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Comparison of fecal coliform enumeration methods for field analysis in resource limited regions

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ABSTRACT

Fecal enumeration is a key factor in determining the quality of a water source. Accuracy and reliability of fecal analysis is dependent upon several variables, viz. incubation temperature, specific conductivity, pH, nutrient availability, population density, suspended solids concentration, etc. The goal of this study was to determine if a correlation exists between the three common field water testing methods (Easy-Gel method, Millipore^{TM} Membrane Filtration method and the 3M^{TM} Petrifilm method) of fecal coliform enumeration and temperature fluctuation or population density. Field test methods were examined using varying incubation temperatures and population densities. Results of fecal enumeration from each of the field test methods were compared to a standard test method (MF-8074) under standard operating conditions. Regression correlations for each of the field test methods were establish to determine method reliability under non-ideal conditions. Study results indicated that temperature fluxuations and population density did adversely affect the accuracy of fecal enumeration on all field test methods.

Keywords: drinking water, emerging regions, fecal coliform, surface water, water quality testing

INTRODUCTION

Water quality is a key aspect in human health and hygiene around the world, especially in regions without access to safe drinking water. According to the World Health Organization [1], globally, diarrheal diseases are the cause of two million deaths annually as a result of poor water quality [1]. Therefore, it is essential that drinking water be tested for diarrheal disease causing microorganisms and the quality improved to reduce the number of deaths. Waterborne disease is a result pathogenic bacteria from infected warm-blooded animals like humans, domesticated animal, farm animals, and wildlife entering the environment [1, 2]. A common indicator of pathogenic bacteria is fecal coliform. Fecal coliform are microorganisms that originate from the intestines of human or animal and can indicate the possible presence of disease causing microorganisms. Fecal contamination can arise from a variety of sources such as contaminated irrigation or runoff, animal feedlots, and other sources. *Escherichia coli* (*E. coli*) is a key organism that makes up fecal coliform and its presence can indicate the potential for existence of pathogenic organisms [2, 3]. Fecal contamination can washed into lakes, rivers, ponds and other forms of surface water and even ground water during rainfall, snow melts and other forms of precipitation [2]. Therefore, *E. coli* infection is usually transmitted through consumption of contaminated water or food [1]. When *E. coli* is ingested it has the common side effects of abdominal cramps, fever, vomiting and diarrhea which can be bloody. Some patients recover within 10 days, but in many cases, the disease may become life-threatening and ultimately lead to death [1].

In resource-limited regions, access to costly water quality testing instrumentation, reliable electrical power or highly variable pathogen loading; it is important to understand the limitations of fecal coliform test methods so that accurate pathogen counts can be reported. When trained members of a NGOs or student group travel to remote areas to complete water quality analysis; variability in the results mislead the groups into either over- or under-reporting the pathogen threat. Therefore, the goal of this study was to determine the impact of non-ideal incubation and environmental conditions, viz., incubation temperature (variability that might occur as a result of unreliable electricity), inoculum concentration (variability that

might occur as a result of variable pathogen concentration), and water composition (variability that might occur under different field conditions). In this study, we examined at three common water-testing methods (Easy-Gel method, Standard Methods 9222SD Millipore™ Membrane Filtration method and the 3M™ Petrifilm method). These test methods are classified as enumeration methods, which by definition, are distinctly different from presence/absence (P/A) methods. While P/A methods only identify the potential presence of potentially pathogen microorganisms, enumeration methods are used to quantify the concentration of potentially pathogenic microorganism. The Standard Methods 92222D Millipore™ Membrane Filtration (SM-9222D) method is the most recognized and accepted industry standard for potential pathogens detection in the United States. The Petrifilm (PF) method and Easygel (EG) method are simple, user-friendly methods, and individuals can be trained to use these methods to check the water quality on a regular basis [4]. When first introduced, these methods were reviewed by several researchers for accuracy under ideal laboratory conditions [5-7] as well as ideal field applications [8-11]. Herein, the United States Environmental Protection Agency's Membrane Filtration Method (MF-8074), was used as a control method and compared to the three test methods to in order to determine the most reliable and accurate water testing method in emerging regions.

