

## Comparison of the presence and partitioning behavior of indicator organisms and *Salmonella* spp. in an urban watershed

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### ABSTRACT

The appropriateness of indicator organisms as surrogates for human pathogens in water quality modeling is dependent on similarities in both presence and transport behavior; however, very little data relating indicator and bacterial pathogen transport behavior in receiving waters is available. In this study observations of presence, partitioning behavior (i.e. association with settleable particles) and removal by upland detention basins were used to assess the suitability of six indicator organisms as surrogates for *Salmonella* spp. bacteria in an urban watershed. The fecal indicator bacteria (fecal coliforms, *E. coli* and enterococci) were most closely correlated with *Salmonella* in terms of presence and partitioning behavior (25–35% associated with settleable particles on average); however, further resolution of the variability associated with *Salmonella* partitioning is required. Higher removal of particle-associated microbes relative to the total microbial concentration by the detention ponds suggests that sedimentation may be an important removal mechanism. However, large fluctuations in pond performance between storm events and occasional net microbial exports in effluents indicate that these best management practices (BMPs), as currently implemented, will be unlikely to achieve water quality objectives.

**Key words** | indicator organisms, partitioning, *Salmonella*, stormwater

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### INTRODUCTION

Storm runoff can mobilize and transport fecal microorganisms from various nonpoint sources to receiving waters, resulting in elevated downstream microbial concentrations (Hunter *et al.* 1992; Noble *et al.* 2003; Kim *et al.* 2005). Increased precipitation has also been linked to outbreaks of waterborne disease (MacKenzie *et al.* 1994; Rose *et al.* 2000; Gaffield *et al.* 2003) and exposure to waters receiving stormwater discharges has been correlated with an elevated risk of illness (Haile *et al.* 1999; Gaffield *et al.* 2003). Development of successful watershed protection or remediation plans to protect the public health requires identification of microbial sources and their transport pathways, with microbial fate and transport models often

playing a prominent role (Ferguson *et al.* 2003). These models require accurate inputs related to microbial transport behavior (e.g. settling velocity), as well as improved estimates of the effectiveness of various best management practices (BMPs) for reducing upland microbial loadings.

Despite the relatively large impact of nonpoint source pollution on instream microbial concentrations, quantitative data describing microbial transport in surface water is sparse and limits the reliability of modeling efforts (Jamieson *et al.* 2004a; Dorner *et al.* 2006; Pachepsky *et al.* 2006; Arnone & Walling 2007; Benham *et al.* 2008; Keller & Cavallaro 2008). Specifically, microbes are generally modeled as individual free cells of near-neutral buoyancy,

even though increasing evidence suggests that many microorganisms partition between a particle-associated and free (unassociated) phase (Ferguson *et al.* 2003). Quantifying the relative fractions of organisms associated with particles is essential to modeling efforts as particle-microbe aggregates settle more quickly from the water column and thus travel shorter distances in receiving waters (Gannon *et al.* 1983; Jeng *et al.* 2005). Previous work also links microbial association with sediments to prolonged survival (Gerba & Schaiberger 1975; Gerba & McLeod 1976; Howell *et al.* 1996; Jamieson *et al.* 2004b) and/or regrowth (Hendricks & Morrison 1967; LaLiberte & Grimes 1982; Desmarais *et al.* 2002; Lee *et al.* 2006).

