PARTICLE-ASSOCIATED MICROORGANISMS IN STORMWATER RUNOFF

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Abstract---This research investigated the effects of blending and chemical addition before analysis of the concentration of microorganisms in stormwater runoff from a single summer storm to determine whether clumped or particle-associated organisms play a significant role. The standard membrane filtration method was used to enumerate the microorganisms. All organisms, except for *Escherichia coli,* showed an increase in the measured concentration after blending samples at 22,000 rpm with or without the chemical mixture. Other than fecal streptococci, the organism concentrations decreased with the addition of the Camper's solution in both blended and unblended samples before analyses. There was a statistically significant interaction between the effects of Camper's solution and the effects of blending for all the organisms tested, except for total coliform. Blending did not alter the mean particle size significantly. The results show no correlation between increased total coliform, fecal coliform, and fecal streptococcus concentrations and the mean particle size.

Key words---stormwater runoff, particle-associated microorganisms, blending, Camper's solution, particle size, chemical addition

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INTRODUCTION

According to the EPA's 1998 National Water Quality Inventory Report to Congress, about 40% of assessed U.S. streams, lakes, and estuaries did not support the criteria for locally-designated uses such as fishing and swimming. High bacteria concentrations in stormwater runoff from agricultural and urban areas are a leading cause in the failures to meet designated use criteria (USEPA, 2000). Investigators have documented large concentrations of fecal coliform and pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* in urban stormwater (Oliveri *et al.*, 1977). Schillinger and Gannon (1982) reported that about 15 to 20 percent of fecal coliform cells present in untreated stormwater are adsorbed to larger suspended particles, most of which were greater than 30μm in diameter. They further noted that more than half the organisms were not attached and remained suspended in water. Traditionally, monitoring and research programs quantify the microorganism concentrations in samples using standard methods (e.g., membrane filtration or multiple tube fermentation). By design, these methods target public health and do not completely measure either clumped organisms or organisms associated with particles; therefore, they may not fully enumerate the organism concentrations. This research gauges the degree that clumped and particle-associated organisms exist in stormwater runoff by using sample pretreatments such as blending, adding a mixture of chemicals believed to help separate organisms from particulates, or both to estimate the degree that these phenomena affect measurements of bacteria in stormwater.

The literature supports the phenomena of clumped/aggregated and particle-associated organisms in drinking and municipal wastewaters and outlines general procedures to document the severity by treating samples before traditional analysis. Conceptually, the pretreatment processes use mechanical or chemical techniques to free bacteria from either particulate matter or other bacteria in the sample. Each separated organism forms a separate colony during incubation, allowing more complete enumeration.

Researchers and practitioners have selected various blending conditions and studied different organisms. Early investigations on primary clarified combined sewer overflow (CSO) by Glover and Herbert (1973) and Moffa *et al.* (1975) established mechanical separation as a technique to more completely enumerate the bacteria concentrations. Camper *et al.* (1985)

evaluated microorganism desorption from granular activated carbon using a mixture of chemicals resulting in concentrations of 10^{-6} M Zwittergent 3-12, 10^{-3} M EGTA, and 0.01 M Tris buffer with 0.01 wt% peptone and about pH 7. In these desorption experiments, blending samples at 16,000 rpm for 3 minutes with the chemical mixture gave the highest recovery of heterotrophic plate count (HPC) organisms from the spent carbon. This process increased the measured HPC concentration as much as 50-fold and coliform concentrations as much as 1200-fold in drinking water treated with carbon, compared with hand shaken samples. Parker and Darby (1995), using multiple tube fermentation (MTF) methods to study secondary effluent disinfection, found that blending samples with the same chemical mixture used by Camper *et al.* at 19,000 rpm for 1.5 minutes produced the greatest recovery of particle-associated coliform organisms. They concluded that particle association and organism shielding significantly affect MTF coliform density measurements, and effluents may contain many more coliform bacteria than measured using the standard enumeration procedure.

