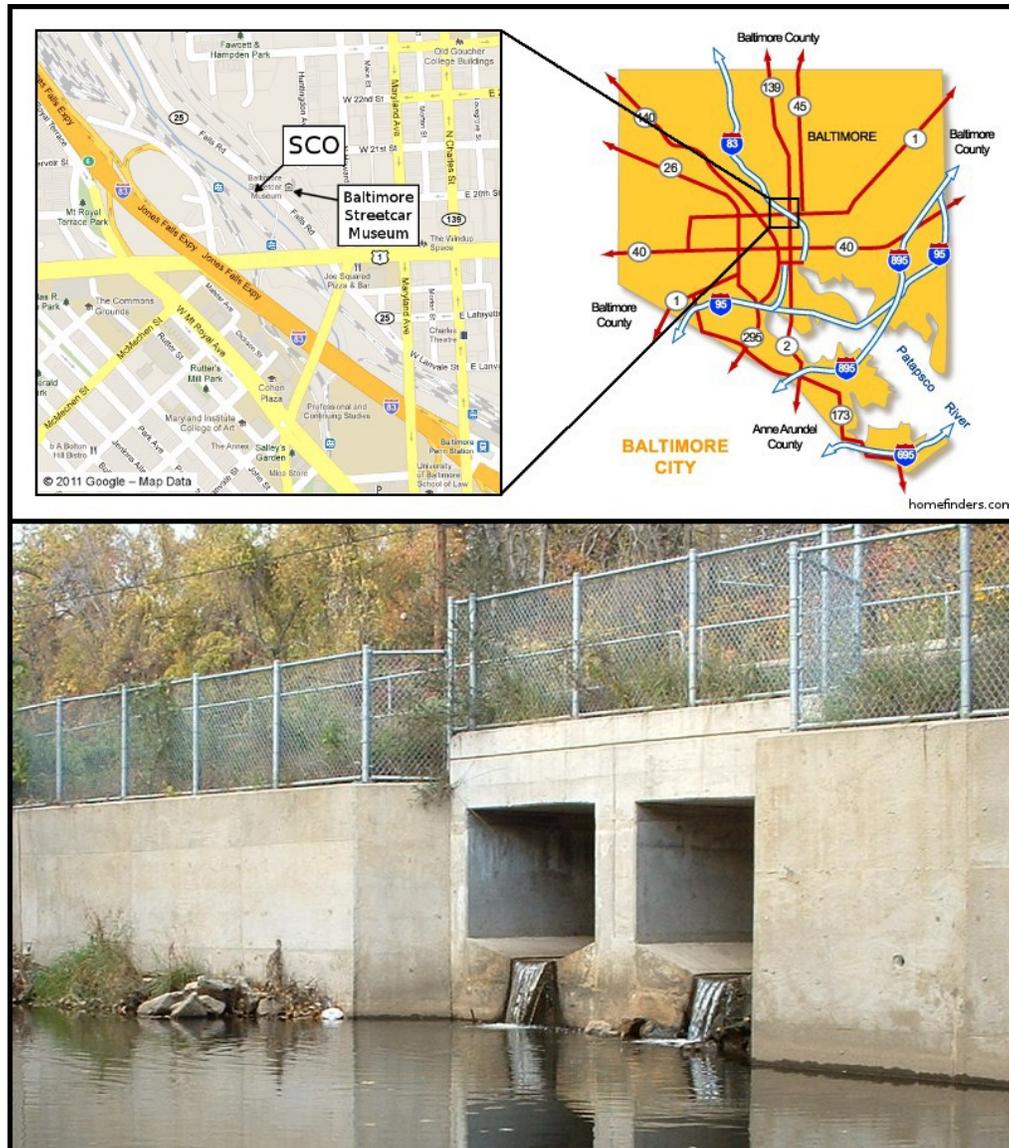


Figure 1. Storm drain outfall (SCO) at the Baltimore Streetcar Museum on Falls Road. Contamination with high numbers of *E. coli* (>10,000 cfu/100 ml of water) are regularly observed in the run off of this outfall.
Top panel: Location of the outfall;
Bottom panel: photograph of the outfall taken in November 2010.