Considerable debate surrounds the continued use of traditional indicator organisms as surrogates for human pathogens, as several studies have recorded differences in incidence and survival between the two groups in the natural environment (Savichtcheva & Okabe 2006). In particular, modeling results describing indicator organism transport behavior, which often play a prominent role in regulatory decisions and watershed restoration plans, will only be applicable to pathogens if both exhibit similar partitioning behavior. If larger fractions of specific pathogens are particle-associated relative to indicator organisms, stormwater loadings of these pathogens would be expected to settle out of the water column more quickly, resulting in shorter distances of downstream travel and perhaps higher removal rates by sedimentation-based BMPs (e.g. detention ponds). While there have been several lab and field-scale examinations of indicator organism partitioning (Schillinger & Gannon 1985; Characklis *et al.* 2005; Jeng *et al.* 2005; Krometis *et al.* 2007), the majority of investigations of pathogen-particle association have been conducted under laboratory conditions. Previous studies have also limited their focus to protozoan parasites without the concurrent examination of indicator behavior (Medema *et al.* 1998; Dai & Boll 2003; Searcy *et al.* 2005). Laboratory techniques used to separate and enumerate particle-associated and free-phase microorganisms differ widely, with no study employing the same technique to identify both particle-associated indicator organisms and pathogens. Because each partitioning technique uses a different operational definition to identify particle-associated microorganisms (e.g. cells removed by a filter of given pore size, cell

retention on antibody-coated filter, removal of cells via sedimentation, etc.) results from studies describing indicator organism partitioning are not directly comparable with those describing pathogen partitioning (i.e. a particle-associated cell may be removed by filtration but not sedimentation, etc).

A recent study of stormwater loadings of *Cryptosporidium* and *Giardia* in the New York Kenisco Reservoir directly compared the partitioning behavior of these protozoans with the behavior of several indicator organisms (Cizek *et al.* 2008). Observations suggested that 15–30% of fecal indicator bacteria and over 50% of (oo)cysts are associated with settleable particles. While these results are interesting, there was no examination of the relationship between microbial particle association and potential removal by upland BMPs designed to treat stormwater. Association with settleable particles would be expected to enhance microbial removal by sedimentation-based BMPs, though previous investigations of indicator organism partitioning and BMP effectiveness have been inconclusive (Borden *et al.* 1998; Davies *et al.* 2003). In addition, while protozoans are important from a public health perspective, waterborne outbreaks of bacterial etiology remain a substantial concern in the United States as well (Craun *et al.* 2005). *Salmonella* bacterial infections alone are responsible for over 40,000 cases of illness yearly (CDC 2008), and though the disease is most commonly foodborne, a recent salmonellosis outbreak linked to drinking water in Colorado indicates that waterborne transmission is still of concern (Berg 2008).

This study compares the incidence and partitioning behavior of waterborne *Salmonella* spp. with that of six indicator organisms (fecal coliforms, *E. coli*, enterococci, *C. perfringens* spores, somatic coliphage and male-specific coliphage) in field samples. Water samples were collected from an impaired freshwater stream in an urban watershed and partitioned into “settleable” and “suspended” fractions using a calibrated centrifugation technique (Characklis *et al.* 2005; Fries *et al.* 2006; Krometis *et al.* 2007; Cizek *et al.* 2008). Use of a single separation technique allows for a direct comparison of the partitioning behavior of indicators and *Salmonella* spp. bacteria. Inflow and outflow samples from suburban detention ponds in the impaired watershed were also analyzed to determine whether these structures

provide similar reductions in indicator and *Salmonella* loadings and whether higher rates of microbial particle association result in higher rates of microbial removal. Results from this study should be useful in the evaluation of the suitability of various indicator organisms as surrogates for *Salmonella* in urban watershed modeling and provide information useful in the design of stormwater treatment BMPs.

## METHODOLOGY

### Site selection

In accordance with the Total Maximum Daily Load (TMDL) provision of the 1972 United States Clean Water Act, states are required to identify water bodies that do not meet water quality standards for their designated uses (e.g. drinking water, swimming, fishing, etc.). These waters are classified as “impaired” and the state is required to develop watershed restoration plans, or TMDLs, in order to bring water quality into compliance with designated standards. Northeast Creek, located near Durham, NC (Figure 1), is currently included on the state’s impaired waters list due to the presence of high concentrations of indicator organisms (fecal coliforms), high turbidity, impaired biological integrity and low dissolved oxygen (NCDWQ 2006). Water quality is of particular concern because the creek is a tributary to Jordan Lake, a drinking water source and popular recreational area. Land-use data from the 2004 USGS dataset characterizes the approximately 116 km<sup>2</sup> watershed as 30% urban, 50% forest, 9% wetland, 8% pasture/rangeland, 2% cropland and 1% barren. Given rapid development in the watershed, the present urban fraction is now likely to be somewhat higher.