Perdek and Borst (2000) evaluated blending CSO samples and diluted sanitary sewage to release particle-associated microorganisms before measuring microbial indicator concentrations by membrane filtration (MF). After screening the samples to remove solids greater than 2 mm, the samples were pretreated to reduce the concentrations of free-swimming microorganisms by ultraviolet irradiation. The analysis of blended samples showed as much as a 10-fold increase in measured fecal coliform and enterococcus concentrations, compared with concentrations measured in the unblended samples without Camper's solution. These experiments showed that a blending speed from 14,500 to 22,000 rpm and a blending time from 0.5 to 3 minutes affected the measured concentrations. Samples blended for about 2 minutes at the highest speed evaluated, 22,000 rpm, showed the greatest concentration increases. These results also showed that the blending decreased mean particle size, but showed no correlation between increased indicator microorganism concentration and decreased particle size. The New York City Department of Environmental Protection (NYCDEP) blended raw treatment influent, primary effluent, and chlorinated primary effluent and reported an increase in concentration with an increase of blending time in the range of 0 to 60 seconds, with peak total coliform counts within the first 10 seconds of blending (CDM, 1997). Ridgway and Olson (1982) found that particle-associated bacteria in drinking water were found mostly on particles greater than 10 : m in

diameter. Scheible *et al.* (1986), as part of a disinfection study, selected much shorter blending times.

Although several researchers documented the release of particle-associated or clumped organisms in drinking and wastewaters using blending and chemical addition before analysis, none have focused on stormwater runoff, and few have studied a varied collection of organisms. This research uses both techniques to decide whether clumped or particle-associated organisms play a significant role in stormwater samples and if sample pretreatment using these techniques will give a more thorough picture of the organism concentration. The research uses both techniques (blending with and without the Camper's solution) because of the inconsistent treatments reported in the literature and the lack of a clear mechanism for the release process.

MATERIALS AND METHODS

Sample collection

An automatic sampler (Model #900 max, American Sigma, Loveland, CO) collected a flow-weighted stormwater sample from a 15-inch diameter, concrete storm sewer outfall. The storm sewer drains a small, slightly sloping, high-density residential area in Monmouth County, New Jersey. Earlier evaluations following the procedures developed by Pitt *et al.* (1993) showed the storm sewer was unlikely to have sanitary cross connections. The automatic sampling began when the flowing water depth in the storm sewer reached 2.54 cm. The sampler collected one 1-L sample after each 1,350 L of stormwater flow was measured by the attached flow meter (Model #960, American Sigma, Loveland, CO). A calibrated peristaltic pump transferred the samples to a precleaned (Standard Methods 9040), 5-gallon HDPE container. The sample was collected during a rain event on July 10, 2000. The event produced 1.8 mm total rainfall over 74 minutes. Rainfall was recorded using a tipping bucket rain gage (Model #RGD-04, Environmental Sensors, Inc., Escondido, CA), positioned near the sampler within the drainage area. The runoff was slightly acidic (pH from 6.03 to 6.86), with a conductivity of 0.1 to 0.2 mS, and a temperature from 20.5 to 23.8^{o}C. The gage recorded no rain at the site during the preceding 140 h. The nearly 6-day dry period should be sufficient for normal terrestrial build-up processes, the net effect of time-dependant deposition and loss processes, to reach equilibrium

(Sartor and Boyd, 1972). The sample was recovered, placed in a cooler with ice and transported to the laboratory for processing.

Experimental methods

In the laboratory, the sample was thoroughly mixed by shaking the container and was divided into six subsamples. The subsamples were prepared using a three-by-two experimental design (Refer to Figure 1). The design evaluates adding the Camper's solution (yes or no) and three blending times (0, 1, or 2 minutes) with a fixed blending speed (22,000 rpm). Camper's solution is a mixture of chemicals (Zwittergent 3-12, ethyleneglycol-bis-($ amino-ethyl ether)-N,N'-tetra acetic acid (EGTA), and Tris buffer). Work by Camper *et al.*, (1985) showed that the mixture enhances bacteria dissociation from solids. The chemicals were added to final concentrations of 10^{-6} M Zwittergent 3-12, 10^{-3} M EGTA, 0.01 M Tris buffer, and 0.01 wt% peptone, buffered to a pH of about 7 before blending.

When called-for in the experimental plan, the samples were blended at 22,000 rpm (Manufacturer's reported speed) in a 7-speed commercial laboratory blender (Blend Master # 57199, Hamilton Beach, New Hartford, CT) for the designated time as measured with a stopwatch. The 1.2-L blender has 4 mixing blades: 2 rounded blades pointing upward and 2 pointed blades tilting downward. Each blade is approximately 2.5-cm long and 1-cm wide at the base. The blender jar was washed with soap between uses and autoclaved at 15 psi pressure for 15 minutes to assure sterility. The plastic lid was rinsed with isopropyl alcohol. The sample temperature increase during blending was monitored during separate studies and found negligible for the 2-minute period.

The resulting samples were analyzed for four indicator organisms (total coliform, fecal coliform, fecal streptococcus, and *Escherichia coli* (*E. coli*)). These organisms are commonly used or proposed bacterial indicators in water quality monitoring.