METHODS

Colony Enumeration using USEPA Membrane Filtration Method

To determine the accuracy of each method, a control method (MF-8074) was also used. Fecal contamination analysis using MF-8074 [12] for inoculum loading, incubation temperature and enumeration was completed simultaneously with the three test methods. Raw waste water, collected from the Norman Water Reclamation Facility (Norman, Oklahoma, USA), was used with the MF-8074 as a controlled pathogen group. The use of raw waste water eliminates/reduces the unknown parameters except for concentration which could be determined via serial dilution. The final dilution set had a concentration of colonies between 20 to 80 colony-forming units (CFU) [13]. Each sample was incubated for 24 ± 2 hours at 44.5˚C ± 0.2˚C using a solid state incubator. When the CFU was too numerous to count (TNTC), such in the experiments of overloading, additional analyses were subsequently completed with serial dilutions until the concentration was within the required range.

Impact of Temperature on Enumeration of Coliform

The recommended temperature for both PF and EG is 35 ℃; while SM-9222D requires an incubation temperature of 44.5 ± 0.2 ℃. In the case of EG an extended incubation time is recommended when an incubator is not available with the assumed temperature of 25 ℃ [5]. Therefore, four temperatures were selected to develop a correlation based on incubation temperatures. For each temperature the SM-9222D, PF and EG methods were analyzed and compared to MF-8074 counts at standard temperature and time. Each analysis was completed in three sets of triplicates (i.e., nine unique experiments) with three plates per method counted for an average CFU/100mL [13]. Therefore, 27 CFU plates were examined for each experimental temperature (25˚C, 30˚C, 35˚C, and 44.5˚C) using a convection oven calibrated at ±0.5˚C. The control group was also completed in three sets of triplicate, and incubated at standard temperature in a solid state incubator calibrated at ±0.2 ˚C. All plates were enumerated for CFU/100mL.

Impact of Concentration of Fecal Coliform on Enumeration of Coliform

The recommended sample loading for both PF is 1 mL; while EG uses 0.5 mL to 5.0 mL of sample. SM-9222D specifies a CFU range similar to that of the control method. To evaluate the impact of inoculum concentration, four different concentrations were analyzed for the SM-9222D, PF and EG methods. Completed in three sets of triplicates, water samples using 0.5X, 1.0X, 1.5X, 2.0X (value multiplier of standard inoculum loading) were examined. The control method was MF-8074 with 1.0X inoculum. Fresh samples were collected for this series of analyses and the fecal coliform was enumerated using MF-8074. For each inoculum multiplier, the SM-9222D, PF, and EG methods, along with the control group, were incubated for 24 hours at 44.5˚C ±0.2˚C or for 24 hours at 35.0˚C ±0.2˚C (depending on each method's requirement) using a solid state incubator. CFU/100mL counts were analyzed and compared to the controlled group.

Impact of Composition of Surface Water on Enumeration of Coliform

The impact of total suspended solids (TSS) of surface water sources on enumeration of coliform was examined. Similar to the concept of bacterial mass loading, TSS within the sample could also reduce the ability of a single bacterium to make contact with the media, thereby resulting in inaccurate CFU. To examine this possible impact, three different types of surface water were sampled. The three types of surface water include lentic and lotic water sources. The surface water samples were collected as grab samples from a large reservoir (>21 km^2); a small detention pond (<1 km^2) and a second-order stream (Norman, Oklahoma,

USA). TSS was measured in three sets of triplicate (i.e., nine unique experiments) following Standard Methods 2540 [14]. Only a correlation between the fecal coliform test methods was examined because MF-8074 does not compensate for TSS concentration. The TSS levels were the same during each test. Incubation temperature and inoculum concentration for all test methods followed standard incubation and standard inoculum values.

