Flow cytometry is a promising and rapid method for differentiating between freely suspended *E. coli* and *E. coli* attached to clay particles

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Abstract

Aim: A standard procedure does not exist to distinguish between attached and unattached microorganisms. In this study, we compared two methods to quantify between *E. coli* attached to clay particles and *E. coli* freely suspended in solution: flow cytometry (attachment assay and viability assay) and settling (or centrifugation followed by settling).
Methods and Results: Methods were tested using three environmental strains collected from swine facilities (A, B, and C) and one purchased modified pathogenic strain (ATCC 43888); four clay particles: Hectorite, Kaolinite, Ca-Montmorillonite, Montmorillonite K-10; and a range of surface area ratios (particle surface area to *E. coli* surface area). When comparing the two methods, the percent attached obtained from the flow cytometry was lower but not significantly different from the percent attached obtained from the settling method for all conditions except when the particle was Hectorite or Montmorillonite K-10; when the strain was C; and when the surface area ratio was below 100. Differences between the methods are likely because traditional culture-based methods cannot detect the viable but non-culturable (VBNC) population whereas flow cytometry can detect the fraction of VBNC with intact membranes.
Conclusion: Our results indicate that flow cytometry is a rapid and culture-independent method for differentiating between attached and unattached microorganisms.
Significance and Impact of Study: Flow cytometry is useful for laboratory-based studies of microorganism-particle interactions.
Keywords: flow cytometry, *E. coli*, clay, attachment, settling.

Introduction

Currently, 72,305 miles of impaired streams are contaminated due to elevated levels of pathogens or pathogen indicators (USEPA 2009), and pathogens are the leading cause of water quality impairments in rivers and streams in the United States. While pathogens are present in the environment due to a variety of point and nonpoint sources, understanding the mechanisms of transport in waters is needed to improve modeling for watershed assessments and to predict when a risk to human health is present. In 1986, *E. coli* were recommended by the U.S. EPA as the primary organism used to indicate the presence of fecal contamination in fresh waters in the United States (USEPA 1986). Therefore, improved mechanistic understanding of environmental *E. coli* transport is needed for modeling bacteria fate and transport and is essential to develop meaningful plans to reduce bacterial contamination of waters.

Microorganisms can move freely in water in a planktonic state or attach to suspended soil and organic particles (Jeng et al. 2005; Hipsey et al. 2006;

Pachepsky et al. 2008). A previous study determined that 10-20% of fecal coliform cells are adsorbed to suspended particles in untreated stormwater runoff (Schillinger and Gannon 1985). Bacterial attachment to soil particles results in an increased settling velocity of the cell, and sedimentation of attached bacteria is often a critical removal mechanism in surface waters (Schillinger and Gannon 1985). The importance of this process is emphasized by the many studies citing concentrations of fecal bacteria in stream sediment that are 10-10,000 times higher than the concentrations in the overlying water column (Song et al. 1994; Davies and Bavor 2000; Bai and Lung 2005). Bacteria in bottom sediments are protected from ultraviolet radiation (Bitton et al. 1972; Schillinger and Gannon 1985), resulting in an extended life expectancy and increasing the likelihood of resuspension back into the water column when stream bottom sediments are disturbed during changes in flows (Song et al. 1994; Davies and Bavor 2000; Jamieson et al. 2005). A variety of separation techniques have been proposed to quantify the attached and unattached fractions of bacteria, including settling, filtration, and centrifugation (Schillinger and Gannon 1985; Henry 2004; Characklis et al. 2005; Jeng et al. 2005; Muirhead et al. 2005). In the aquatic environment, particles with larger density should settle out more quickly than particles with smaller densities. A study by Liu et al. (2011) used Stoke's equation to determine the settling time for quartz to separate freely-suspended *E. coli* from quartz-attached *E. coli* (Liu et al. 2011). Filtration is characterized by its ability to remove particles via a sieving mechanism based on the size of the membrane pores relative to that of the particulate matter (USEPA 2005). Qualls et al. defined the unattached bacteria as cells able to pass through an eight-micron screen (Qualls et al. 1983). Krometis et al. (2009) used 8 µm filters to partition unattached *E. coli* from soil particles in aquatic environment. In 2008, Soupir et al. utilized a series of screens and filters (35 mesh screen, 230 mesh screen, 8 µm and 3 µm) (Soupir et al. 2008) to quantify the *E. coli* attachment percentage in surface runoff samples (Soupir and Mostaghimi 2011). Since a typical *E. coli* cell is 1.1 to 1.5 µm wide by 2 to 6 µm long (Grismer 2006), filtration procedures may include not only free bacteria but also those sorbed to very small particles or even small bioflocculated clumps (Soupir et al. 2008). Centrifugation is another frequently used method for determining the amount of bacteria that are sediment-attached (Faegri et al. 1977; Schillinger and Gannon 1985). In these studies, *E. coli* in the supernatant were considered suspended, and the difference between this concentration and the total concentration is assumed to be the attached bacteria fraction (Soupir et al. 2008). However, centrifugation has some drawbacks as well. Since unattached bacteria have a similar diameter as small-sized particles, determining proper centrifuge speed and time to partition attached and unattached bacteria can be challenging (Henry 2004). Although the above separation techniques have been used by researchers for more than a decade, they are time-consuming and labor-intensive. Furthermore, the attached and unattached fractions are typically assessed via plate counting techniques, which involves serial dilutions and manual colony counting.