Particle size distributions of the samples before and after blending the sample for one or two minutes were measured using a Coulter Particle Characterization Unit (Model # Delsa 440 SX, Beckman Coulter, Miami, FL).

Analysis of microorganisms

All samples were analyzed using membrane filtration methods following Standard Methods for the Examination of Water and Wastewater (APHA *et al.*, 1998). Three serial sample dilutions were prepared in sterile phosphate buffered solution before filtration. A series of three dilutions were selected for each organism using analytical results of samples previously collected at the same outfall, in order to obtain a colony count in the preferred 20 to 60 colony range. Quadruplicate analyses for each organism at each dilution were completed to monitor the analytical variability. Reference cultures were used in the laboratory to evaluate the test procedures, including media and reagents. Blanks were run before and after each analytical set.

Total coliforms were determined by incubation on M-Endo agar for 24 h at 35°C and confirmed by gas formation in lauryl tryptose broth and brilliant green lactose broth. Fecal coliform was incubated on M-FC agar for 24 h at 44.5^{o}C and confirmed by gas formation in lauryl tryptose broth and EC broth. *E. coli* levels were measured by transferring the membrane from the Endo-type medium to a nutrient agar containing 4-methylumbelliferyl-$-D-glucuronide (NA-MUG), incubating for 4 h at 35°C, and checking for blue fluorescence on the colony periphery under long-wavelength UV. Similarly, *E. coli* levels can also be measured by transferring the membrane from the fecal coliform positive sample to a nutrient agar containing NA-MUG. Fecal streptococci concentrations were determined by incubation on m-Enterococcus agar for 48 h at 35°C. Colonies were transferred to brain heart infusion (BHI) agar incubated for 24-48 h at 35°C. Transfers were made to BHI broth and incubated at 35°C for 24 h, with confirmations made by retransfer to bile esculin agar incubated at 35°C for 48 h, BHI broth incubated at 45°C for 48 h, and BHI with 6.5% NaCl incubated at 35°C for 48 h.

Chemicals

Zwittergent 3-12 was purchased from Calbiochem-Novabiochem Corp. (LaJolla, CA). M-Endo agar, M-PA agar, Baird-Parker agar, NA-MUG, and M-Endo media were obtained from Difco Labs (Detroit, MI). M-FC media and lauryl tryptose broth were purchased from Beckton Dickinson (Sperks, MD). All other chemicals were obtained from Sigma Chemical Corp. (St Louis, MO). All the chemicals were stored according to manufacturers' recommendations.

Data analysis and statistical methods

Organism concentrations were calculated and expressed as colony forming units per 100 mL (CFU/100 mL). Previous evaluation at this outfall (data not shown) supported a log-normal distribution ($\log_{10}$-transformed) of organism concentrations as suggested by other researchers (APHA *et al.*, 1998; USEPA, 1983). Organism concentrations were $\log_{10}$-transformed before data analysis. Raw colony counts obtained from the set of plates with enumerations in the preferred 20- to 60-count range were used for data analysis. When no set of plates contained countable colonies in the target range, countable plates on both sides were used for data analysis.

Multiple analysis of variance was used to compare data groups. Statistical significance was set at the 95% level of confidence. Analysis was done using Statistica software (StatSoft, Inc., 1998).

RESULTS

Analytical variability

Analytical variability of microorganisms was calculated using the standard deviation of the $\log_{10}$-transformed data from the four replicate analyses in the dilution sets used. The standard deviation of the $\log_{10}$-transformed data is relatively constant and generally less than 0.25 units.

Effects of blending on microorganism concentrations

Total Coliform: Table 1 summarizes the results for the total coliform analyses. Adding Camper's solution decreased the measured total coliform concentration in both blended and unblended samples ($p<0.01$). Blending the sample before analysis increased the measured concentrations in both the samples with Camper's solution and without Camper's solution. The 0.26-log increase in mean concentration of samples analyzed without Camper's is not significant ($p=0.56$). The 0.67-log difference in samples analyzed with Camper's solution is significant ($p=0.02$). The interaction between the effects of Camper's solution and the effects of blending is not significant ($p=0.15$). The difference between 1- and 2-minute blending time is not significant ($p=0.25$). Figure 2 presents the concentrations of total coliform in both blended and unblended samples with and without Camper's solution.

