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METHOD TO PARTITION BETWEEN ATTACHED AND UNATTACHED *E. COLI* IN RUNOFF FROM AGRICULTURAL LANDS[1]

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ABSTRACT: Remediation of waters impaired by bacterial indicators is usually dictated by total maximum daily load plans, which are heavily dependent on fate and transport modeling of bacterial indicators. Nonpoint source pollution models are most frequently used to assess bacterial transport to surface waters and most models typically simulate bacterial transport as a dissolved pollutant. Previous studies have found that cells preferentially attach to sediments; however, a variety of techniques have been used to assess attachment including filtration, fractional filtration, and centrifugation. In addition, a variety of chemical and physical dispersion techniques are used to release attached and bioflocculated cells from particulates. Here we developed and validated an easy-to-replicate laboratory procedure for separation of unattached from attached *E. coli* which will also identify particle sizes to which *E. coli* preferentially attach. Physical and chemical dispersion techniques were evaluated and a combined hand shaker treatment for 10 min followed by dilutions in 1,000 mg/l of Tween 85 significantly increased total *E. coli* concentrations by 31% when compared with a control. In order to separate unattached from attached fractions, two commonly used techniques, fractional filtration, and centrifugation were combined. The filtration and centrifugation treatments did not reduce *E. coli* concentrations when compared with a control ($p > 0.05$), indicating that damage was not inflicted upon the *E. coli* cells during the separation procedure.

(KEY TERMS: *E. coli;* microbial partitioning; nonpoint source pollution.)

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INTRODUCTION

The three most common pathogen indicators in the United States include fecal coliform, *E. coli,* and enterococci. Although fecal coliform have been traditionally used to detect the presence of pathogens in surface waters, *E. coli* and *Enterococcus* are thought to have a higher degree of association with outbreaks of gastrointestional illness (USEPA, 1986) and *E. coli* is typically the indicator preferred in freshwater systems. In an attempt to reduce pollutant loading to the nation's water bodies, total maximum daily loads (TMDLs) are being developed to assess water quality problems, identify pollution sources, and determine pollutant reductions needed to restore and protect rivers, streams, and lakes. A TMDL is a calculation of the maximum amount of a pollutant that can be discharged to a water body, while still meeting the water quality standards, and an allocation of that

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amount to the pollutant's sources (USEPA, 2005). Nonpoint source (NPS) pollution models are most frequently used to assess bacterial transport to surface waters and most models typically simulate bacterial transport as a dissolved pollutant (Paul *et al.*, 2004) primarily because sufficient data on bacterial partitioning are not available (Jamieson *et al.*, 2004). Models that do partition fecal bacteria into the attached and unattached phases typically use a linear partitioning relationship to represent initial release from manure (Benham *et al.*, 2006).

Previous studies have determined that fecal bacteria preferentially attach to particulate matter (Auer and Niehaus, 1993; Ling *et al.*, 2002; Henry, 2004) and as a result their survival time may be increased (Gerba and McLeod, 1976; Burton *et al.*, 1987). *E. coli* attachment to particulates ranges from 20 to 35% during stream base flow conditions and 30-55% during storm events (Characklis *et al.*, 2005; Jeng *et al.*, 2005). Attachment to particulates averaged 8% when cowpats were placed on trays beneath a rainfall simulator (Muirhead *et al.*, 2005). The variety of techniques used to assess partitioning between the unattached and attached phases could lead to differences in results and thus partitioning coefficients implemented into NPS models.

Most frequently, a separation technique such as filtration, fractional filtration or centrifugation is used to separate the unattached from the attached cells (Schillinger and Gannon, 1985; Auer and Niehaus, 1993; Henry, 2004; Characklis *et al.*, 2005; Jeng *et al.*, 2005; Muirhead *et al.*, 2005; Fries *et al.*, 2006). The unattached and total fractions are enumerated and the attached fraction is assumed to be the difference between the two. Chemical or physical dispersion techniques are often employed to separate sorbed bacteria from sediments to assess the total concentration. Dispersing the indicator organisms from sediments and organic particles allows each bacterium to form a separate colony; therefore, achieving a better approximation of the total number of both free cells and those associated with particulates. Many studies have examined impacts of dispersion techniques on total bacteria or fecal coliforms (McDaniel and Capone, 1985; Yoon and Rosson, 1990; Trevors and Cook, 1992; Epstein and Rossel, 1995; Craig *et al.*, 2002) and some researchers have obtained optimal results by using a combination of dispersion techniques (Yoon and Rosson, 1990). Fecal coliforms include the genera *E. coli*, but these previous studies have not examined the effects of different dispersion techniques specifically on mixed environmental strains of *E. coli* present in runoff samples.