Despite the need to quantify attached and unattached cells, a standard procedure to partition between unattached and attached *E. coli* is currently nonexistent. This lack of a procedure and information has resulted in a standard assumption in water quality models that bacteria are freely suspended. Flow cytometry is a technique used for measuring and analyzing multiple parameters of individual particles (Vital et al. 2010), including microorganisms, nuclei, and latex beads (Brown and Wittwer 2000). The flow cytometer performs simultaneous multiparametric analysis by passing thousands of cells and particles per second through a laser beam and capturing the light as each cell or particle emerges. Flow cytometry can also record the number of cells that pass through and report physical and/ or chemical characteristics such as relative size, relative granularity or internal complexity, and relative fluorescence intensity (Brown and Wittwer 2000). Flow cytometry can

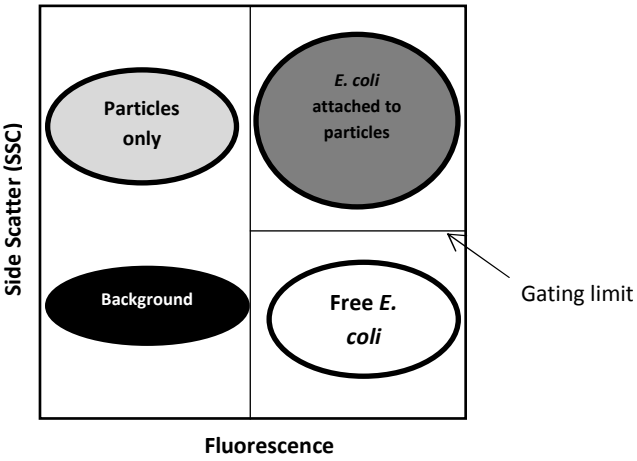

Figure 1. Representation of the flow cytometry as a technique to distinguish between freely suspended and *E. coli* attached to particles.

potentially be used to partition and quantify attached and unattached *E. coli* populations. In flow cytometry, light measured approximately at a 90˚ angle to the excitation line is called side scatter. The side scatter channel (SSC) provides information about the granularity or internal complexity. In Figure 1, soil particles have a high side scatter signal but do not bind to fluorescent dye. After binding with a fluorescent dye, *E. coli* will fluoresce, as shown in the right bottom corner of the plot. Moreover, after attaching to particles, the *E. coli* attached to clay will be recognized with a high side scatter signal (from the particle) and a high fluorescent signal (from the labeled *E. coli*), thus they present in the upper right corner of the plot. The gating limit is determined between *E. coli* attached to clay and freely suspended *E. coli*. The goal of this study was to compare flow cytometry and a settling/centrifugation separation method as techniques for partitioning between *E. coli* attached to clay particles and *E. coli* freely suspended in solution.

Materials and Methods

A newly developed flow cytometry method was compared with a settling method, a more standard technique for partitioning between *E. coli* attached to clay particles and freely-suspended *E. coli*. While bacterial attachment is thought to depend on many properties, we tested the separation techniques with various *E. coli* strains, particles, and particle ratios (particle surface area to *E. coli* surface area). Properties of the bacteria were considered by using different environmental strains from our own library, as described below and a known modified pathogenic strain (strain A, B, C, and ATCC 43888); experiments were conducted on four different pure clay particles (Kaolinite, Hectorite, Ca-Montmorillonite, and Montmorillonite K-10); and environmental conditions such as ionic strength, temperature and pH were held constant for each clay type at specific surface area ratios (1, 2, 50, 100, 200, and 500) for all samples.