Fecal Coliform: Figure 3 and Tables 2 and 3 give the results of the analyses. Adding Camper's solution decreased the measured concentration of fecal coliform in both blended and unblended samples ($p<0.01$). Blending the sample before analysis increased the measured concentrations in both the samples with Camper's solution and without Camper's solution. The 0.87-log difference without Camper's is significant ($p<0.01$) and the 1.42-log difference in samples analyzed with Camper's solution is also significant ($p<0.01$). An interaction exists between Camper's solution and blending ($p<0.01$). Increasing the blending time from 1 to 2 minutes increased the measured concentration. The 0.27-log increase in mean concentration without Camper's solution is not significant ($p=0.11$), but the 0.33-log increase with Camper's is significant ($p<0.01$).

Fecal Streptococcus: Adding Camper's solution decreased the measured concentration of fecal streptococcus in blended samples, but increased the concentration in unblended samples ($p<0.01$) (Table 4). Blending the sample before analysis increased the measured concentrations in both the samples with and without Camper's solution. The 0.82-log difference in mean concentrations without Camper's is significant ($p<0.01$). The 0.06-log difference in the means of samples analyzed with Camper's solution is not significant ($p=0.67$). The interaction between the effects of Camper's solution and the effects of blending is significant ($p<0.01$). Increasing the blending time from 1 to 2 minutes does not increase the measured concentration in samples without Camper's solution. Figure 4 presents the concentrations of fecal streptococcus in both blended and unblended samples with and without Camper's solution.

E. coli: Adding Camper's solution decreased the measured *E. coli* concentration in unblended samples ($p<0.01$), but increased the concentration in blended samples ($p<0.01$) (Table 5). Blending the sample before analysis decreased the measured concentrations in samples without Camper's solution ($p<0.01$), but increased the measured concentration in samples with Camper's solution ($p=0.12$). The 2.29-log difference without Camper's is significant ($p<0.01$). The 0.94-log difference in the means of samples analyzed with Camper's solution is not significant ($p=0.12$). The interaction between the effects of Camper's solution and the effects of blending is significant ($p<0.01$), and increasing the blending time does not affect the measured sample concentration. Figure 5 presents the concentrations of *E. coli* in both blended and unblended samples with and without Camper's solution.

Effects of blending on particle size

Table 6 lists the mean particle size of the samples before and after blending for 1 and 2 minutes. The mean particle size remains essentially constant with 1 and 2 minutes blending. The results show no correlation between increased total coliform, fecal coliform, and fecal streptococcus concentrations and the mean particle size. Earlier studies by Perdek and Borst (2000) with CSO samples and diluted sanitary sewage showed decrease in mean particle size with blending. However, no correlations between increased fecal coliform and enterococcus indicator concentrations and decreased mean particle size were observed.

Summary

Blending samples before analysis increased the measured concentration of all bacteria except for *E. coli*. Adding Camper's solution to the sample but not blending decreased the concentration of all measured bacteria concentrations other than fecal streptococcus. Adding Camper's solution before blending decreased all measured concentrations other than *E. coli*, however, these decreases were smaller than the decreases observed with Camper's alone. An interaction exists between blending and adding Camper's solution. Blending the sample does not affect the measured mean particle size.

DISCUSSION

In many, perhaps most measurements, including the particle-associated organisms in the measured aqueous bacterial load will have little direct effect on the intended use of the analytical result. The comparative difference in concentration measured in raw combined or sanitary sewage accounted for by attached organisms, for example, will generally be negligible if considered with the magnitude of the measurement and the application. Increasing the reported concentration of a few orders of magnitude in these applications say from 10^6 to 10^9CFU/100 mL will not effect decisions based on the data even if the differences are statistically significant. In

these conditions, understanding of the propensity of organisms attached to particles may have little more than academic interest. In selected applications, however, the relative differences can be not only statistically significant, but also physically significant. Evaluating of the full or partial disinfection processes of these same two streams after pretreatment may require consideration of the associated organisms to fully understand the process effectiveness.

Stormwater runoff in municipal separate storm sewer systems (MS4s) can become a major part of the total stream flow in some low-order receiving waters. In these applications, the fully-diluted stormwater flow can readily raise the bacteria concentration in the monitored receiving water to approach water quality standards. Including the particle-associated organisms can shift the analysis from "pass" to "fail." Similarly, when stormwater flows through retention controls, the particles and associated organisms can, depending on effective holding time and settling velocity, settle and accumulate. Knowing that the organism decay processes have long time constants in sediment (Schillinger and Gannon, 1982), may suggest that sediment removal frequencies should increase to prevent microorganism-rich washouts to receiving waters. Similarly a stormwater management strategy could be developed promoting aqueous conditions that induce passive particulate attachment making retention an effective substitute for stormwater disinfection to protect receiving waters. These results, although based on a single event from a single outfall, support the supposition that particle-associated bacteria exist in stormwater. Watershed managers and supporting practitioners must consider the potentially advantageous and disadvantageous effects that result.