Samples were collected at four locations in the Northeast Creek watershed: two points in the creek itself and two nearby detention ponds, both of which are permitted as stormwater treatment structures (Figure 1). Two potentially significant sources of microbial loadings exist between the two in-stream sites: a wastewater treatment plant (permitted to discharge 23 million liters/day) and a waterfowl impoundment. The wet ponds are located less than a kilometer apart in similar suburban communities.

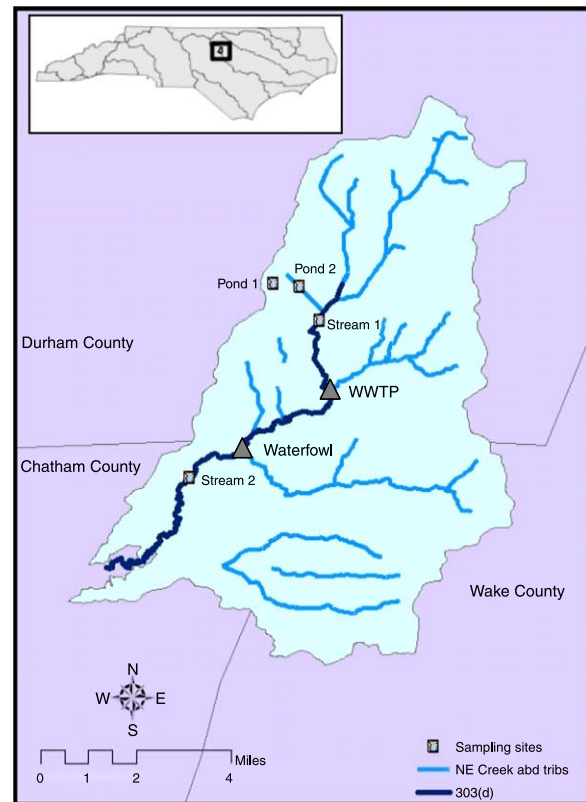


Figure 1 | Northeast Creek watershed sampling points.

In order to characterize potential changes in partitioning and to evaluate pond removal of microorganisms, samples were collected from the inflow and outflow of each pond. During storms, samples were also collected from stormwater runoff in the street gutters directly feeding the storm sewer leading to each pond’s inflow point, thereby allowing concentrations and partitioning behavior to be tracked at several locations along the stormwater “transport chain” leading from upland sources (e.g. lawns, streets) to eventual discharge into receiving waters.

Samples were collected during both dry (background) and wet (storm) conditions during the summer and fall of 2006. Background conditions were defined as existing after a minimum of three days with no appreciable precipitation (i.e. no changes in streamflow). Storm events were defined as precipitation resulting in at least a fourfold flow increase over baseflow. All samples were stored on ice for transport to the laboratory and then refrigerated at 4°C until analysis. Bacterial and particle analyses were initiated within

24 hours of sample collection. Bacterial spore (*C. perfringens*) and phage analyses were completed within 48 hours. Total organic carbon (TOC) concentrations were determined within one week.