E. coli strain selection and preparation

Three environmental strains and one modified pathogenic strain of *E. coli* were used for testing the flow cytometry method. Two hundred and three isolates were collected from swine facilities in Iowa in 2008 and 2009 via membrane filtration, EPA Method 1603, on modified mTEC agar (USEPA 2002) and preserved in 25% glycerol stock solution at -80˚C. The environmental strains used in this study were selected because in previous work attachment fractions exceeded 99% to quartz particles (63 to 125 μm) when sufficient surface area was available (unpublished data). It was necessary to utilize *E. coli* strains with a high propensity for attachment to ensure that positive attachment results would be obtained when testing the two methods. A pathogenic strain was also considered, ATCC^{TM} 43888, a genetically modified version of *E. coli* O157:H7, with Shiga-like toxin I and II producing genes removed. Growth in a nutrient broth simulates development of cell appendages and attachment abilities when compared to growth on an agar media (Schillinger and Gannon 1985). Therefore, the four *E. coli* strains were grown in stock suspension of Luria-Bertani broth or Tryptic Soy broth (BD Biosciences; San Jose, CA) for 18 hours at 37 ˚C, for environmental or pathogenic strains, respectively, to reach the stationary stage of the growth curve. *E. coli* were centrifuged for 3 min at 2,000 rpm (Eppendorf, Hauppauge, NY) at 4 ˚C. The supernatant was discarded, and the cells were resuspended to a 0.5 McFarland standard (approximately 1.0×10^8 CFU ml^{-1}) according to the Clinical and Laboratory Standards Institute (2006) in phosphate-buffered saline (PBS) (HACH, Loveland, CO; pH 7.4) that was autoclaved at 121˚C for 15 minutes for sterilization. The cell suspension was diluted to 1.0×10^7 CFU ml^{-1} with clay suspension, PBS and/or SYTO 11 dye (5 mmol l^{-1} solution in DMSO; Life Technologies; Grand Island, NY) to fall within the recommended range: 10^4 to 10^7 CFU ml^{-1} for optimal performance by the flow cytometer (Hussein et al. 2002). The cell concentration was confirmed to be approximately 1.0×10^7 CFU ml^{-1} by using BD viability kits with beads (BD, Franklin Lakes, NJ) via flow cytometry.

Clay particles

Clay particles were selected because *E. coli* has previously been shown to be more likely to attach to small particles and because particles smaller than 30 microns in diameter are recommended for use by the flow cytometer (BD 2000). Clay particles are the smallest among fine-grained soils.

Four clays with different particle sizes and mineral groups were selected for testing (Table 1). For each particle type, a suspension of the highest clay concentration (surface area ratio of 500) was used to determine the sufficient settling time. The clay suspension was made with clay particles and 1 l

of deionized water in a 1 l glass jar and then autoclaved at 121˚C for 15 min.

Table 1. Clay particle sources and surface area

Clay Particles	Source	Surface area[1] (m^2/g)
Hectorite	San Bernadino County, California The Clay Mineral Society, SHCa-1	63
Kaolinite	Acros Organics, #211740010	11.2
Ca-Montmorillonite	Gonzales County, Texas The Clay Mineral Society, STx-1b	84
Montmorillonite K-10	Acros Organics, #233170050	240

1. Surface area of each particle was provided by the manufacturer.

Surface area ratios

The surface area of *E. coli* was estimated as 6×10^{-12} m^2 (The CyberCell Database 2003) and the surface area of the clay particles was calculated using the surface area values provided by the suppliers (Table 1). We include 6 clay particle surface area to *E. coli* surface area ratios (1, 2, 50, 100, 200, and 500) for method comparison testing.

***E. coli* Partitioning Techniques**

Flow cytometry

The flow cytometer technique presented here is suitable for laboratory studies to improve understanding of bacterial attachment mechanisms but not yet appropriate for application to environmental samples. PBS was filtered through a 0.45 µm filter paper. The filtrate was then centrifuged at 2500 rpm for 5 minutes at 4ºC three times and the supernatant was saved for further use. SYTO 11 green fluorescent nucleic acid stain was filtered through a 0.2 µm filter to remove any potential particles. Fixed volumes of *E. coli*, clay, PBS, and SYTO 11 were combined to a volume of 250 µl (BD Biosciences; San Jose, CA). Six controls were required for each test: PBS only, PBS+SYTO dye, PBS+ *E. coli*, PBS+*E. coli*+ SYTO dye, PBS+ clay, and PBS+ clay+ SYTO dye. The *E. coli* concentration was 1.0×10^7 CFU ml^{-1} and clay concentration was based on surface area ratio. Samples were shaken by hand for 10 min to increase bacteria and particles interaction. 2µL of SYTO 11 was added after the other components were in the test tubes and immediately before analysis by the flow cytometer to prevent exposure to light. The samples were tested at 488 nm wavelength on FACSCanto flow cytometer (BD, Franklin Lakes, NJ) and analyzed by BD FACSCanto Clinical Software (BD, Franklin Lakes, NJ). The instrument was factory-modified from the base FACSCanto 6-color configuration to an 8-color configuration. For SYTO 11, FL1 channel was used as the optical detector and the bandpass filter was 530/30 nm. In this part of the analysis, the distinction between free *E. coli* and those attached to clay particles was established by examining the SSC of cells identified in gate "Free". "Free" *E. coli* (i.e. not attached to particles) are defined as those cells with low SSC, as demonstrated in Figure 2-A wherein *E. coli* are treated with SYTO 11 in the absence of clay particles. *E. coli* "Attached" to clay particles are defined as those cells with high SSC, as demonstrated in Figure 2-B wherein a mixture of *E. coli* and clay particles is treated with SYTO 11. SYTO 11 events with high SSC are only detected in samples containing a mixture of live *E. coli* and clay particles. In our case, the "Gating limit" was set by minimizing the percentage of cell events in gate "Attached" when *E. coli* are treated with SYTO 11 in the absence of clay particles in multiple samples with the same *E. coli* strain.