Filtration is one technique used to separate unattached from attached bacteria. Typically, bacteria passing through the filter are assumed to be unattached, previously defined as cells able to pass through an eight-micron screen (Qualls *et al.*, 1983; Mahler *et al.*, 2000; Henry, 2004). Since a typical *E. coli* cell is 1×3 μm (Madigan *et al.*, 2000) in size, the filtration method assumes that free bacteria, those sorbed to very small particles, or even bioflocculated clumps are able to pass through the eight-micron filter. Mahler *et al.* (2000) validated this method by passing a known suspension of *E. coli* through the eight-micron filter. The authors successfully recovered 99% of the unattached *E. coli*, demonstrating that only a small proportion of the free cells that were retained would have been incorrectly classified as attached. Fractional filtration has been used to identify the bacteria attached to different particles sizes (Schillinger and Gannon, 1985; Auer and Niehaus, 1993; Jeng *et al.*, 2005). Fractional filtration of urban stormwater was performed by Schillinger and Gannon (1985) and Auer and Niehaus (1993) to separate sediment particle sizes; however, both studies also assumed that all cells retained by screens were associated with particulates of that size. It is possible that bioflocculated or unattached bacteria could attach to the filters (Henry, 2004) or particulates could clog filters, retaining free cells and resulting in incorrect classification.

Centrifugation is another method frequently used by researchers to determine partitioning between unattached and sediment sorbed bacteria (Sayler *et al.*, 1975; Schillinger and Gannon, 1985; Huysman and Verstraete, 1993; Dumontet *et al.*, 1996; Characklis *et al.*, 2005; Muirhead *et al.*, 2005). The percent attached is typically determined by subtracting the total count from the concentrations remaining in the supernatant following centrifugation. Use of centrifugation to separate microbial fractions assumes that microbial sorption to particulates is not affected even though interactions between cells and particles are increased during the process (Characklis *et al.*, 2005). In addition, unattached bacteria can be similar in size to clay particles, so determining proper centrifuge settings to separate sediments from nonattached bacteria can be difficult (Henry, 2004). Previous research has not identified a separation technique that will also identify particle sizes to which *E. coli* preferentially attach without the assumption that all unattached cells are able to pass through filtration devices (Mahler *et al.*, 2000; Henry, 2004; Jeng *et al.*, 2005).

A method to disperse and separate unattached from attached forms of environmental *E. coli* present in runoff is needed to improve consistency between research results which will ultimately improve bacterial transport modeling. The goal of this study was to develop an easy-to-replicate laboratory procedure for separation of unattached from attached *E. coli* which will also identify particle sizes to which *E. coli* preferentially attach. The first objective was to compare

previously employed methods for dispersing attached *E. coli* from sediments and suspended particles and the second objective was to validate a sequence of previously employed methods for separation of unattached and attached *E. coli*.

MATERIALS AND METHODS

Sample Collection

A single, fresh cowpat was collected from the confined stalls at the Virginia Tech Dairy Farm and was placed on sloped pastureland of Grosclose silt loam soils (Creggar *et al.*, 1985) with particle size distribution of 38% sand, 54% silt, and 8% clay. A cowpat fecal source was selected because previous research results indicated higher concentrations of indicator organisms and total suspended solids (TSS) in runoff from pastureland plots treated with cowpats when compared with poultry litter and liquid dairy manure (Soupir *et al.*, 2006). The selected cowpat was representative of a standard cowpat as defined by a diameter of approximately 20.3 cm (8 inches), a height of 2.54 cm (1 inches), and a weight of 0.9 kg (2.0 lbs) (Thelin and Gifford, 1983). The cowpat was placed on bare soils to ensure sufficient transport of both sediment and organic particulates. High concentrations of particulates increased availability of bacterial attachment sites and likelihood of *E. coli* attachment. The cowpat was hand irrigated with water from a local well and the runoff sample was collected approximately 0.30 m (1-ft) from the base of the cowpat in a 140 ml sterile bottle. High concentrations of organic matter from the cowpat and sediments were observed in the runoff samples. Particles were dislodged at a higher rate than what would typically be observed in runoff from pastureland at the edge-of-the-field; however, this could be representative of a scenario where cattle are allowed direct access to streams. A cowpat deposited directly on the streambank would allow for little or no settling of particulates before entering surface waters because of the short distance between the fecal source and surface water. Cowpat runoff samples were used to compare the effectiveness of physical and chemical dispersion techniques and to develop the separation technique.