April 23, 2012

In the Coliscan method (Figure 2), for each of the 3 plates (triplicates), all purple/dark blue colonies were counted and reported as *E. coli*. All pink colonies were counted and reported as “other coliforms”. The number of *E. coli* and “other coliforms” were added up to obtain the number of total coliforms. All blue-green, light blue, yellow, and white colonies were also counted and reported as non-coliform bacteria.

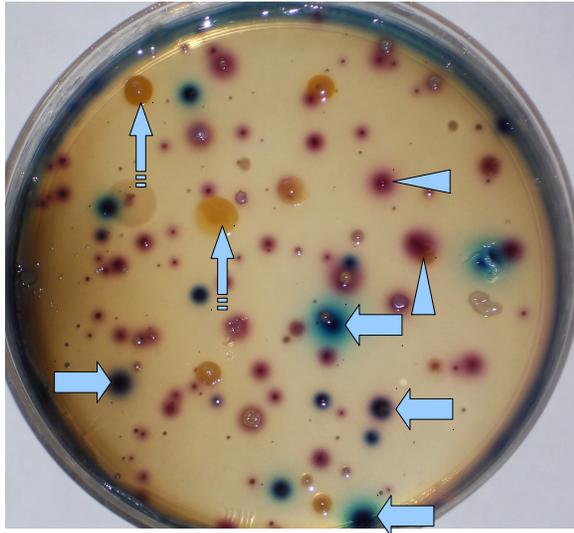


Figure 2. In this Coliscan Easygel agar plate, blue colonies presumably represent *E. coli*. Pink colonies represent “other coliforms” and white/yellow colonies represent non-coliform bacteria.

Arrows: examples of dark blue/purple colonies

Triangles: examples of pink colonies

Striped Arrows: examples of non-coliform bacteria

In the 3-M™ Petrifilm *E. coli*/Coliform Count (EC) Plates (Figure 3), for each of the 3 plates (triplicates), with the aide of a counting light box with a grid system, all blue colonies that produced gas bubbles were counted and reported as *E. coli*. All pink colonies that produced gas bubbles were counted and reported as coliforms. Those blue/red colonies that did not produce gas bubbles were separately counted and reported as non-coliform bacteria. The number of *E. coli* and coliforms were added up to obtain the number of total coliforms.

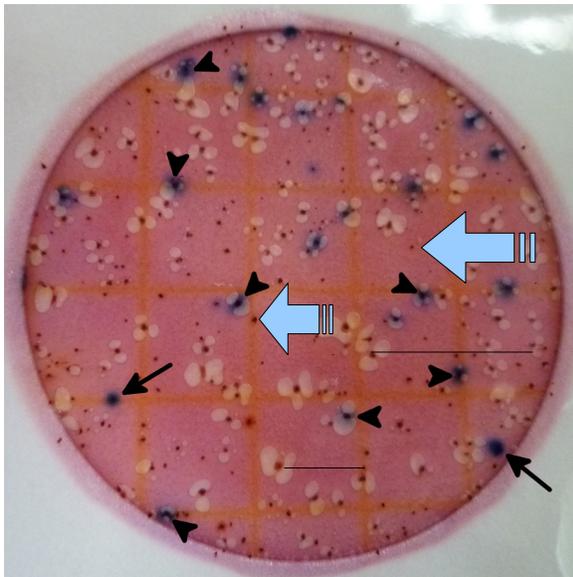


Figure 3. Blue colonies with gas bubbles presumably represent *E. coli*. Blue colonies without gas formation represent non-*E. coli* bacteria.