The live cell percentage was analyzed to address the inability to distinguish live/dead cells when only using one stain. SYTO 11 is a cell-impermeant dye that labels both live and dead cells, enabling discrimination of cells from background electronic noise and debris. Propidium iodide (PI) is impermeable to cells with intact membranes, but permeates dead cells. Fixed volumes of *E. coli*, PBS, PI dye, and SYTO 11 were combined to a total volume of 250 µl, in triplicate for each *E. coli* strain. Five controls were used as the gating references: PBS+PI dye+ SYTO dye, PBS+ *E. coli*, PBS+*E. coli*+ PI dye, PBS+ *E. coli*+ SYTO dye, 30% ethanol +*E. coli* + PI dye+ SYTO dye (dead cell staining). The culturing condition and concentration of *E. coli* are held constant with the conditions in the attachment assay, and the volume of SYTO 11 and/or PI added was 2 µL. For PI, FL3 channel was used as the optical detector and the bandpass filter was 610/20 nm. In this part, the gate for identifying *E. coli* is based on FSC signal and SYTO 11 fluorescence. Within this gate, the area for "live" *E.*

coli is defined based on control samples of *E. coli* stained with SYTO 11 only and the area for "dead" *E. coli* is defined based on control samples of ethanol-fixed *E. coli* stained with both SYTO 11 and PI. Moreover, performing the attachment assay and viability test simultaneously did not work well as the

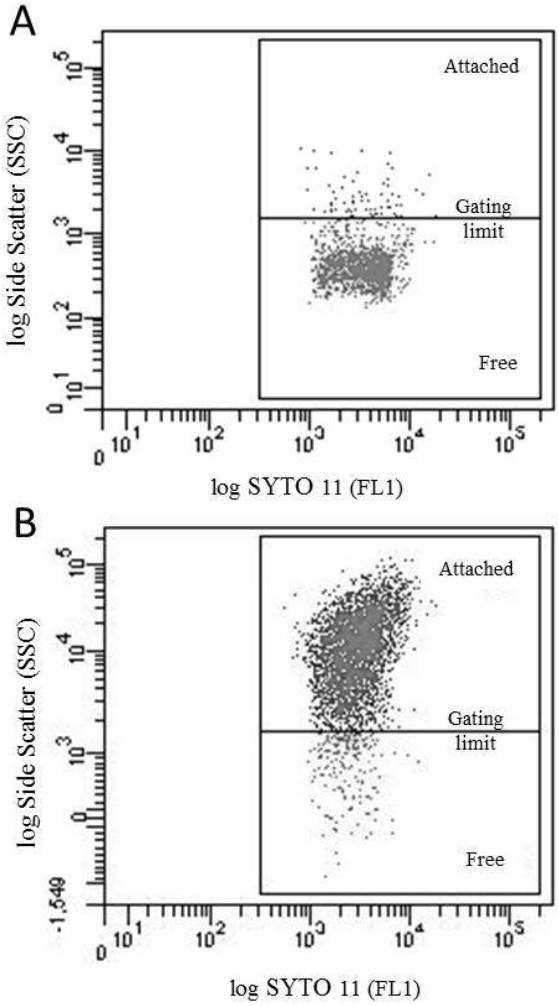

Figure 2. Distinction between free *E. coli* strain A and those attached to Hectorite. "Free" *E. coli* are defined as those cells with low SSC treated with SYTO 11 in the absence of clay particles (2-A). *E. coli* "Attached" to clay particles have high SSC (2-B), and are a mixture of *E. coli* and clay particles treated with SYTO 11. The "Gating limit" was established using the SSC of the cells in the control sample.

dead cell percentages were determined to be up to 95% in this case. There are two possible explanations: (1) after attaching to a particle, the membrane integrity of *E. coli* could be disrupted and PI may recognize it as dead cell, or (2) the particles are as bright as the bacteria in the PI channel: the particles have a higher level of auto-fluorescence in the PI channel or they are taking up some PI dye. Therefore, attachment percentage and live cell percentage were obtained separately. The actual attachment fraction for each "*E. coli* strain+ clay+ surface area ratio" combination was calculated as

$$\frac{\text{attachment percentage from attachment assay}}{\text{live cell percentage from viability assay}}$$

where we assume only live *E. coli* with intact membranes can attach to particles. A confirmation experiment using ethanol to fix *E. coli* was used to prove the assumption. In the presence of clay particles, after adding ethanol, all *E. coli* events appeared in "Free" *E. coli* zone. Thus, dead cells stained with SYTO 11, but did not attach to clay particles.