CONCLUSIONS

Stormwater runoff contains organisms not readily identified using standard MF analysis. Each tested organism, except for *E. coli,* showed an increase in measured concentration after blending. The relative increases varied with the specific organism. The chemical mixture developed by Camper *et al.* (1985) for releasing organisms from activated carbon did not consistently promote, and appears to usually suppress, the release for later enumeration. In some cases, the apparent suppression was two orders of magnitude. There is a statistically significant interaction between the effects of Camper's solution and the effects of blending for all the

organisms tested, except for total coliform. Blending had negligible effects on mean particle size.

Although based on a single storm event from a single outfall, these results suggest that particle-associated microorganisms play an important, if often unmeasured, portion of the total organism count in stormwater.

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DISCLAIMER

Use of trade, brand, or firm names in this report is for identification purposes only and does not constitute endorsement by the U.S. Environmental Protection Agency.

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Table 1. Summary of Results for Total Coliform Analysis

Camper's Added	Unblended Samples			Blended Samples			Significance
	Concentration, C (CFU/100 mL)	Log C	Number of Samples N	Concentration, C (CFU/100 mL)	Log C	Number of Samples N	p
No	3.7×10^4	4.56	5	6.7×10^4	4.82	5	p=0.56
Yes	3.1×10^2	2.50	4	1.5×10^3	3.17	6	p=0.02
Significance		p<0.01			p<0.01		

Table 2. Effects of Blending Time on Fecal Coliform Concentrations

Camper's Added	1-Minute			2-Minute			Significance
	Concentration, C (CFU/100 mL)	Log C	Number of Samples N	Concentration, C (CFU/100 mL)	Log C	Number of Samples N	p
No	7.3×10^4	4.86	4	1.3×10^5	5.13	3	p=0.11
Yes	2.0×10^2	2.31	4	4.3×10^2	2.64	4	p=0.01
Significance		p<0.01			p<0.01		

Table 3. Summary of Results for Fecal Coliform Analysis

Camper's Added	Unblended Samples			Blended Samples			Significance
	Concentration, C (CFU/100 mL)	Log C	Number of Samples N	Concentration, C (CFU/100 mL)	Log C	Number of Samples N	p
No	1.3×10^4	4.11	10	9.5×10^4	4.98	7	$p<0.01$
Yes	1.1×10^1	1.05	2	3.0×10^2	2.47	8	$p<0.01$
Significance		$p<0.01$			$p<0.01$		

Table 4. Summary of Results for Fecal Streptococcus Analysis

Camper's Added	Unblended Samples			Blended Samples			Significance
	Concentration, C (CFU/100 mL)	Log C	Number of Samples N	Concentration, C (CFU/100 mL)	Log C	Number of Samples N	p
No	1.0×10^3	3.00	5	6.6×10^3	3.82	8	$p<0.01$
Yes	2.7×10^3	3.43	5	3.1×10^3	3.49	9	$p=0.67$
Significance		$p<0.01$			$p<0.01$		

Table 5. Summary of Results for *E. coli* Analysis

Camper's Added	Unblended Samples			Blended Samples			Significance
	Concentration, C (CFU/100 mL)	Log C	Number of Samples N	Concentration, C (CFU/100 mL)	Log C	Number of Samples N	p
No	1.7×10^4	4.23	5	8.8×10^1	1.94	4	$p<0.01$
Yes	2.0×10^1	1.29	4	1.7×10^2	2.23	17	$p=0.12$
Significance		$p<0.01$			$p<0.01$		

Table 6. Mean Particle Size

Condition	Mean Particle Size (: m)
Unblended	0.768
Blended for 1 minute	0.758
Blended for 2 minutes	0.775