### Partitioning technique

Three-liter samples were collected at each of the sampling sites and partitioned via a calibrated centrifugation technique. While this method has been discussed in detail in several other publications (Characklis *et al.* 2005; Fries *et al.* 2006; Krometis *et al.* 2007; Cizek *et al.* 2008), a brief discussion is included here. A one-liter subsample was centrifuged at 1,164 g (2,000 rpm) for 10 min at 4°C (Sorvall RC-3B centrifuge with a H-6000A rotor) with a brake of 4 (approx 5 min deceleration time). These settings were calibrated to remove microbes associated with “settleable” likely inorganic particles, while leaving free-phase or cells associated with lighter organic particles in suspension, and are based on experiments using standardized particle suspensions detailed in Characklis *et al.* (2005). The procedure was designed to distinguish between microbes that will exhibit different transport properties, primarily by virtue of different settling velocities. Glass particles (density = 2.65 g/cm<sup>3</sup>, diameters = 5–60 µm) were used as a surrogate for inorganic particles (e.g. clays, silicates) while latex particles (density = 1.05 g/cm<sup>3</sup>, diameter = 5–40 µm) were used as a surrogate for organic particles and associated microorganisms (density = 1.01–1.2 g/cm<sup>3</sup>) and/or free-phase microorganisms (density = 1.05–1.3 g/cm<sup>3</sup>). Centrifugation via this regimen removed over 97% of the glass particles from suspension, while over 80% of the latex particles remained in suspension (including essentially all latex particles less than 10 µm in diameter). This suggests that the vast majority of inorganic particles and associated microbes in water samples will be removed at these centrifugation settings, while free-phase microorganisms (generally < 5 µm) will remain in suspension.

Following centrifugation, the top 700 mL of supernatant was removed via a vacuum pipette. Both raw and supernatant subsamples were analyzed separately for the six indicator organisms, *Salmonella*, particle concentration and total organic carbon (TOC). Particles and particle-associated organisms removed via the centrifugation

procedure are operationally defined as “settleable”, while those remaining in the supernatant are described as “suspended”. It is important to note that the microorganisms in the “suspended” (supernatant) fraction may actually be associated with lighter, likely organic material; however, these microorganisms would still be unlikely to settle out of the water column more quickly than truly planktonic, unassociated cells. Settleable concentrations of microorganisms and particles are calculated as the difference between the raw and suspended concentrations. For the purpose of analysis in this study, for each microorganism targeted, only those samples with a raw concentration greater than three microbes per 100 mL were included in partitioning calculations. Additionally, in samples with a supernatant concentration that exceeded the total concentration value the settleable fraction was assumed to be zero (i.e. no “negative” fraction associated). Only 10–15% of bacterial samples fell in this category, while 30–50% of coliphage-suspended concentrations exceeded the corresponding total concentrations.

Subsequent application of this procedure to stormwater samples led to the removal of approximately 90% of particles, but less than 5% of the organic carbon. This suggests that the vast majority of the particles removed were inorganic, while most organic material, including free-phase cells, likely remained in suspension (Characklis *et al.* 2005; Krometis *et al.* 2007).

Earlier work (Fries *et al.* 2006) used a mass balance approach involving analysis of the raw environmental samples, centrifuge supernatant and resuspended centrifuge pellets to confirm that microbial population sizes and particle size distribution were essentially unaltered by the procedure. Results indicated no significant loss of microorganism culturability as a result of the centrifugation regimen. The analysis by Fries *et al.* also estimated the potential effects of “clumping” (i.e. multiple cells/particle) on estimates of settleable fraction determined via this technique. Because particle-associated clumps of microorganisms would result in an underestimation of total concentration, this source of bias could result in a consistent underestimation of the actual settleable concentrations. The settleable fractions presented in the present work are therefore best regarded as conservative estimates of particle association. Estimates by Fries *et al.* indicate that

the actual settleable fraction of cells would be 5–20% greater if each positive well was actually populated by an aggregate of eight cells.