Settling/centrifugation followed by settling

A spectophotometer (HACH, Loveland, CO) was used to determine sufficient times for clay particles to settle from solution. The concentrations of clay suspensions with the greatest surface area ratio 500 (clay surface area to E. coli), were selected to test the settling time. Clay suspensions were placed in 15 ml centrifuge tubes on a polystyrene foam holder after hand-shaking. 1 ml of the supernatant was removed from the tube every 0.5 h and the absorbency values were then tested at a single wavelength of 400nm. At wavelength of 400nm, higher absorbency values appeared to be associated with higher sediment concentration (Karabulut and Ceylan 2005). The suitable concentration range of the used spectrophotometer is 5- 750 mg l^{-1}. The suitable settling time was set based on two subsequent readings of the same absorbency value. The determined settling times were: 60 min for Montmorillonite K-10, 150 min for Ca-Montmorillonite, and 5760 min (2 days) for Kaolinite. Samples with Kaolinite were pre-treated by centrifugation at 300 rpm for 5 min and the settling time was shortened to 1080 min (18 hr).

The clay concentrations were calculated according to the surface area ratio. Figure 3 shows a flow chart of the experimental procedures. Calculated volumes of *E. coli* suspensions (final concentration 1.0×10^7 CFU ml^{-1}), clay suspensions (final concentration as listed in Table 1), and PBS were made up to 50 ml in 250-ml Erlenmeyer flasks and the samples were shaken at 80 rpm for 10 min on an orbital shaker to increase bacterial particle interactions and attachment (Dimkpa et al. 2011). After shaking, the samples were transferred to 50-ml conical tubes and the tubes were placed vertically in racks to allow clay particles to settle via gravity for specified settling times except for Kaolinite samples as pre-centrifugation, as described above, was

required. The samples were placed at 4°C to reduce the possibility of *E. coli* regrowth. After settling 25 ml of supernatant was extracted and placed in a new conical tube. After vortexing for 10 s, 3 ml of supernatant was removed and diluted in 27 ml phosphate-buffered saline and then 1 ml was serially diluted in 9 ml phosphate-buffered saline four times. The final concentration was within the countable range recommended for the membrane filtration techniques (APHA 1999). 1 drop of Tween 85 (Fisher Scientific, Fair Lawn, New Jersey) was added to the remaining 25 ml and shaken at 300 rpm for 10 min with a handshaker (Fisher Scientific, Asheville, NC) (Soupir et al. 2008). The serial dilution procedure was the same as described for the supernatant. The total *E. coli* concentration in the supernatant and remainder were enumerated by membrane filtration in triplicate on Luria-Bertani agar or Tryptic Soy agar for environmental or pathogenic strains, respectively.

Analysis

Statistical analysis of data was performed using R project software (version 2.14.1). One sample t-tests were conducted to determine the method variability between the settling and flow cytometry separation techniques. The significant level was adjusted using Bonferroni method according to the number of samples used for comparison. Furthermore, three-way Anova tests were conducted to test the impacts from each variable (strain, clay type, or surface area ratio).

Results

The settling method has been employed to distinguish between unattached and attached *E. coli* in previous research. The average percentages of attached *E. coli* for all variables are shown in Table 2. The percent attached obtained from the flow cytometry was lower but not significantly different

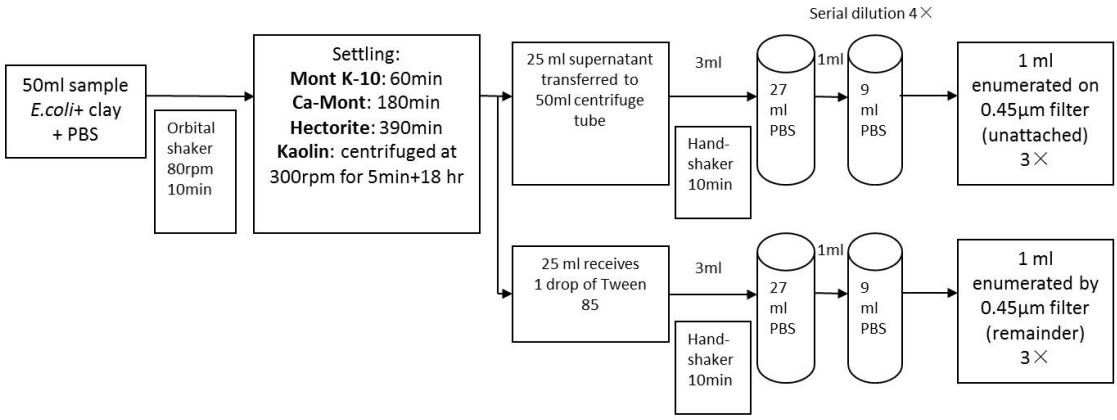

Figure 3. Flow chart describing the settling method procedure. (Mont is the abbreviation for Montmorillonite)

Table 2. Average percent attached for each clay, *E. coli* strain, and surface area ratio.