Statistical Analysis and Correlation Development

Colony counts from triplicate plates for each experimental set completed in triplicate were converted to CFU/100 mL of sample and used in an analysis of variance and Student-Newman-Keuls test to determine significant differences among the test methods. The statistical analysis used to develop correlation between the MF-8074 and the three experimental groups (SM-9222D, PF, and EG) were determined using the Pearson Product Moment Correlation Coefficient, (*r*). This is a measure of the strength of the linear relationship between the control group and the three experimental groups. If the value of *r* is near or equal to zero then it can be determined that there is no linear relationship, viz., there is no correlation. If the value of *r* is closer to 1 or -1, then there is a stronger relationship between the variables. Positive values for *r* imply that as the *y* value increases, the *x* value decreases. Similarly, negative values for *r* imply that *y* value decreases as *x* value increases [15].

RESULTS AND DISCUSSION

Impact of Temperature

Colony counts for each temperature (growth at 25˚C, 30˚C, 35˚C, and 44.5˚C) using each test method for both a 24 hour (*Figure 1*) and 48 hours (*Figure 2*) incubation period were compiled and the mean and standard deviation of each method with each temperature was determined [13, 16]. For a 24 hour incubation time, there was no observed growth for CFU at 25˚C for PF (*Figure 1*). Based on an analysis of variance between the control (MF-8074 at 44.5˚C) and the various test methods at different incubation temperatures, there was a significant difference ($P<0.05$) among the incubation temperature and methods as compared to the control except for SM-9222D-44.5˚C. This is a result of the methods' similarity. Using a Pearson Product moment and a correlation significance of 0.01 level (2-tailed) and 99% confidence interval, no correlation was observed with incubation temperature of any of the test methods. However, a significant positive correlation for EG germane to increased

incubation temperature was observed. Based on these results, it was concluded that these two test methods viz., EG and PF, were significantly influenced by incubation temperatures and thereby reducing their accuracy in field studies where constant incubation temperature is not possible. For EG and PF, the most accurate incubation temperature was at 35˚C; which corresponds to the manufactures' recommendation. These results are consistent with other studies [17, 18]. Aziz et al., [17] noted that at incubation temperatures below 30˚C, the growth is under-report; and above 45˚C, the temperature is too high for accurate counts.

As a result of slower growth at lower temperature, another experimental set was examined with an incubation time of 48 hours. Although additional growth was observed for PF-25˚C, no significant difference (P<0.05) was observed as compared with the 24 hour incubation time. However, increased variability in enumeration was observed. From this, it was concluded that additional incubation time could not compensate for lower growth temperature and may lead to inaccurate reporting. Therefore, if incubation ovens were not maintained at a mid-to-high mesophilic temperature range, additional incubation time could not be used to compensate. Although lower temperature conditions can be compensated by rich growth media over a wider range accuracy is compromised [19].

Impact of Inoculum Concentration

The impact of inoculum concentration relative to requirements established for each method was determined. Each test method was modified using an inoculum multiplier viz. 0.5X, 1.0X, 1.5X and 2.0X of that required for the method. For all test methods, a 2.0X multiplier resulted in overloading and the plates were TNTC. TNTC results were not included in the ANOVA analysis. There was a significant difference (P<0.05) between EG and PF test methods as compared to the control method (*Figure 3*). However, as shown in Figure 1, differences in colony counts can be attributed to incubation temperatures established for each method. Herein, it is important to examine the significance within each test group rather than comparison to the control method. Due to the similarity in SM-9222D and the control method, colony counts did not significantly differ (P<0.05) for 1.0X multiplier. However, EG and PF methods significantly underestimated the total CFU/100 mL.

Within each test method, only PF colony counts did not significantly differ (P<0.05) among the inoculum loading multipliers. This result illustrates the impact of high fecal coliform concentration on the accuracy of each test method. These results are consistent with field studies that showed decreased accuracy for EG at higher bacterial density [20].

Variability based on bacterial density (both planktonic and sessile) using membrane filtration based enumeration methods [21-23] as well as over-lay enumeration methods [24] is documented. When field analyses do not consider bacterial density in the sample, microbial and/or consortium competition results in inaccurate enumeration values [23]. Although confidence bounds and rarity index utilized in Most Probable Number methods is not practical for field enumeration; a basic consideration of bacterial density is important when selecting method and sample volume.