### Microbiological analysis

In order to maximize the comparability of results, all bacterial microorganisms were recovered and enumerated via culture-based most probable number (MPN) techniques. Fecal coliform, *E. coli* and enterococci are the most frequently used indicators in recreational and drinking water quality guidelines and standards (USEPA 1986; Pruss 1998). Concentrations of these bacteria were determined using the Colilert and Enterolert defined-substrate methods (IDEXX, Westbrook, ME). Colilert quantitrays for fecal coliform/*E. coli* analysis were incubated at 37°C for 2 h followed by incubation at 44.5°C for 19 ± 3 h to select for fecal (thermotolerant) coliforms (Yakub et al. 2002). The Enterolert procedure was not modified and all quantitrays were incubated at 41°C for 21 ± 3 h. Concentrations were determined using two quantitrays per sample, thereby doubling the number of wells and narrowing the MPN confidence intervals by roughly 15% (Hurley & Roscoe 1983).

*Clostridium perfringens* spores have been recommended as potentially more useful surrogates for protozoan pathogens such as *Cryptosporidium* and *Giardia* and for monitoring impacts of discharges on receiving waters (Medema et al. 1997b; Lisle et al. 2004; Wiedenmann et al. 2006). In the present study, *C. perfringens* concentrations were determined using the multiple-tube fermentation method with iron milk medium as substrate (St John et al. 1982; AOAC 1995). Prior to addition to the iron-milk medium, sample aliquots for *C. perfringens* spores analysis were heated to 65°C for 20 min to inactivate any vegetative bacteria. Inoculated tubes were incubated at 41°C for 21 ± 3 h before examination for stormy fermentation and/or gas production.

Male-specific (F+) and somatic coliphage concentrations were determined via the single-agar layer method detailed in EPA Method 1602 (USEPA 2000). Both types of coliphage have been recommended as surrogates for human enteric viruses (Havelaar et al. 1993; Wiedenmann et al. 2006). Samples for male-specific coliphage analysis were

combined with tryptic soy agar supplemented with streptomycin-ampicillin and *E. coli* F<sub>amp</sub> as a host and poured into Petri dishes to solidify. Samples for somatic phage analysis were combined with tryptic soy agar supplemented with nalidixic acid and *E. coli* CN13 as a host. After 18 ± 2 h of incubation at 37°C, the solidified agar was inspected for clear zones of lysis (plaques) in the dense host bacteria growth.

*Salmonella* concentrations were determined for each water sample using a multiple-tube method similar to that described in work by Hill & Sobsey (2001). Triplicate sets of buffered peptone water bottles were inoculated with four sample volumes (100 mL, 10 mL, 1 mL and 0.1 mL) and incubated at 37°C for 21 ± 3 h as a pre-enrichment to recover and propagate injured cells. After incubation, 100 µL of each bottle of enriched sample was transferred to a 10 mL tube of selective Rappaport–Vassilades broth and incubated at 41°C for 24 h. One loopful of liquid from each tube was streaked onto *Salmonella-Shigella* (SS) agar and incubated for 24 h at 37°C. Suspect colonies (circular, black, surrounded by a clear ring of lysis) were confirmed as *Salmonella* spp via the Enterotube biochemical test (Beckton, Dickinson & Co., NJ). The presence of one or more *Salmonella* colonies on the SS agar indicated a positive tube. Four-tube MPN tables were used to determine concentration (lower detection limit = 0.4 MPN/100 mL).

### Physical analysis

Particle concentration and size distribution were determined using a Coulter Multisizer I with a 100 µm aperture tube (measurement range: 2–60 µm; Beckman Coulter Inc.). Total organic carbon (TOC) was determined using a Shimadzu TOC-5000 Combustion-Infrared analyzer according to Standard Method 5310B (Standard Methods 1998).

### Statistical analysis

The Shapiro–Wilk test confirmed that the observed data were not normally distributed ( $p < 0.001$ ). This was anticipated, as nonparametric statistics are generally considered most appropriate for the analysis of microbial data from environmental samples (Tillett et al. 2001). As in

several previous studies (Lemarchand & Lebaron 2003; Horman *et al.* 2004; Brookes *et al.* 2005) the Spearman rank correlation coefficient was used to assess potential correlations between *Salmonella* and indicator organism presence. Matched groups (i.e. influents and effluents from detention ponds) were compared using the Wilcoxon matched-pairs signed-rank test. All analyses were completed using SAS Statistical Software (SAS Inc., Cary, NC) with significance defined as  $\alpha = 0.05$  (i.e. 95% confidence).