	Variable	Number of samples	Flow cytometry Percent attached	Settling Percent attached	p-value[2]
Clay	Hectorite	72	15(±2)[1] a[3]	34(±2) b	2.13×10^{-12}
	Kaolin	72	48(±5) a	37(±3) a	8.46×10^{-4}
	Ca-Montmorillonite	72	16(±2) a	23(±2) a	1.03×10^{-2}
	Montmorillonite K-10	72	5(±1) a	20(±2) b	$<2.20\times10^{-16}$
Strain	A	72	26(±4) a	29(±2) a	0.51
	B	72	18(±3) a	24(±2) a	6.66×10^{-3}
	C	72	16(±3) a	30(±2) b	2.54×10^{-9}
	ATCC 43888	72	22(±4) a	31(±2) a	1.49×10^{-2}
Particle: *E. coli* ratio	1	48	1(±0) a	17(±1) b	2.54×10^{-14}
	2	48	2(±1) a	18(±2) b	8.90×10^{-13}
	50	48	14(±2)a	24(±2) b	2.23×10^{-5}
	100	48	27(±4) a	30(±2) a	0.42
	200	48	35(±5) a	35(±3) a	0.90
	500	48	46(±5) a	47(±4) a	0.87

1 Standard error for each percent attached is listed in parenthesis.

2 The p-value of attachment difference between the two methods.

3 Within each row, values with the same letter are not significantly different. The significant level is determined by Bonferroni method: the adjusted α=α/repeated times with α=0.05. For comparison for clay or strain, the repeated times was 72, thus $\alpha_{adj}=6.94\times10^{-4}$. Similarly, for comparison for surface area ratio: $\alpha_{adj}=1.04\times10^{-3}$; for the overall comparison, $\alpha_{adj}= 1.74\times10^{-4}$.

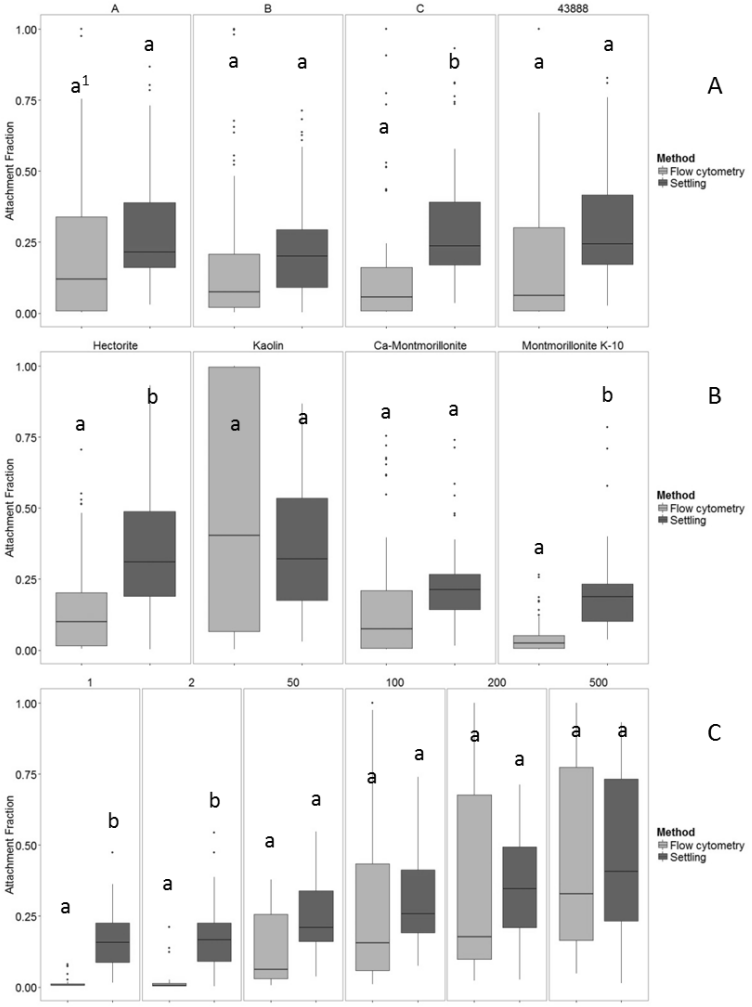

Figure 4. Boxplots[2] of attachment fraction from flow cytometry and the settling partitioning technique analyzed by each strain, clay type and surface area ratio.

1. Within each subplot, values with the same letter are not significantly different at the significant level. The significant level is determined by Bonferroni method: the adjusted α=α/repeated times with α=0.05. For comparison for clay or strain, the repeated times was 72, thus $\alpha_{adj}=6.94\times10^{-4}$. Similarly, for comparison for surface area ratio: $\alpha_{adj}=1.04\times10^{-3}$.