Arrow heads: examples of colonies with gas bubbles

Arrows: examples of blue colonies w/o gas bubbles

Lines: examples of pink colonies with gas bubbles

Striped Arrows: examples of pink colonies w/o gas bubbles

DNA Extraction and PCR amplification. Sampling was done in October when dark blue or purple colonies were less plentiful on plates. After enumeration, selected colonies were isolated, resuspended in sterile PCR grade water (100 µl), and lysed by boiling and freeze-thawing. Lysates were tested for *E. coli* with both the *E. coli uidA* gene (only found in *E. coli*) and the multiplex PCR assays targeting the *lacZ* and *uidA* genes regions. To verify whether the *uidA* negative samples contained enough DNA template and/or no PCR inhibition prevented them from amplifying, these isolates were tested using microbial small subunit (SSU) assay that amplifies the 16S rRNA ribosome gene of bacteria and therefore can be used to detect bacteria in a sample. PCR amplification was performed by using a DNA thermal cycler and Choice™ *Taq* DNA Polymerase (Denville Scientific Inc., South Plainfield, NJ). The PCR solution used contained **1x PCR reaction** (10x buffer contains 100 mM KCl, 100 mM HCl [pH 9.0], final concentration MgCl₂ of 15 mM), 200 µM of each dNTP, 0.5 µM of each of the primers, 0.5 Units (U)/µl of *Taq* (*Thermus aquaticus*) DNA polymerase, and nuclease-free PCR grade dH₂O. The template volume used was 2 µl in a final PCR reaction volume of 20 µl.

Universal primers 8-Fm (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492-Rm (5'-GNTACCTTGTTACGACTT-3') located on the 16S rRNA gene were used to test for the presence of amplifiable microbial small subunit (SSU) DNA. The PCR cycling conditions were: template DNAs were denatured at 94 °C for 5 min, followed by 1 cycle each of 94 °C for 1 min, 65-55 °C for 1 min, 72 °C for 3 min for a total of 10 cycles, followed by another 20 cycles of 94 °C for 1 min, 65-55 °C for 1 min, 72 °C for 3 min, and a final extension step of 72 °C for 7 min. The positive control was 11/072911 and nuclease-free water was the negative PCR control. The expected amplicon size was ~1500 bp.

Primers UAL-1939 (5'-TATGGAATTCGCCGATTTT-3') and UAR-2105 (5'-TGTTTGCCTCCCTGCTGCGG-3') were used to amplify *E. coli uidA* gene. A GC clamp was attached to the 5' end of primer UAL-1939, leading to UAL-1939GC (Farnleitner et al. 2000). The PCR cycling conditions were a touchdown approach of 30 cycles as follows: genomic DNA was denatured at 94°C for 5 min, followed by 5 cycles of 92°C for 1 min, 65°C for 1 min, and 72°C for 1 min 30 sec, 5 cycles of 92°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 sec, and 20 cycles of 92°C for 1 min, 50°C for 1 min, and 72°C for 1 min 30 sec with a final extension step of 72°C for 7 min. The positive control was 11/072911 and nuclease-free water was the negative PCR control. The expected amplicon size was ~147 bp.

For the multiplex PCR, a pair of 24-mer primers ZL-1675 (5'-ATGAAAGCTGGCTACAGGAAGGCC-3') and ZR 2025 (5'-GGTTTATGCAGCAACGAGACGTCA-3') located within the coding region of the *lacZ* gene of *E. coli* and a pair of 20- and 21-mer primers UAL-754 (5'-AAAACGGCAAGAAAAAGCAG-3') and UAR-900 (5'-ACGCGTGGTTACAGTCTTGC G-3') located within the *uidA* structural gene of *E. coli* were used. The PCR cycling conditions used were: template DNAs were denatured at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, primer annealing at 50°C for 1 min, and DNA extension at 72°C for 1 min 30 sec with a final extension step of 72°C for 7 min. The expected amplicon sizes were ~147 bp (coding region of the *E. coli uidA* gene) and ~264 bp (shorter region of the *E. coli lacZ* gene). The positive control was the known *E. coli* isolate and nuclease-free water was the negative PCR control.

Detection of amplified DNAs. PCR-amplified DNAs were detected using 1.5% agarose gel electrophoresis. Environmental samples for 1x PCR reaction were run in a 0.5x TAE buffer (5 mL of 50x TAE buffer [242 g Tris Base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH 8.0), adjusted with dH₂O to 1 L, and autoclaved at 15 psi for ~20 minutes] and 495 mL of dH₂O). The gels were stained in ethidium bromide solution (0.5x TAE buffer with a final concentration 0.5 mg/L EtBr), PCR products were visualized with a UV transilluminator to confirm the presence of amplicons with appropriate bp size, and photographed.