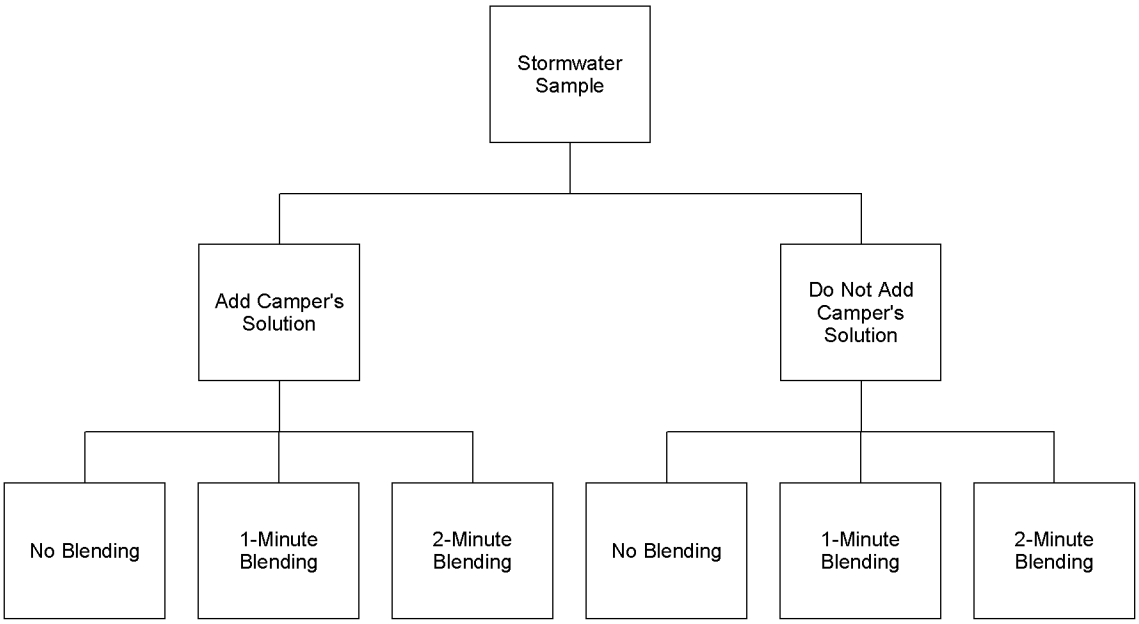

Figure 1. Test Design for Blending Study

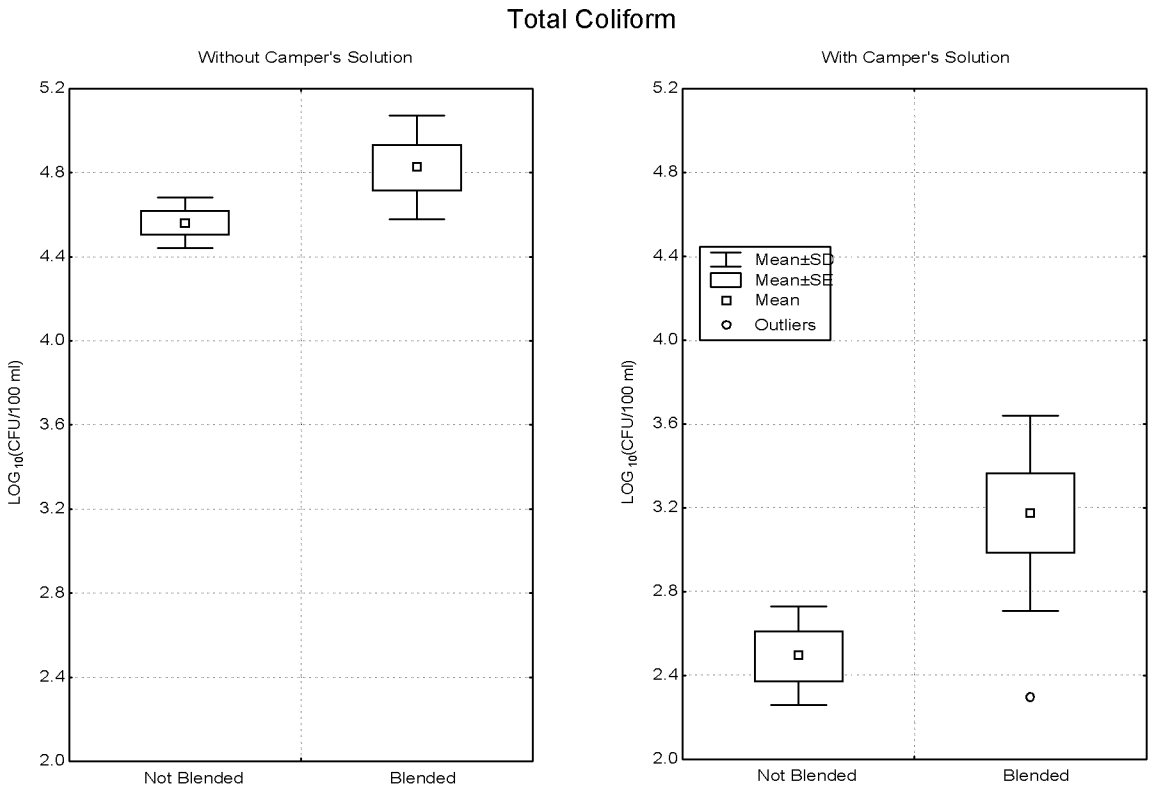