2. Each plot is using five-number summaries: the smallest observation (Q1=Q2-1.5(Q4-Q1), the low end of the line), 25% quartile (Q2, low boundary of box), median (Q3, the band near the middle of box) , 75% quartile (Q4, high boundary of box), and largest observation (Q5=Q4+1.5(Q4-Q1), the up end of the line); and also consider outliers (dots) if any.

from the percent attached obtained from the settling method for all conditions except when the particle was Hectorite or Montmorillonite K-10; when the strain was C; and when the surface area ratio was below 100.

Comparing the two partition techniques, the average attachment fraction achieved from the flow cytometry method was 25.7% lower than the average attachment fraction from the settling method over all variables. Under most conditions, the attachment fractions from the flow cytometry were not statistically significantly different from what were obtained from the settling method, while the difference was significant under the condition of strain C (p-value=2.54×10^{-9}), clay Montmorillonite K-10 (p-value=$<2.2\times10^{-16}$), and clay Hectorite (p-value=2.13×10^{-12}). From the boxplots in Figure 4-C, it was observed that as the surface area ratio increased from 1 to 500, the difference between the two methods became less significant and when the surface area ratio was greater than 50, the results from the two methods were not significantly different.

The attachment fraction of *E. coli* to Hectorite detected by flow cytometry was on average 56.8% lower than the attachment fraction achieved from the settling method. For example, for strain A: the average percent attached as detected by flow cytometry was 69.8% lower than the percent attached as detected by the settling method. Hectorite was the smallest particle tested, with an average diameter 1 μm; much smaller than an average *E. coli* (1.1 to 1.5μm wide by 2 to 6 μm long). Attachment to Hectorite particles did not result in a significant size change, making identification of the gating limit between attached and unattached cells difficult. *E. coli* attached to Hectorite could not be differentiated from unattached *E. coli* as was the case with the other three clay particles. This resulted in a lower attachment fraction value by the flow cytometer method and appears to be a limitation of this flow cytometry application. Moreover, as recommended, suspended particle or cell sizes smaller than 30 μm are most suitable for

analysis by the flow cytometer (BD 2000). This recommendation also suggests that the Montmorillonite K-10 particle may be unsuitable, since its diameter at 90% is 41.3 μm, tested by CILAS 1190 (Cilas, Madison, WI). Larger-sized or aggregated particles would likely clog the inlet of the flow cytometer, and therefore, there is also an upper limit on the particle sizes suitable for this analysis.

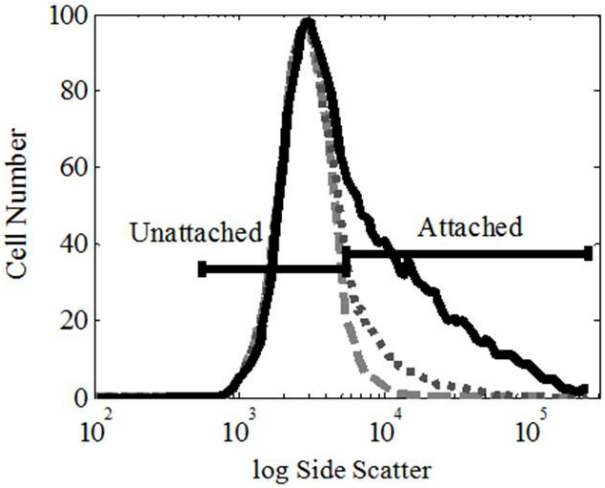

Figure 5A. Histogram of cell number vs. log side scatter for *E. coli* strain A attaching to Montmorillonite K-10 over three different surface area ratios. The long dashed line represents surface area ratio 2, the short dashed line shows surface area ratio 100 and the continuous line is for the surface area ratio of 500.

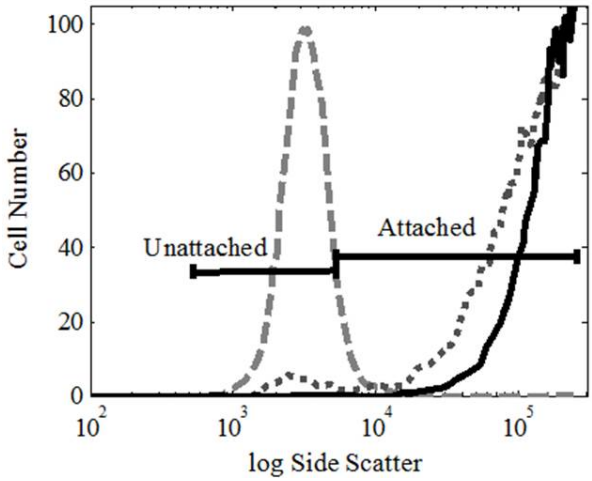

Figure 5B. Histogram of of cell number vs. log side scatter for *E. coli* strain A attaching to Kaolinite over three different surface area ratio. The long dashed line represents surface area ratio 2, the short dashed line shows surface area ratio 100 and the continuous line is for the surface area ratio of 500.