Impact of Composition

To examine the impact of water composition, water samples from different surface water sources were used. Geometric mean (N=243) of fecal coliform was measured from three surface water sources including lentic (large reservoir and small detention) and lotic (first-order stream) water using each testing method following manufacture's direction or standard protocol (*Figure 4*). Given that in an emerging region one of the main sources of drinking water is surface water, it was ideal to use the lentic and lotic water sources to examine reliability of testing method. As was similarly observed in previous field studies in Nigeria (data not shown), method SM-9222D was not significantly different from the control (P<0.01). However, at the same significance level, the other two test methods viz., EG and PF methods resulted in significantly different (P<0.01) fecal coliform counts for lentic water sources. The variability can be attributed to the reduction of direct media contact (and therefore, reduced nutrient exchange) arising from elevated suspended solids.

With a decrease in the confidence interval, there was no significant difference between the control group and EG (P<0.1). Studies have shown that the PF method gives a reasonably accurate fecal coliform count [25]. Herein, PF was the least accurate when compared to a control method. It was also noted that this method resulted in the largest CFU variability. Variations in water composition have been shown to alter fecal enumeration [9, 26, 27]. Similarly, using cluster analysis, Simeonov et al. [28] showed fecal enumeration were dependent on the physicochemical characteristics and pollution levels of the studied water systems. Due to the higher variance in PF, and considering the inoculation method (direct application of sample); sample homogeneity was an important variable in the results.

CONCLUSION

The purpose of this study was to elucidate the variables that may reduce the accuracy of coliform enumeration during field analysis in resource-limited regions. Three common fecal coliform enumeration methods under different incubation and environmental conditions, viz., incubation temperature, inoculum concentration, and water composition were examined. It was shown that when constant temperature incubation is not practical; the three test methods yield inaccurate and unreliable coliform enumeration. The manufacture of EG cautions users that opt to incubate at room temperature should constantly monitor the ambient temperature. Unfortunately, this is not always practical and may be overlooked. In addition, monitoring without corrective action may result in inaccurate enumeration counts. Similarly, without detailed analysis of inoculum concentrations; over-loading the enumeration plates will result in inaccurate enumeration counts. Although this studied showed the most reliable (as compare to the control of ideal conditions) was SD-9222D; this method requires resource-intensive equipment and thus may not be practical for all field applications. It is not the intention of this study to promote one method; but rather identify some important variables that may lead to inaccurate reporting.

In addition to determining correlations between the test methods, it was also important to determine which of the three common water testing methods was easiest to use and most cost efficient. *Table 1* is used to illustrate a summary of the cost, difficulty level and the standard temperature, incubation time, and inoculum amount for each of the methods. The easiest method to use was the Easygel method and the most difficult method was the Membrane Filtration method. In regions with not enough access to water testing equipment, the standard temperature of 35˚C or 44.5˚C will be difficult to maintain. Therefore, keeping the plates or the films in a closet or desk drawer will be the most ideal if an incubator is not available.

Figures and tables

Table 1. Overall procedure and conclusion for each method

	Standard Inoculum Amount	Standard Temperature	Standard Incubation Time	Difficulty Level	Cost
Membrane Filtration	-	44.5 ℃	24 hrs	Medium-High	$$$
Easygel	0.5-5.0 mL	35 ℃	24-48 hrs	Low	$$
Petrifilm	1.0 mL	35 ℃	24-48 hrs	Low-Medium	$