Figure 2. Total Coliform Concentrations

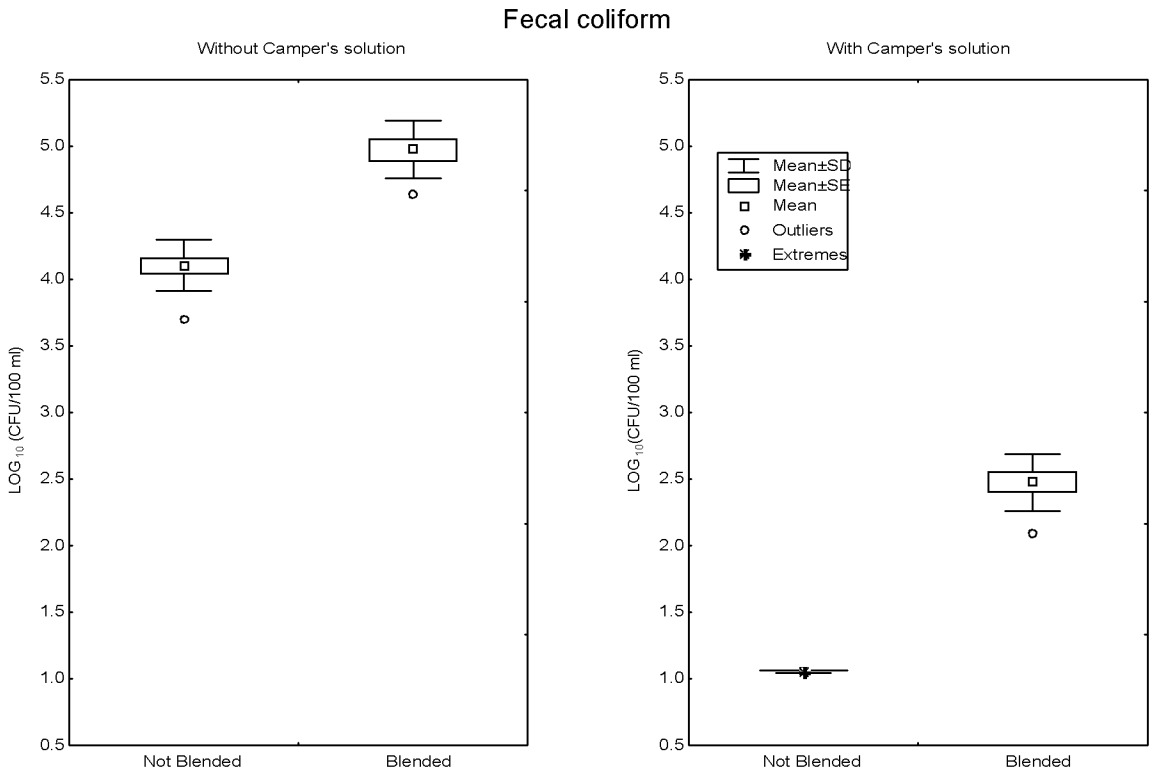

Figure 3. Fecal Coliform Concentrations

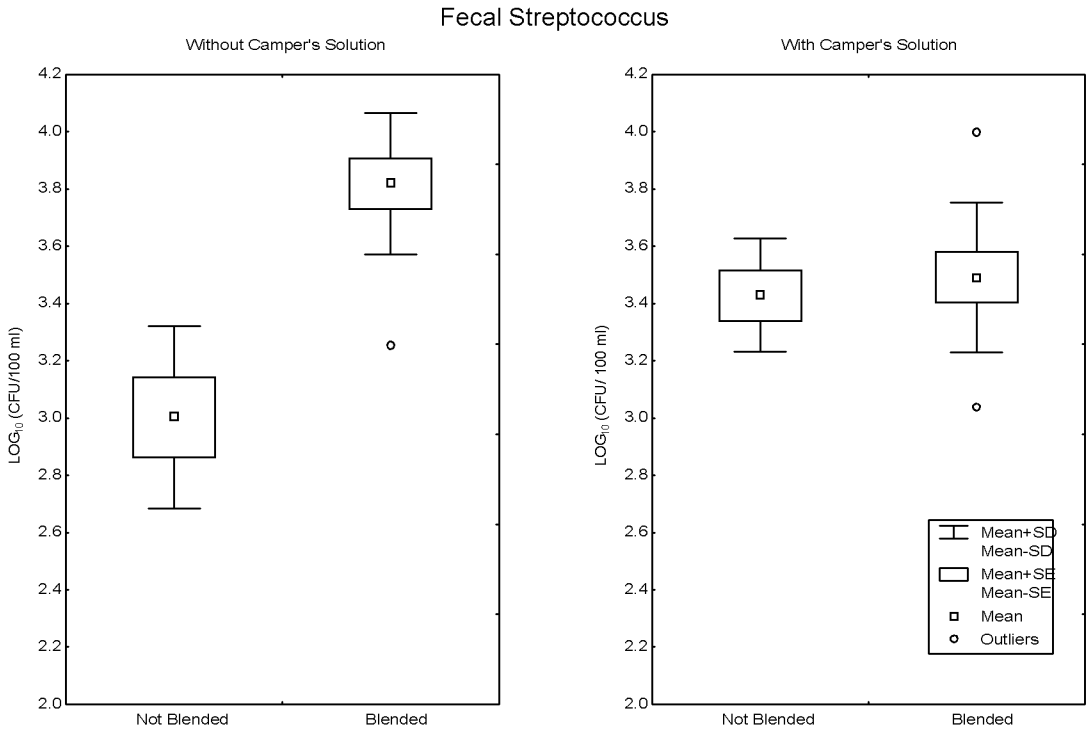