Comparison of Dispersion Treatments

A comparative study was conducted to identify the best method of dispersing environmental strains of *E. coli* from sediment and organic matter particles present in runoff samples. Chemical and physical dispersion techniques were tested separately in preliminary studies before the optimal techniques were combined to evaluate if a combination of methods would further increase enumeration.

Based on findings from previous studies, selected chemical dispersion treatments included Tween 80 (Fisher Scientific, Fair Lawn, New Jersey; Yoon and Rosson, 1990) and Tween 85 (Fisher Scientific, Fair Lawn; Auer and Niehaus, 1993; Henry, 2004) at concentrations of 10, 100, 1,000, and 10,000 mg/l and sodium pyrophosphate (NaPP) combined with 1% glycerol, 1% peptone, and deionized water (Trevors and Cook, 1992). Tween 80 is polyoxyethylene sorbitan monooleate (Fisher Scientific, 2008a) and Tween 85 is polyoxyethylene sorbitan trioleate (Fisher Scientific, 2008b). Tween 80 added to water at 0.02% concentration is commonly used to disperse microorganisms prior to inoculation (BD, 2008). Runoff samples were diluted in the chemical solutions and enumerated on Modified mTEC agar (USEPA, 2000) using membrane filtration procedures (standard method number 9222 D, Clesceri *et al.*, 1998). The control samples were diluted in phosphate buffer solution (HACH Company, Loveland, Colorado) with a pH of 7.2 ± 0.5 and enumerated by membrane filtration. All samples were vortexed for approximately two seconds to ensure mixing prior to membrane filtration.

Three physical dispersion treatments: hand shaker (Wrist Action Shaker, Burrell Corporation, Pittsburg, Pennsylvania) treatment for 10 min, ultrasonic bath (Fisher Scientific, 50/60 Hz, 55W) treatment for 30 s, 2 min, 6 min and 10 min, and a one minute vortex (Touch Mixer Model 231, Fisher Scientific, Pittsburg, Pennsylvania) were also compared. After the runoff samples were collected, a 1 ml control subsample was extracted and diluted in phosphate buffer solution (HACH Company, Loveland, Colorado) and enumerated by membrane filtration (Clesceri *et al.*, 1998). The remaining sample was treated with a dispersion technique prior to dilution and membrane filtration.

A runoff sample was microscopically inspected to validate the selected dispersion technique. Samples were diluted in phosphate-buffered water, stained with fluorochrome (acridine orange), and viewed using the epifluorescence microscopic method number 9216 B as described by Clesceri *et al.* (1998).

Development of Separation Technique

Multiple screen filtration separated suspended solids into particle sizes while centrifugation was used to separate attached from unattached cells. A flow chart of the separation process is presented as Figure 1. A Mini-Sieve Microsieve Set (Bel-Art