Statistical Analyses. To compare the PCR results between both methods for accuracy, statistical analysis using the Fisher's Exact Test was performed. To compare *E. coli* counts, a t-test was performed. A P value ≤ 0.05 was used as the indicator of significance.

RESULTS AND DISCUSSION

Testing for accuracy in both methods in truly identifying putative *E. coli* colonies (PEC) using molecular methods (i.e. PCR-based assays specific for *uidA* and *lacZ* genes (only present in *E. coli*) and microbial SSU DNA)

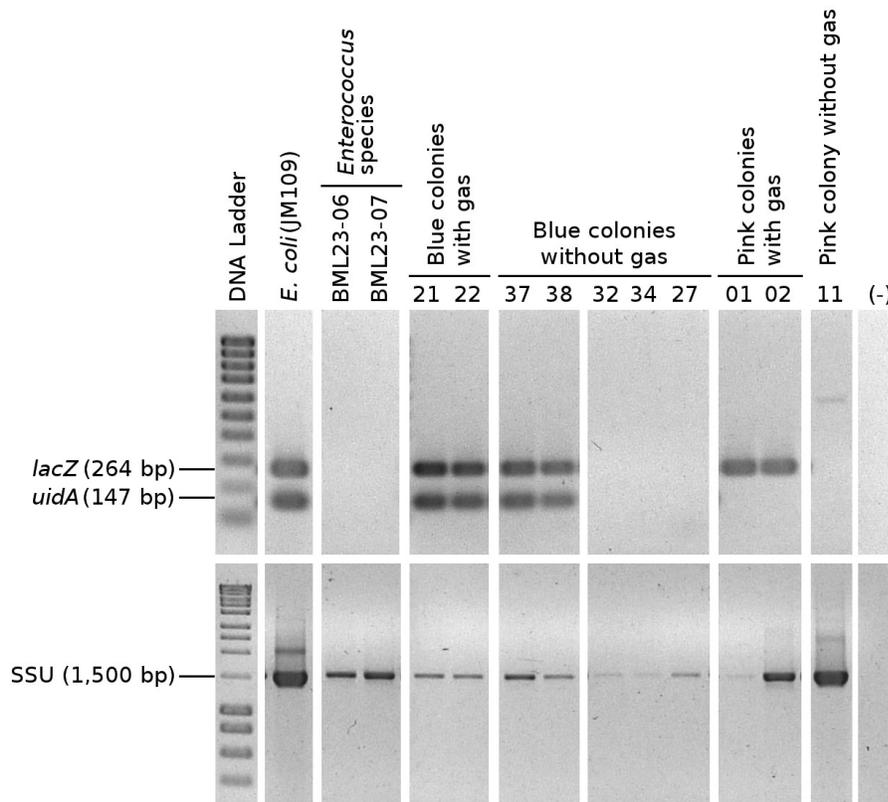


Figure 4. In the top gel, isolates from the 3-M Petrifilm method, with and without the production of gas bubbles, were tested for *E. coli*. The second lane (JM109) is bonifide *E. coli*, and as expected, it amplified at both the *E. coli uidA* (~147 bp) and *lacZ* (~264 bp) genes and is truly *E. coli*. In the next two lanes no amplification is seen (as expected) because these samples are *Enterococcus* species and not *E. coli*. The next two samples (21 & 22) are blue colonies with gas, and as expected, amplifications are seen at both bp. Therefore, these isolates are truly *E. coli*. The next five isolates are blue colonies w/o gas, and according to the manufacturer's instructions, they should not be *E. coli*. However, amplifications are seen at both bp for 2 of the 5 isolates. Therefore, these isolates (37 & 38) are truly *E. coli* and good examples of underestimation of *E. coli* counts or false negatives. To verify whether those isolates (32, 34, & 27) that did not amplify at all had enough template DNA and/or no PCR inhibition prevented them from amplifying, they were tested using microbial small subunit (SSU) assay that amplifies the 16S rRNA ribosome gene of bacteria. As seen in the bottom gel, all isolates, in the same order, amplified (some strong and some faint or weak) at the expected bp of ~1,500 bp. This implication is important because since these three isolates tested negative with the *uidA* and multiplex PCR assays, but tested positive for microbial SSU DNA, indicating that enough DNA template is present, and since it was determined from previous results (not shown) that the SSU PCR assay is less sensitive than the multiplex, these samples are truly not coliforms and *E. coli*. The next 2 isolates (01 & 02) are pink colonies with gas, and since all coliforms contain the *lacZ* gene, only 1 amplification is seen at ~264 bp supporting the fact that these samples are truly coliforms (as expected). The next sample (11) is a pink colony w/o gas bubbles. It is a non-coliform bacterium because a very faint or weak amplification was seen of larger bp size. This template should not have amplified at all and why it did is unknown and needs to be investigated. To determine this isolate's genus, it must be sent for DNA sequencing. The last lane is the negative control that contain only PCR grade dH₂O and no amplification is seen (as expected).

3-M PETRIFILM COUNT

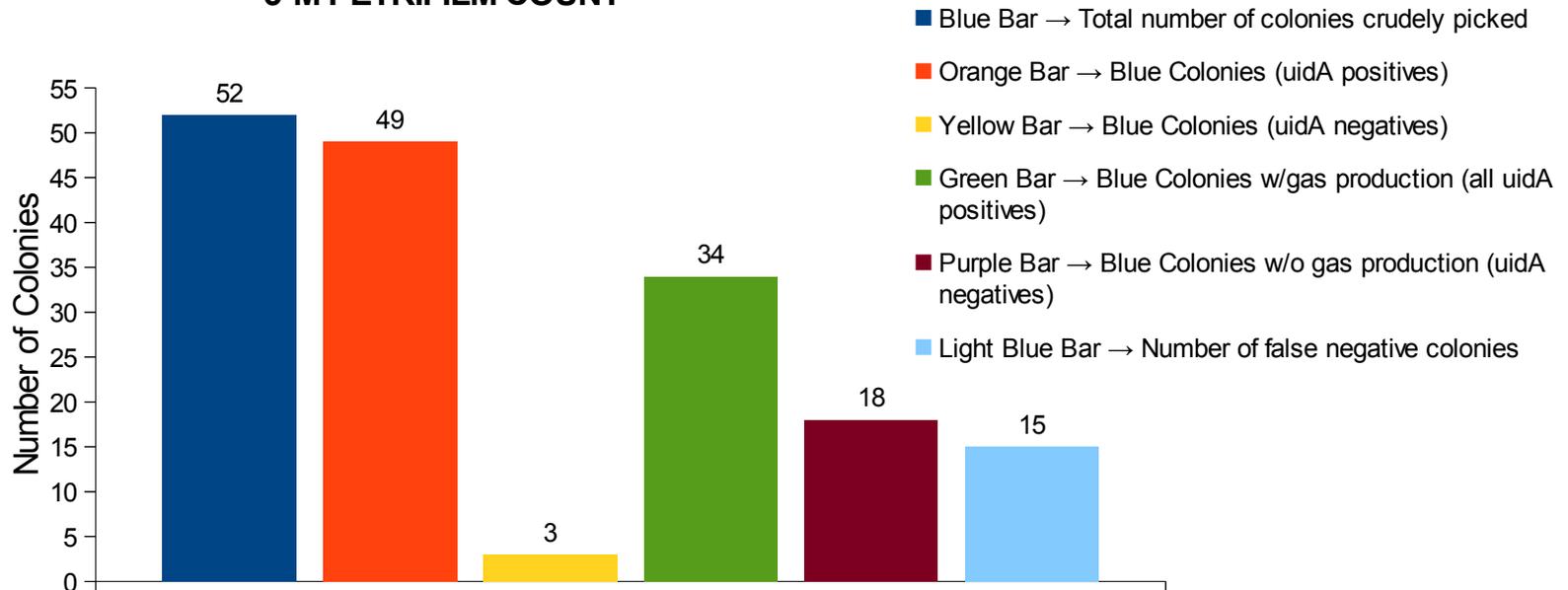


Figure 5.