Figure 4. Fecal Streptococcus Concentrations

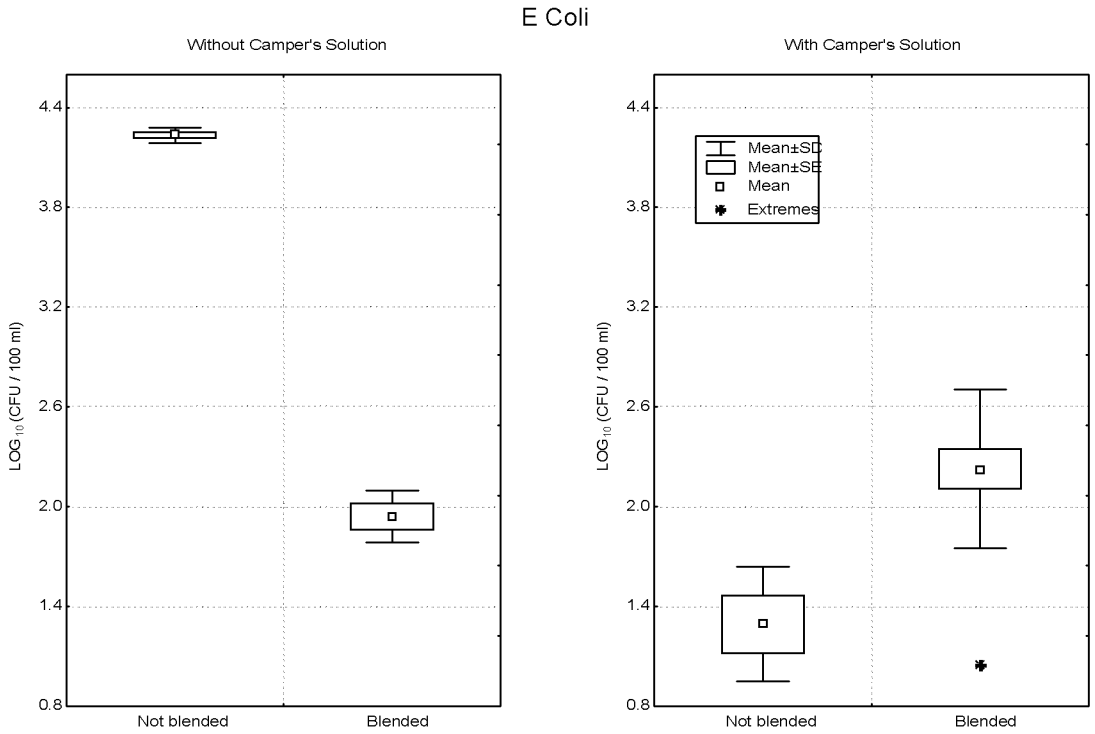

Figure 5. *E. coli* Concentrations