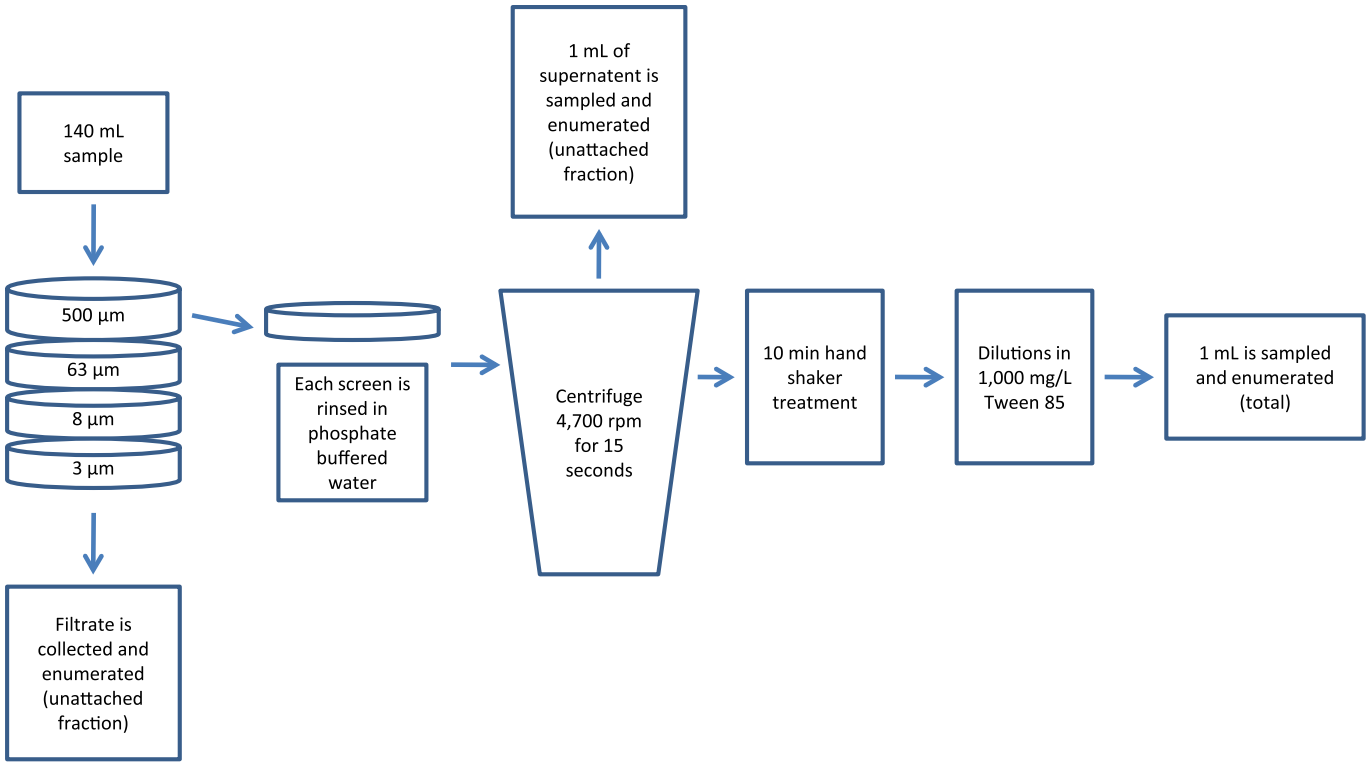

FIGURE 1. Flow Chart Depicting the Separation Technique.

Products, Pequannock, New Jersey) containing a number 35 mesh screen was used to retain particles larger than coarse sand (>500 μm) and a number 230 mesh screen was used to retain medium, fine, and very fine sand (63-500 μm). An eight-micron filter (Poretics, Polycarbonate, GE Water and Processes Technologies, Trevose, Pennsylvania) was used to retain fine, medium, and coarse silt particles (Qualls *et al.*, 1983; Mahler *et al.*, 2000; Gordon *et al.*, 2002; Henry, 2004) and a three μm filter (Nuclepore Track – Etch Membrane Filtration Products, Whatman, Florham Park, New Jersey) was used to retain clay and very fine silt particles. Gravity flow was augmented with vacuum application.

In order to determine the recovery of *E. coli* in filtrate passing through eight-micron and three-micron filters, *E. coli* DH2 1030 was collected from the stationary phase of the growth curve. *E. coli* cells were diluted in phosphate-buffered water to approximately 10^5 cfu/ml and 10 ml of suspended cells were filtered through the eight and three-micron filters. The initial concentration and the concentration in the filtrate were enumerated by membrane filtration. Eighty eight percent of cells passed through the eight-micron filter while only three percent of the cells present in the control passed through the three-micron filter (n = 5). This significant reduction indicated that an alternative method would be necessary to account for unattached cells retained on the filter surface or trapped by sediments and organic particles. Therefore, the mesh screens and filters were aseptically removed, placed in phosphate-buffered solution, and gently rinsed to remove sediments from the filters. A preliminary study monitored the change in mass of the four screens before and after rinsing. The change in screen mass was divided by the mass of the rinsed solids to determine the percent of particulates remaining on the filter during this process. The treatment and rinsing of screens resulted in a gain of 0.9, 6.6, 0.4, and 0 mg in the 500, 63, 8, and 3 μm screens, respectively. More than 97% of all particulates were removed from the screens. Samples were then centrifuged at 4,700 rpm ($3{,}043 \times g$) for 15 s to separate unattached cells from suspended particles (Lago, 2005). To validate the centrifugation technique, approximately 10^5 cfu/ml *E. coli* DH2 1030 was centrifuged to separate unattached cells from suspended particles (Lago, 2005). No reduction of unattached concentrations was observed (n = 12). While centrifugation could increase interactions between cells and particles, possibly strengthening existing bonds, we assumed that the application of the dispersion technique prior to enumeration of the total concentration should disperse any increased attachment that results from centrifugation.