- As seen in **Figure 5.**, a total of 52 blue colonies were tested.
- 34 (65%) of the 52 blue colonies produced gas.
- 18 (35%) of the 52 blue colonies did not produce gas.
- 49 (94%) of the 52 blue colonies were *uidA* positive and confirmed as *E. coli*.
- 34 (100%) of the 34 gas producing blue colonies were *uidA* positive.
- 15 (83%) of the 18 non-gas producing blue colonies were *uidA* positive and 3 (17%) were *uidA* negative.
- The use of color and gas as the discriminatory criteria to identify *E. coli* colonies would result in **15 (31%) false negative** colonies.
- If color alone were to be used, 3 (6%) colonies would be false positive.

Coliscan Easygel Count

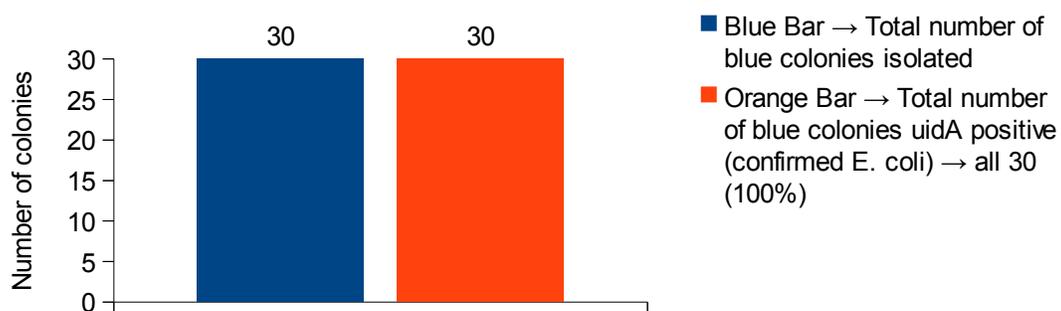


Figure 6.

- As seen in **Figure 6.**, a total of 30 blue colonies were tested.
- 30 (100%) of the 30 blue colonies were *uidA* positive and confirmed as *E. coli*.

The production of gas bubbles should not be used to identify *E. coli* since an underestimation of *E. coli* counts may result. Comparing the PCR results of both methods using the Fisher's Exact Test, the results revealed that the association between the methods and *E. coli* and non-*E. coli* colonies is considered to be not statistically significant. If color alone is used as the discriminatory criterion, both methods have the same accuracy in detecting *E. coli*.

Microbial Analysis Tables

From 3M Plate Method

| Plate# | Pink colonies (coliform bacteria) with gas | Pink colonies (coliform bacteria) without gas | Blue colonies (<i>E. coli</i>) with gas | Blue colonies (<i>E. coli</i>) without gas | Total Count |
|--|--|---|---|--|-------------|
| 1 | 75 | 100 | 29 | 5 | 209 |
| 2 | 84 | 152 | 22 | 7 | 265 |
| 3 | 75 | 173 | 26 | 11 | 285 |
| Average (if color + gas production are used) | 78 | 141.67 | 25.67 | 7.66 | 253 |

| | | | | |
|--|---------|---------|--------|-------|
| Average (only color is used) | 219.67 | | 33.333 | |
| Cfu/100 ml* (if color + gas production are used) | 78,000 | 141,667 | 25,667 | 7,660 |
| Cfu/100 ml* (only color is used) | 219,667 | | 33,333 | |

(# of colonies)*(10x dilution factor)*(100 ml/volume plated)

From Coliscan Method

| Plate# | Pink colonies (coliform bacteria) | Blue/Purple colonies (<i>E. coli</i>) | Total Count |
|-------------|-----------------------------------|---|-------------|
| 1 | 230 | 18 | 248 |
| 2 | 224 | 24 | 248 |
| 3 | 233 | 28 | 261 |
| Average | 229 | 23.333 | 252 |
| Cfu/100 ml* | 229,000 | 23,333 | |