## RESULTS AND DISCUSSION

### Indicator organism and *Salmonella* incidence

*Salmonella* spp. were recovered from 23 of 35 (66%) dry weather samples and 45 of 48 (94%) stormwater samples. This storm-related increase in the frequency of *Salmonella*-positive samples was significantly significant (Fischer's exact test,  $p = 0.001$ ). All sampling points, including street runoff, detention basin inflows and outflows, and in-stream sites were positive for *Salmonella* on multiple occasions. Of the 25 samples that met the EPA recommended criterion of 235 *E. coli* per 100 mL for recreational contact (USEPA 1986), 14 (56%) were positive for *Salmonella* (concentration range: 0.5–93 MPN/100 mL) (Figure 2), suggesting that waters in compliance with current indicator organism-based water quality standards may still pose some health risk. All samples with concentrations less than 235 *E. coli* per 100 mL were collected during dry weather conditions, i.e. all storm samples exceeded this threshold.

Geometric mean concentrations of indicator organisms and *Salmonella* spp. during dry weather sampling were mostly at least one order of magnitude less than concentrations observed during storms (Table 1), suggesting that stormwater loadings are responsible for the majority of microbial loadings to this receiving water. Spatial variability in the concentration of all microorganisms was lower across sample sites during dry weather, but higher during storms, with storm concentrations particularly high in the upland detention ponds. During storm events, sample concentrations of all indicator bacteria and *Salmonella* were often higher in the effluent of both suburban pond sites than in samples from stream site 1 just downstream. This difference was statistically significant when comparing matched pairs for individual storm events (Wilcoxon,  $\alpha = 0.05$ ) for fecal coliform, *E. coli*, enterococci, *C. perfringens* spores and *Salmonella* in pond 1 and for fecal coliforms and *C. perfringens* spores when comparing pond 2 effluent and stream site 1. In contrast to the bacteria, both somatic and male-specific coliphage concentrations were generally higher in the stream site samples than in any of the upland sites.

*Salmonella* spp. concentrations at stream site 2 were expected to be particularly high relative to the other sites, as this site is just downstream from a waterfowl impoundment and avian species are often suspected carriers of *Salmonella* bacteria (CDC 2008). Although mean *Salmonella* concentrations increased as the creek flowed downstream, the increase was not statistically significant (Wilcoxon,  $\alpha = 0.05$ ). Geometric mean concentrations of all indicator

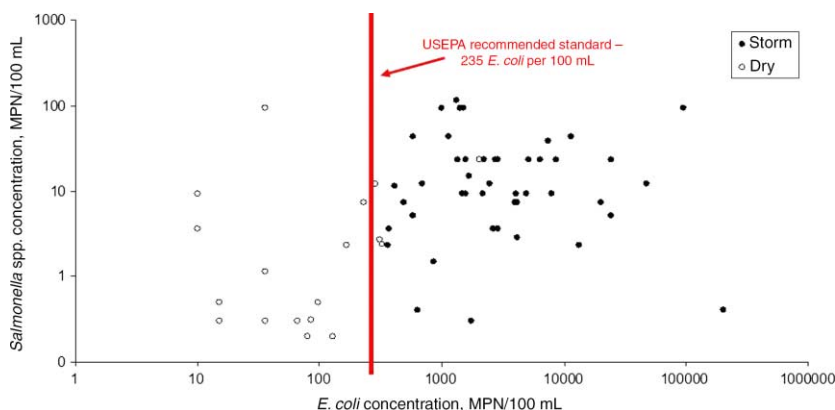


Figure 2 | *Salmonella* spp. vs. *E. coli* incidence in the Northeast Creek watershed.