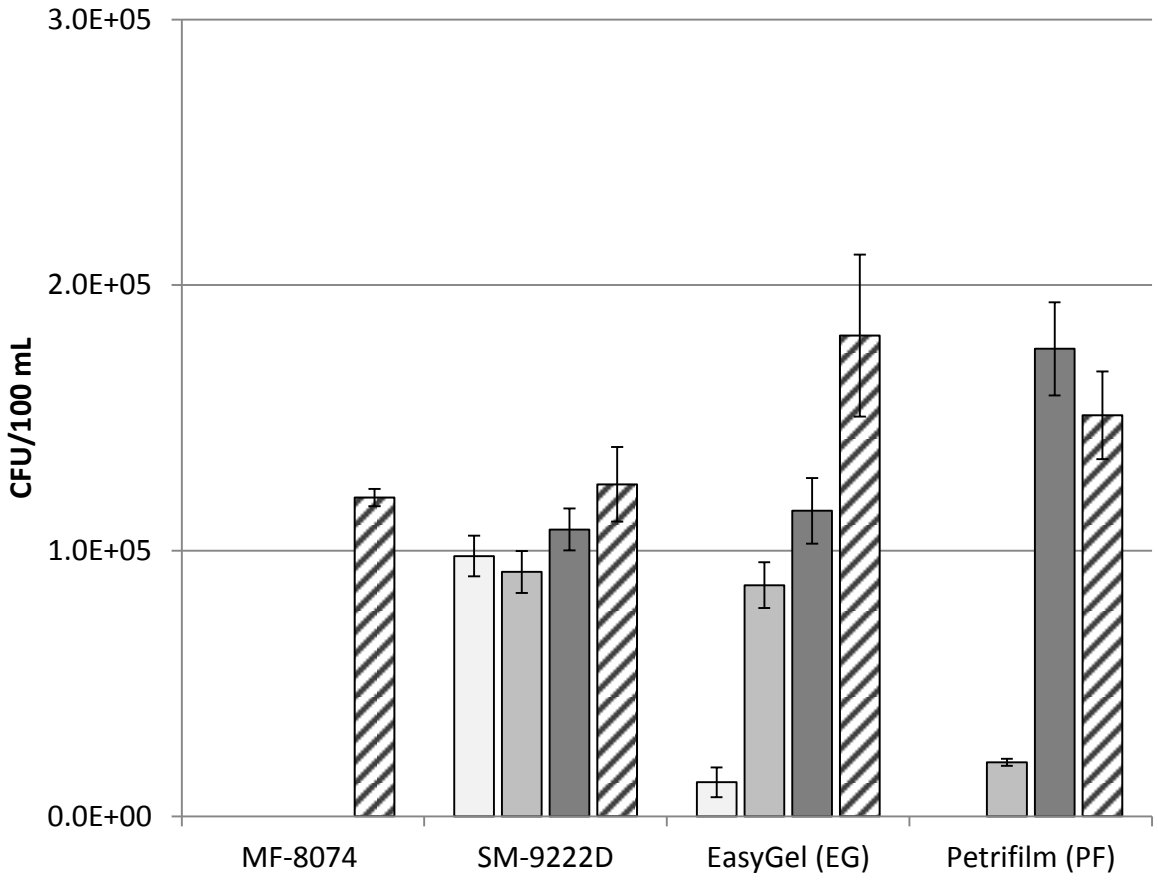

Figure 1. The mean CFU/100mL and standard deviation for ☐ 25˚C, ▒ 30˚C, ■ 35˚C, and ▨ 44.5˚C incubated for 24 hours period. The standard temperature for the MF-8074 is 44.5˚C.