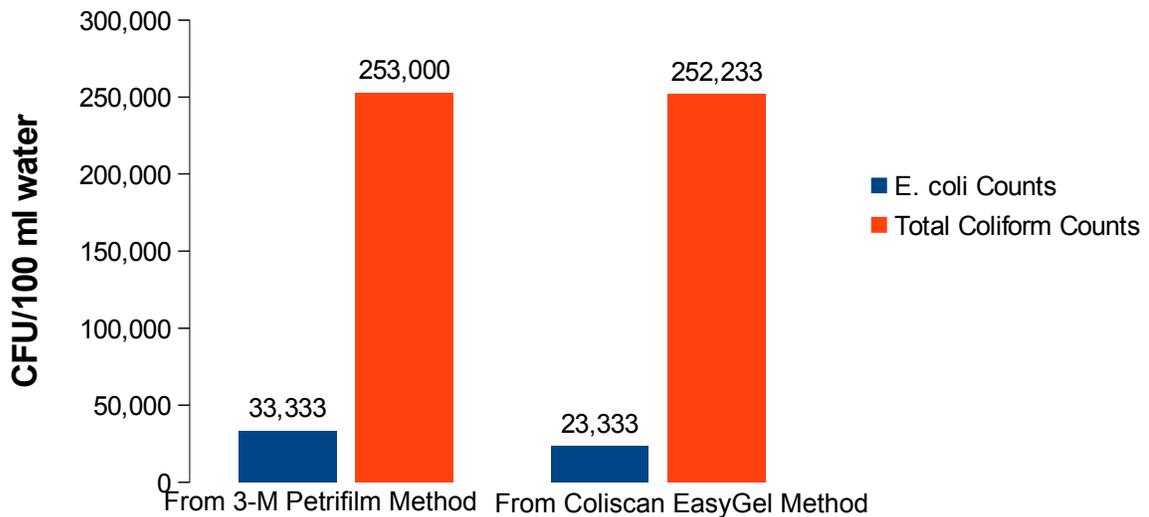
(# of colonies)*(10x dilution factor)*(100 ml/volume plated)

Figure 7.

Figure 7. shows total coliforms and *E. coli* counts table for both methods and **Figure 8.** shows its graph.

Figure 8.

COMPARISON OF *E. coli* and TOTAL COLIFORM COUNTS



April 23, 2012

- Seen in **Figure 7.**, the average sample mean (\bar{x}_1) from the triplicate plates (102411, 10 fold dilution) of the 3-M™ Petrifilm method, based on color and gas bubbles, was 33.333. The sample variance (s^2_1) was 16.3 and the cfu/ml was 33,333 cfu/100 ml of water.
- The average sample mean (\bar{x}_2) from the triplicate plates (102411, 10 fold dilution) of the Coliscan® EasyGel® method was 23.333. The sample variance (s^2_2) was 25.3 and the cfu/ml was 23,333 cfu/100 ml of water.
- After performing a t-test to compare the *E. coli* counts, the results revealed that the 3-M Petrifilm method identified more *E. coli* (10,000 cfu/100 ml water) than the Coliscan Easygel method and statistical difference exists between the methods for the given degree of freedom (df) = 5 → $t = 2.67$, $p < 0.05$, $df = 5$
- However, both methods have very close total coliforms counts (**Figure 8.**): 3-M Petrifilm → 253,000 cfu/100 ml water and Coliscan EasyGel → 252,333 cfu/100 ml water – a difference of only 667 cfu/100 ml water. After performing a Fisher's Exact Test, the results revealed that this difference is not statistically significant.
- Some possible explanations as to why the 3-M Petrifilm method identified more *E. coli* colonies than the Coliscan Easygel could be:
 - Maybe some dark pink colonies counted as coliforms could be potential *E. coli* with low *uidA* gene activity.
 - Either the *E. coli* counts in 3-M Petrifilm method were overestimated or they were underestimated with the Coliscan Easygel method.
 - It could also be that the agar media in the Coliscan Easygel method did not promote the growth of *E. coli* as well as the 3-M Petrifilm method did.
 - Also, the sample was taken straight from the storm drain (a good source of contamination), but not from the stream meaning that it is not really an environmental sample.