The supernatant and filtrate passing through the three-micron filter were enumerated for *E. coli* concentrations on Modified mTEC agar using membrane filtration (Clesceri *et al.*, 1998) to assess

the unattached bacterial concentrations. The sediment and organic particles associated with each screen size were resuspended in phosphate-buffered water, treated with the optimal dispersion technique and enumerated by membrane filtration to assess the total bacterial concentration retained by each screen size. Total and unattached concentrations were converted to a mass basis based on filtration volumes and the difference between the total and unattached masses was assumed to be the attached portion. The attached fraction associated with each screen size was divided by the total suspended solids associated with each screen size to obtain the cfu per gram of particulates and identify the particle sizes to which *E. coli* preferentially attach.

To validate the separation technique, runoff samples collected from large cowpat-treated field plots were enumerated for *E. coli* concentrations by treatment with the dispersion technique followed by membrane filtration (control samples) and compared with *E. coli* concentrations that were pre-treated by screen filtration and centrifugation followed by dispersion technique treatment and membrane filtration. A mass balance was conducted to examine the separation technique for loss of cells during the filtration and centrifugation process. Field plots were constructed at the Virginia Tech Prices Fork research farm on the Grosclose silt loam soils examined in the dispersion portion of this study. Additional details about plot construction, rainfall simulation, and sample collection are presented by Soupir (2008). A total of 68 samples were collected at the base of the plots, two samples collected at each sampling interval were used for the two different sets of analysis. The first sample was analyzed for total *E. coli* concentrations by treatment with the hand shaker for 10 min followed by membrane filtration. The second sample was analyzed for unattached and attached concentrations by the screen filtration and centrifugation procedure followed by treatment with the hand shaker for 10 min and enumeration by membrane filtration. The mass of attached and unattached *E. coli* associated with each screen size and the mass of *E. coli* present in the filtrate were summed to determine the total for the separation technique.

Data Analysis

The experimental design for the comparison of dispersion techniques was a randomized block design with preliminary comparisons of either chemical or physical treatments conducted in triplicate to identify the treatments most likely to disperse attached cells and increase *E. coli* counts. Five replicates were used when the best chemical and physical dispersion techniques were combined. Percent increase or decrease from the control was calculated by subtracting the control average concentration from the treatment average concentration and then dividing the result by the control average concentration. Experimental data were log-transformed and analyzed using the Mixed procedure of SAS (SAS Institute, 2004) and least square means were compared using Tukey's pairwise comparison (Ott and Longnecker, 2001). Statistical differences between total *E. coli* pretreated with screen filtration and centrifugation and total *E. coli* analyzed by the dispersion technique and membrane filtration were determined by Analysis of Variance (ANOVA) and a probability level of ≤ 0.05 was considered significant.

RESULTS AND DISCUSSION

Comparison of Dispersion Techniques

A comparative study was conducted to identify the best method of dispersing wild strains of *E. coli* from sediment and organic matter particles present in runoff from pasturelands. Previous researchers found a great deal of variation in the methods that result in greatest recovery of viable bacteria. While some methods increase enumeration, care must be taken when exposing the bacteria to either chemical or physical dispersion treatments so that the cells are not damaged, resulting in reduced enumeration.