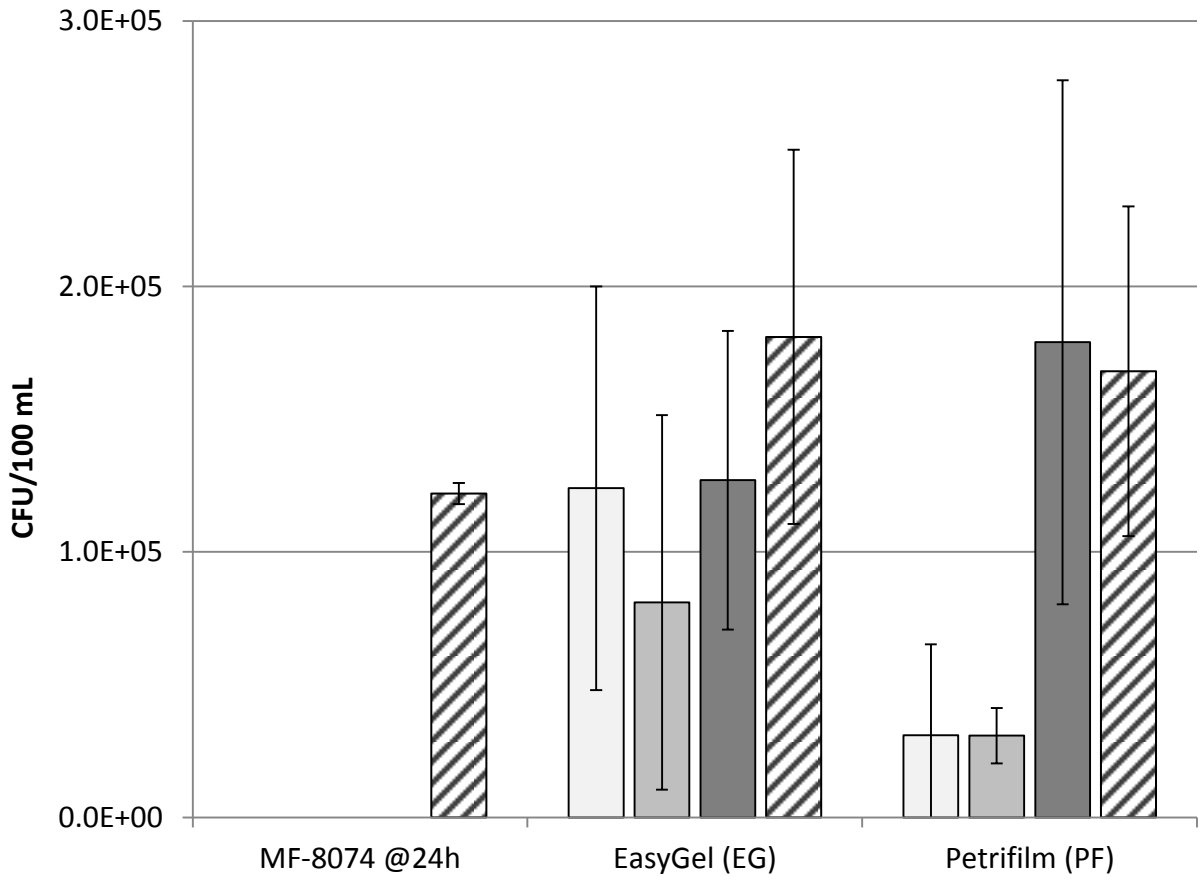

Figure 1. The mean CFU/100mL and standard deviation for ☐ 25˚C, ▒ 30˚C, ■ 35˚C, and ▨ 44.5˚C incubated for 48 hours period. The standard temperature for the MF-8074 is 44.5˚C for a 24 h incubation period.