Conclusion

In conclusion, the results showed that gas production should not be used to identify *E. coli* colonies because an underestimation of *E. coli* counts may result. If color alone is used, both the Coliscan® EasyGel® and 3-M Petrifilm methods are equally accurate in detecting *E. coli* thereby supporting Hypothesis #1. Colonies in the 3-M Petrifilm method, if both color and gas production were used as the discriminatory criteria to identify *E. coli*, yielded 31% (15/49) false negative colonies (underestimation of *E. coli* counts). With color alone there was only 6% false positive colonies and no false negative. In addition, the 3-M Petrifilm method had higher *E. coli* counts (33,333 cfu/100 ml of water) versus 23,333 cfu/100 ml of water with the Coliscan® EasyGel® (a difference of 10,000 cfu/100 ml water). The t-test results revealed that statistical difference exists between the methods. Yet, the total number of coliforms in both methods were approximately the same. Since the *E. coli* colony counts is not the same in both methods, Hypothesis #2 is rejected by the results. To address this issue of difference, the follow up project should be to pick as many pink colonies from the Coliscan method to see if they were misidentified as non-*E. coli* (false negatives) or whether more false positive colonies exist with the 3-M Petrifilm method than previously thought, and compare the results with the EPA-approved mTEC method for similar results. The 3-M Petrifilm method is an economical *E. coli* monitoring kit to assess the quality of local waterways in a cost-effective manner and is much easier to use and interpret when compared to the Coliscan Easygel method.

References

- Bej, A. K., Steffan, R. J., DiCesare, J., Haff, L., & Atlas, R. M. (1990). Detection of coliform bacteria in water by polymerase chain reaction and gene probes. *Applied and Environmental Microbiology*, 56(2), 307-314. doi: 0099-2240/90/020307-08\$02.00/0
- Bej, A. K., DiCesare, J. L., Haff, L., & Atlas, R. M. (1991). Detection of *Escherichia coli* and *Shigella* spp. in water by using the polymerase chain reaction and gene probes for *uid*. *Applied and Environmental Microbiology*, 57(4), 1013-1017. doi: 0099-2240/91/041013-05\$02.00/0

April 23, 2012

- Bej, A. K., McCarty, S. C., & Atlas, R. M. (1991). Detection of coliform bacteria and *Escherichia coli* by multiplex polymerase chain reaction: Comparison with defined substrate and plating methods for water quality monitoring. *Applied and Environmental Microbiology*, 57(8), 2429-2432. doi: 0099-2240/91/082429-04\$02.00/0\$02.00/0
- Belt, K. T., Hohn, C., Gbakima, A., & Higgins, J. A. (2007). Identification of culturable stream water bacteria from urban, agricultural, and forested watersheds using 16S rRNA gene sequencing. *Journal of Water and Health*, 5(3), 395-406.
- Farnleitner, A. H., Kreuzinger, N., Kavka, G. G., Grillenberger, S., Rath, J., & Mach, R. L. (2000). Simultaneous detection and differentiation of *Escherichia coli* populations from environmental freshwaters by means of sequence variations in a fragment of the β -D-glucuronidase gene. *Applied and Environmental Microbiology*, 66(4), 1340-1346. doi: 0099-2240/00/\$04.0010
- Stepenuck, K. F., Wolfson, L. G., Liukkonen, B. W., Iles, J. M., & Grant, T. S. (2011). Volunteer monitoring of *E. coli* in streams of the upper midwestern United States: A comparison of methods. *Environment Monitoring Assess*, 174, 625-633. doi: 10.1007/s10661-010-1483-7

ACKNOWLEDGEMENTS

Special thanks to both Dr. Wolf Pecher and Dr. Stanley Kemp for allowing me to partake in their researches and providing me with all the tools necessary to obtain my results. Moreover, without the invaluable constructive criticism, assistance, and guidance of Dr. Pecher this paper would not be possible.