A preliminary comparison of Tween 80, Tween 85, and sodium pyrophosphate solutions found that the 1,000 mg/l Tween 85 solution increased *E. coli* concentrations by 36% when compared with the control; however, this increase was not statistically significant ($p > 0.05$). All other Tween treatments resulted in a decrease in *E. coli* concentrations that ranged from −3 to −34%. Yoon and Rosson (1990) found that a specific concentration of Tween 80 resulted in greatest recovery of bacteria; however, the study did not specifically enumerate recovery of *E. coli*. They treated turbid seawater samples with Tween 80 concentrations ranging from 0 to 100 mg/l to improve enumeration of bacteria attached to sediments. Recovery was greatest from samples treated with the 10 mg/l concentration and concentrations >25 mg/l increased bacterial die-off. While Trevors and Cook (1992) found that sodium pyrophosphate increased enumeration of total aerobic colony forming units, in this study each treatment significantly ($p < 0.001$) reduced *E. coli* concentrations by an average of 73% when compared with the control. Sodium pyrophosphate might be effective in increasing counts of total

aerobic bacteria; however, we found that when specifically enumerating wild strains of *E. coli* the addition of sodium pyrophosphate contributed to die-off as demonstrated by the decrease in culturable cells.

Others have found that chemical treatments have little or no effect on the dispersion of bacteria (Epstein and Rossel, 1995; Craig *et al.*, 2002); therefore, physical dispersion techniques were also investigated. A preliminary comparison of physical dispersion techniques revealed that the ultrasonic bath treatment resulted in up to 212% increase in *E. coli* concentrations ($p < 0.0001$) when compared with the control with the 30 s treatment in the ultrasonic bath resulting in greatest recovery of *E. coli*. Ten minutes of hand shaking also increased *E. coli* concentrations by 149% ($p = 0.0018$) while a one minute vortex treatment decreased *E. coli* counts by 33%, but this decrease was not statistically significant.

The ultrasonic bath and hand shaker techniques were then combined with 1,000 mg/l dilutions of Tween 85 solution to evaluate if a combination of techniques would further increase cell recovery (Table 1). Contrary to findings from the preliminary studies, the physical treatments did not always increase enumeration of *E. coli*. The ultrasonic treatments differed in *E. coli* concentrations from the control by −19 to 2%; although none of the differences were statistically significant. It is possible that the cells were stressed prior to treatment with the ultrasonic bath and thus damaged by the treatments. The 10-min hand shaker treatment increased concentrations by 24% when compared with the control. Diluting samples treated with the hand shaker in the 1,000 mg/l concentration of Tween 85 resulted in a 45% ($p = 0.0028$) increase in *E. coli* concentrations. The 10-min hand shaker treatment followed by dilutions in 1,000 mg/l Tween 85 was identified as the optimal treatment because this treatment provided the most consistent results during both the preliminary study and when physical and chemical techniques were combined. The 10-min hand shaker treatment followed by dilutions in 1,000 mg/l Tween 85 was the only treatment that resulted in a statistically significant increase in *E. coli* concentrations (Table 1). Preliminary studies found statistically significant increases from the ultrasonic bath treatment, but the response of *E. coli* to the ultrasonic bath treatment was inconsistent. The lower percentage of increase in recovery of all samples may indicate that fewer *E. coli* were bioflocculated or attached to sediment particles during the second sample collection. A previous study found that *E. coli* cells were released from cowpats and transported as single cells (Muirhead *et al.*, 2005), possibly explaining the reduced effectiveness of all dispersion techniques and thus statistical significance. McDaniel and Capone (1985) suggested that response to dispersion techniques may be dependent upon the soil type and it might be necessary to test different techniques for each soil prior to use.

TABLE 1. Comparison of Combined Physical and Chemical Dispersion Treatments on Runoff Samples Collected Below a Dairy Fecal Deposit on Pastureland.

Dispersion Treatments	Concentration cfu/ml (STD)	Average % Change from Control[1]
Ultrasonic bath treatment 30 s	2.5×10^4 (8.75×10^3)	2
Ultrasonic bath treatment 30 s + dilutions in 1,000 mg/l Tween 85	2.56×10^4 (2.30×10^3)	5
Ultrasonic bath treatment 2 min	4.78×10^4 (7.09×10^3)	−19
Ultrasonic bath treatment 2 min + dilutions in 1,000 mg/l Tween 85	6.2×10^4 (1.17×10^4)	5
Ultrasonic bath treatment 6 min	5.72×10^4 (1.01×10^4)	−3
Ultrasonic bath treatment 6 min + dilutions in 1,000 mg/l Tween 85	6.56×10^4 (6.27×10^3)	11
Ultrasonic bath treatment 10 min	5.04×10^4 (5.37×10^3)	−15
Ultrasonic bath treatment 10 min + dilutions in 1,000 mg/l Tween 85	6.12×10^4 (7.09×10^3)	4
Hand Shaker 10 min	9.32×10^4 (9.63×10^3)	24
Hand Shaker 10 min + dilutions in 1,000 mg/l Tween 85	10.9×10^4 (1.08×10^4)[2]	45

[1]A negative value indicates a decrease in cells when compared with the control.
[2]Indicates statistical significance when compared with the control treatment according to Tukey's pairwise comparison.