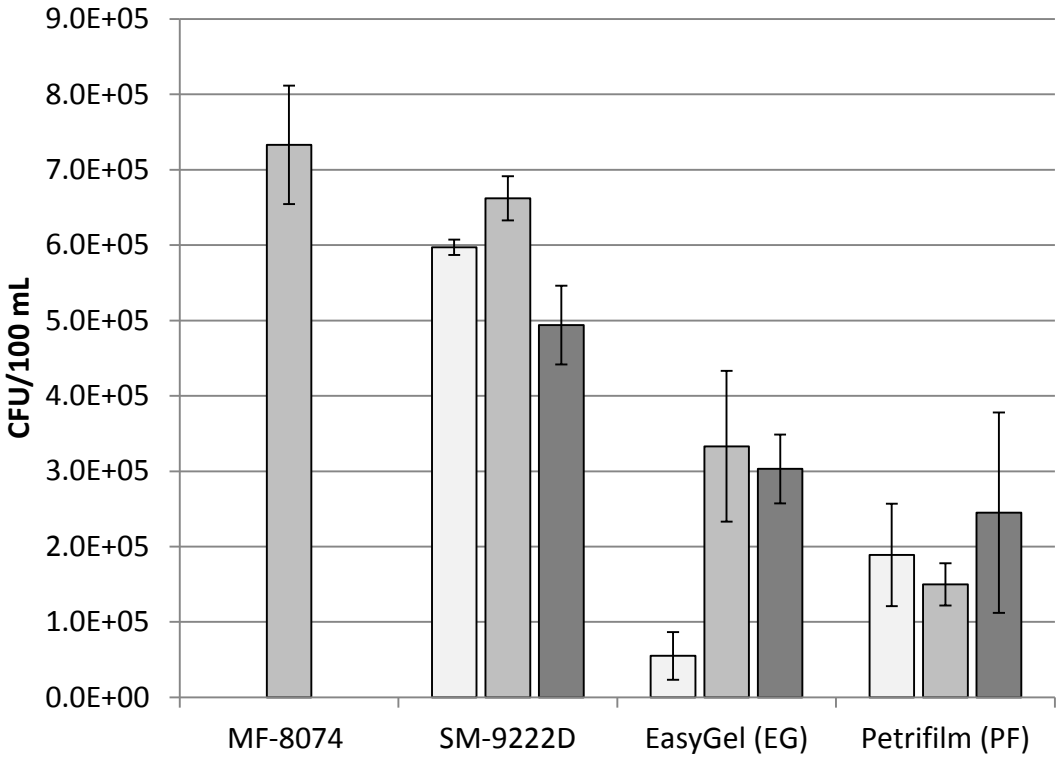

Figure 3. The mean CFU/100mL and standard deviation for ☐ 0.5X, ☐ 1.0X and ☐ 1.5X concentration loading.

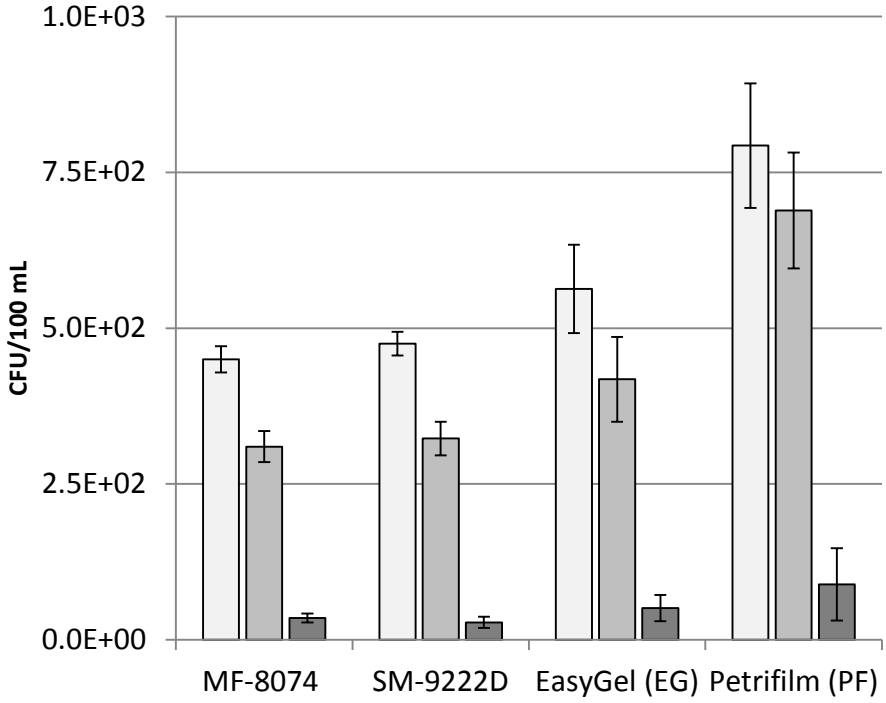

Figure 4. The geometric mean (N=243) CFU/100mL and standard deviation for □ reservoir, ▒ detention pond and ■ stream water sources. TSS (in mg/L) for reservoir, detention pond and stream was 230 ±73, 85 ±13, and 15 ±4; respectively.

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