**Table 1** | Geometric means (in bold) of microbial observations bracketed by 95% confidence intervals (number of observations in parentheses) for all sites in the Northeast Creek watershed

		<b>Fecal coliforms</b> (MPN/100 mL)	<b>E. coli</b> (MPN/100 mL)	<b>Enterococci</b> (MPN/100 mL)	<b>C. pref. spores</b> (MPN/100 mL)	<b>Salm. spp.</b> (MPN/100 mL)	<b>F + coliphage</b> (PFU/100 mL)	<b>Somatic coliphage</b> (PFU/100 mL)
<i>Detention pond 1</i>								
Runoff		141,926	8,846	35,321	497	4	1	868
		<b>47,254 (5)</b>	<b>1,902 (5)</b>	<b>10,801 (5)</b>	<b>183 (4)</b>	<b>1 (3)</b>	<b>0.1 (4)</b>	<b>2 (4)</b>
		15,733	409	3,303	68	0.1	0	0
Influent	Storm	119,889	20,844	71,280	671	28	87	218
		<b>43,082 (8)</b>	<b>5,904 (8)</b>	<b>16,262 (8)</b>	<b>262 (7)</b>	<b>6 (6)</b>	<b>2 (6)</b>	<b>71 (7)</b>
		15,482	1,673	3,710	102	1	0	23
	Dry	1,995	7	128	1		0.2	5
		<b>721 (6)</b>	<b>5 (5)</b>	<b>37 (6)</b>	<b>0.1 (6)</b>	<b>0 (5)</b>	<b>&lt; 0.1 (5)</b>	<b>0.2 (5)</b>
		261	3	10	0		0	0
Effluent	Storm	128,090	11,963	69,938	2,546	75	19	1,184
		<b>57,677 (8)</b>	<b>5,904 (8)</b>	<b>29,974 (8)</b>	<b>712 (7)</b>	<b>3 (6)</b>	<b>1 (7)</b>	<b>633 (7)</b>
		25,971	2,914	12,864	199	0.1	0	339
	Dry	5,063	34	766	234	1	1	39
		<b>2,138 (6)</b>	<b>22 (5)</b>	<b>375 (6)</b>	<b>95 (6)</b>	<b>0.1 (5)</b>	<b>&lt; 0.1 (5)</b>	<b>1 (5)</b>
		902	14	184	38	0	0	0
<i>Detention pond 2</i>								
Runoff		463,128	927	45,955	1,008	27	235	1,047
		<b>95,956 (4)</b>	<b>383 (4)</b>	<b>26,139 (4)</b>	<b>468 (3)</b>	<b>3 (3)</b>	<b>2 (3)</b>	<b>153 (3)</b>
		19,881	158	14,868	218	0.4	0	22
Influent	Storm	210,761	30,521	78,525	2,522	63	42	653
		<b>73,055 (8)</b>	<b>7,112 (8)</b>	<b>30,356 (8)</b>	<b>1,585 (7)</b>	<b>3 (7)</b>	<b>4 (7)</b>	<b>263 (6)</b>
		25,323	1,657	11,735	997	0.2	0	106
	Dry	16,784	134	354	1,467	18	6	20
		<b>7,509 (5)</b>	<b>26 (4)</b>	<b>219 (5)</b>	<b>669 (5)</b>	<b>1 (5)</b>	<b>0.2 (5)</b>	<b>6 (4)</b>
		3,360	5	136	305	0.1	0	2
Effluent	Storm	127,848	10,855	43,942	1,017	18	9	443
		<b>51,519 (8)</b>	<b>4,193 (8)</b>	<b>18,189 (8)</b>	<b>346 (7)</b>	<b>1 (7)</b>	<b>1 (7)</b>	<b>102 (7)</b>
		20,760	1,620	7,529	118	0.1	0	24
	Dry	11,073	188	1,928	1,464	2		
		<b>2,092 (4)</b>	<b>79 (3)</b>	<b>450 (4)</b>	<b>446 (4)</b>	<b>1 (4)</b>	<b>0 (3)</b>	<b>0 (3)</b>
		395	33	105	136	0.2		
<i>Stream site 1</i>								
Storm	Storm	17,973	2,918	16,049	383	5	41	1,159
		<b>13,305 (11)</b>	<b>2,138 (11)</b>	<b>9,326 (11)</b>	<b>233 (10)</b>	<b>3 (10)</b>	<b>20 (9)</b>	<b>850 (10)</b>
		9,850	1,566	5,419	142	1	9	624
	Dry	2,740	248	307	87	1	4	109
		<b>1,322 (10)</b>	<b>86 (9)</b>	<b>149 (10)</b>	<b>59 (10)</b>	<b>0.1 (10)</b>	<b>1 (9)</b>	<b>34 (9)</b>
		638	30	72	40	0	0	11
<i>Stream site 2</i>								
Storm	Storm	38,827	2,963	19,226	177	17	41	1,031
		<b>20,786 (6)</b>	<b>1,700 (6)</b>	<b>8,990 (6)</b>	<b>83 (6)</b>	<b>9 (6)</b>	<b>14 (5)</b>	<b>622 (6)</b>
		11,128	976	4,204	39	5	5	375
	Dry	5,026	246	1,779	103	5	32	102
		<b>2,522 (6)</b>	<b>116 (5)</b>	<b>453 (6)</b>	<b>51 (6)</b>	<b>4 (6)</b>	<b>2 (5)</b>	<b>36 (5)</b>
		1,266	55	115	25	0	0	13