Dispersion Technique Validation

Fluorochrome stained runoff samples are presented in Figure 2. Samples were stained prior to any dispersion treatment (Figure 2a), after receiving the hand shaker treatment (Figure 2b), and after receiving the Tween 85 treatment (Figure 2c). Removal of bacteria from sediment and organic particles by the dispersion technique was confirmed by examining the samples prior to and after each treatment. The presence of dispersed cells, reduced clumping, and fewer cells attached to sediment or organic matter particles following the dispersion treatment validated this technique.

Separation Technique Validation

Multiple screen filtration has been used previously to identify particle sizes to which bacteria are

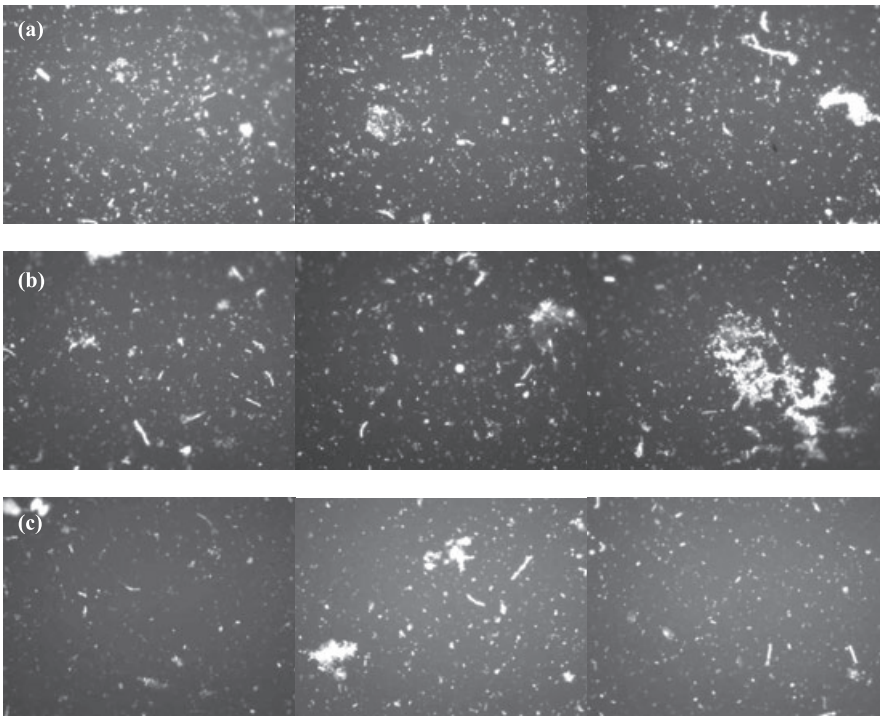

FIGURE 2. Cells Receiving No Dispersion Treatment (a); Cells Treated With a Hand Shaker for 10 Min (b); Cells Treated With a Hand Shaker for 10 Min and Diluted in 1,000 ppm Tween 85 (c).

associated; however, centrifugation is more frequently used to separate unattached from attached cells. We propose combining these two techniques to eliminate concerns associated with filtration techniques. While filtration using a single screen size of eight-micron has been identified previously as a viable method to separate attached and unattached bacteria (Qualls *et al.*, 1983; Mahler *et al.*, 2000; Gordon *et al.*, 2002; Henry, 2004), the presence of sediments and organic particles in runoff from agricultural lands make it very likely that sediments could clog the filters, retaining unattached and bioflocculated cells.