Histograms of *E. coli* attachment to Kaolinite exhibited different patterns when compared to histograms of *E. coli* attachment to Hectorite, Ca-Montmorillonite and Montmorillonite K-10, as shown in Figure 5-A and 5-B. In the figures, three samples with different surface area ratios for strain A attaching to Montmorillonite K-10 and Kaolinite were overlaid in Figure 5-A and 5-B, respectively. From Figure 5-A, the percent attached of strain A to Montmorillonite K-10 increased when the particle ratio increased from 2 to 500, but the three curves shared one peak, which means that the majority of *E. coli* was still in the same optical density range after increasing Montmorillonite K-10 concentration. However, in Figure 5-B, which shows the attachment of strain A to Kaolinite, the peaks of particle ratios 100 and 500 differ from the peak of the particle ratio 2 curve. The shift indicates that the dimension of *E. coli* increased sharply after attaching to Kaolinite as the ratio increased. Kaolinite has the smallest surface area among the four clays (Table 1), so with the same surface area ratio, the Kaolinite suspension had the highest clay particle count. When considering the small diameter and surface area of Kaolinite particles, it is likely that several clay particles attached to the same *E. coli* surface.

Strain type, clay type, and clay concentration (surface area ratio) all impact the *E. coli* attachment to clay particles. Among the four clays, *E. coli* was more likely to attach to Kaolinite, which has the smallest surface area and second smallest diameter. This result is consistent with the previous research, in which *E. coli* preferentially associates with smaller sized particles. Even though the three environmental strains were all isolated from swine manure, they varied in attachment fraction to clay particles. As the surface area ratio increased from 1 to 500, the average percent attached of triplicates increased with greatest attachment occurring at surface area ratio 500 (46% or 47% for flow cytometry or the settling method, respectively). Moreover, when using the flow cytometry technique, the single percent attached reached a maximum value of 100% for *E. coli* attachment to Kaolinite.

Discussion

The greatest advantage of the flow cytometry detection method is the rapidity in which a large numbers of cells can be analyzed (Macey 2007). In this study, using flow cytometry shortened the experimental time from up to 1 day to about 1 hour and a cost comparison of the two methods yielded similar results (Liang 2012).

Viability analysis was performed to address the inability to distinguish live/dead cells when only using one stain in the attachment analysis. An advantage of combining viability and attachment analysis in flow cytometry when compared to more standard culture based methods is the ability to detect part of the viable but nonculturable (VBNC) portion of the total cell population. When released in natural waters, a fraction of fecal bacteria that have been exposed to environmental stresses, such as nutrient starvation and high pressure, lose the ability to recover metabolically active, intact cells (Oliver 1993; Khan et al. 2010). The VBNC state is reversible since the cells can be resuscitated to become culturable again under suitable condition (Barer et al. 1993; Pinto et al. 2011). In the viability analysis, flow cytometry can count all intact membrane cells as live cells, while the culture based methods might potentially miss some intact but VBNC cells. The percent attached obtained from the settling method was always higher than the attachment values obtained from the flow cytometry technique. Some of these differences between the two methods are due to the VBNC portion of cells. From this perspective, the percent attached achieved from the settling method may be considered less reliable.

The flow cytometer application presented here is best suited for laboratory studies to improve understanding of bacterial attachment mechanisms. Further method refinement is required for environmental applications. Environmental water samples contain mixed bacteria populations. Recognizing different strains may be achieved by multiple-staining techniques, but only if the types and properties of the microorganisms are well-known. For example, the Fluorescence in situ hybridization (FISH) technique has previously been applied with flow cytometry for testing the microorganism in environmental water samples (Mosiman et al. 1997; Yee and Fein 2001). Direct detection of *E. coli* O157:H7 in flow cytometry, can be achieved using Fluorescein-labeled antibodies (FITC), which bind only to specific protein expressed by *E. coli* O157:H7 (Wu et al. 2012).

In natural waters, there exist various other microorganisms, which have diverse characteristics. Further, particle surfaces would not exist as the clean mineral surfaces used in this study. Organic compounds and metal oxide are typically found as a coating of the surface structure of particles which would likely impact the attachment from microorganisms. Here, the experiment was designed with ideal conditions to test the flow cytometry method and eliminate the potential impact from other factors when comparing the two methods.

Conclusion

Flow cytometry was compared to a standard settling method to partition between freely suspended *E. coli* and *E. coli* associated with clay particles. In general the method was demonstrated as a rapid technique for distinguishing and quantifying the unattached and attached *E. coli* that can be readily used for laboratory studies of bacteria partitioning behavior. Limitations of the method include environmental strain diversity for application to environmental samples and the diameter of particles suitable for testing. This method is useful for future work on the exploration of waterborne pathogen attachment properties which is needed since current pathogen indicators are not always representative of a potential risk to public health.

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Conflict of interest

No conflict of interest declared.

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