Application of the separation technique to runoff samples collected at the base of 18.3-m long field plots provided an opportunity to conduct a mass balance and examine the technique for potential loss of cells during the filtration and centrifugation process. Results from both analyses were converted to concentrations and are presented in Table 2. Interestingly, the combination of screen filtration and centrifugation, followed by treatment with the hand shaker and membrane filtration, resulted in a 30% increase in average sample concentration per 100 ml when compared with the average concentration in the control. It should be noted that Tween 85 was not added to samples following treatment with the hand shaker for this portion of the study because the large number of samples and unknown *E. coli* concentrations (and thus required dilution levels) make it likely that re-plating would be necessary and the impact of longer term exposure of Tween 85 on *E. coli* viability is unknown. Analysis by ANOVA determined that no statistically significant differences existed between the control and summed (total) separation technique values ($p > 0.05$); however, these results were unexpected. We had hypothesized that screen filtration

TABLE 2. *E. coli* Concentrations Determined by Multiple Screen Filtration and Centrifugation Pretreatment and Then Enumerated by Dispersion and Membrane Filtration Are Compared With Total *E. coli* Concentrations Enumerated by Dispersion and Membrane Filtration (control).

	***E. coli* Mean Concentration cfu/100 ml (STD)**	**Percent of Total *E. coli* Associated With Screen Sizes and Filtrate**
Screen size 500 μm	2.91×10^3 (5.16×10^3)	0.26
Screen size 63 μm	2.49×10^3 (1.87×10^3)	0.23
Screen size 8 μm	3.54×10^4 (7.28×10^4)	3.2
Screen size 3 μm	6.37×10^4 (1.17×10^5)	5.8
Filtrate	9.99×10^5 (3.46×10^6)	90.5
Sum of screen sizes + filtrate	1.10×10^6 (3.48×10^6) a[1]	
Control (no pre-treatment)	8.47×10^5 (9.12×10^5) a	

Note: *E. coli* concentrations retained by each screen size and in the filtrate are also presented.

[1]Means followed by the same letter do not differ at the 5% level of significance according to Tukey's pairwise comparison.

and centrifugation procedures could possibly damage the cells and decrease total concentrations when compared with the samples only treated with the hand shaker. One possible reason for the increased concentrations could be compounded sample variability as a result of summing the bacteria associated with each screen size. The exclusion of Tween 85 from the dispersion technique would not have impacted these results because Tween was not used in analysis of either the samples treated with the separation technique or the control. Regardless, the combination of screen filtration and centrifugation did not decrease concentrations of culturable *E. coli* and the means between the technique and the control did not differ statistically. Ninety one percent of all *E. coli* cells passed through all screens in the fractional filtration device while the three-micron screen retained the highest percentage of cells, 5.8%. These results indicate the *E. coli* are primarily transported in the unattached state or associated with particulates <3 μm in size.

Standard deviations presented in Table 2 show that variability in total *E. coli* concentrations among samples is quite high. Standard deviations of 9.12×10^5 cfu/100 ml exist for control samples while the screen filtration and centrifugation pretreatment increases variability to standard deviations of 3.48×10^6 cfu/100 ml. Splitting cells into fractions based on association with particle size categories increases variability. While increasing the number of screens would improve understanding of distribution of *E. coli* attachment to particle sizes, this would likely introduce additional variability, even when a high number of samples are available for analysis (68 samples were analyzed in Table 2).

SUMMARY AND CONCLUSIONS

The combination of a physical and chemical dispersion treatment followed by fractional filtration resulted in an easy-to-replicate method to separate *E. coli* into attached and unattached phases. This method can be applied to runoff or stormwater samples and is useful to determine partitioning coefficients for NPS models and identify the particle sizes to which *E. coli* preferentially attach. This method was not compared with previously employed methods for separation of pathogen indicators between attached and unattached phases and therefore the performance of this technique compared with others was not assessed. Physical and chemical dispersion techniques were evaluated, and a combined treatment with a hand shaker for 10 min followed by dilutions in 1,000 mg l^{-1} of Tween 85 significantly increased total *E. coli* concentrations by 45% ($p = 0.0028$) when compared with a control. To separate unattached from attached fractions two commonly used techniques, fractional filtration and centrifugation, were combined and validated. Centrifugation of suspended, unattached *E. coli* did not reduce suspended concentrations and the filtration and centrifugation treatments also did not reduce *E. coli* concentrations when compared with a control (p value >0.05). Future research is recommended to ensure that the identified dispersion technique is optimal for samples dominated by different particle sizes. Additional screen sizes could be included in the screen filtration to identify a finer distribution of *E. coli* preferential attachment.

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