organisms, except fecal coliforms, were actually lower at stream site 2 than at the more urbanized stream site 1 (upstream of the impoundment). The highest single sample concentrations of *Salmonella* (115 MPN/100 mL) were detected in samples from the wet ponds. This is notable, as the pond catchment areas are highly urbanized with no wildlife or agricultural areas in evidence, and in keeping with a recent study of microbial contamination in San Francisco wetlands that observed a higher incidence of *Salmonella*-positive water samples in primarily stormwater-contaminated streams than designated wildlife areas (Shellenbarger *et al.* 2008).

*Salmonella* spp. were significantly correlated (Spearman,  $\alpha = 0.05$ ) with the presence of all the indicator organisms examined with the exception of male-specific coliphage (Table 2), suggesting that these indicators are reasonable sentinels of *Salmonella* presence in Northeast Creek. Correlations between the incidence of fecal coliform, *E. coli*, enterococci and somatic coliphage and *Salmonella* were of similar strength ( $\rho_s = 0.46$ – $0.52$ ), while the correlation between *C. perfringens* spores and *Salmonella* incidence was somewhat weaker ( $\rho_s = 0.24$ ), though still significant. Although male-specific coliphage have been identified as strong indicators of fecal pollution and human health risks at recreational beaches (Colford *et al.* 2007), they are not always present at adequate concentrations to serve as pathogen indicators in waters primarily contaminated by stormwater (Ferguson *et al.* 1996). Theoretically, an ideal indicator should always be present at concentrations exceeding pathogen concentrations (Savichtcheva & Okabe 2006), but in Northeast Creek, male-specific coliphage concentrations only exceeded *Salmonella* concentrations in 50% of samples. Additionally, *Salmonella*

was actually recovered from 20 of 26 samples (77%) in which no male-specific coliphage were detected.

No minimum or threshold indicator organism concentration could be identified as an entirely reliable measure of pathogen presence or absence (e.g. consistent absence of *Salmonella* when *E. coli* was below a given concentration): *Salmonella* was detected in samples with *E. coli* concentrations as low as 10 MPN/100 mL. Consequently, despite statistical correlations relating incidence, relatively low concentrations of indicator organisms do not preclude the presence of *Salmonella* bacteria in the Northeast Creek watershed.

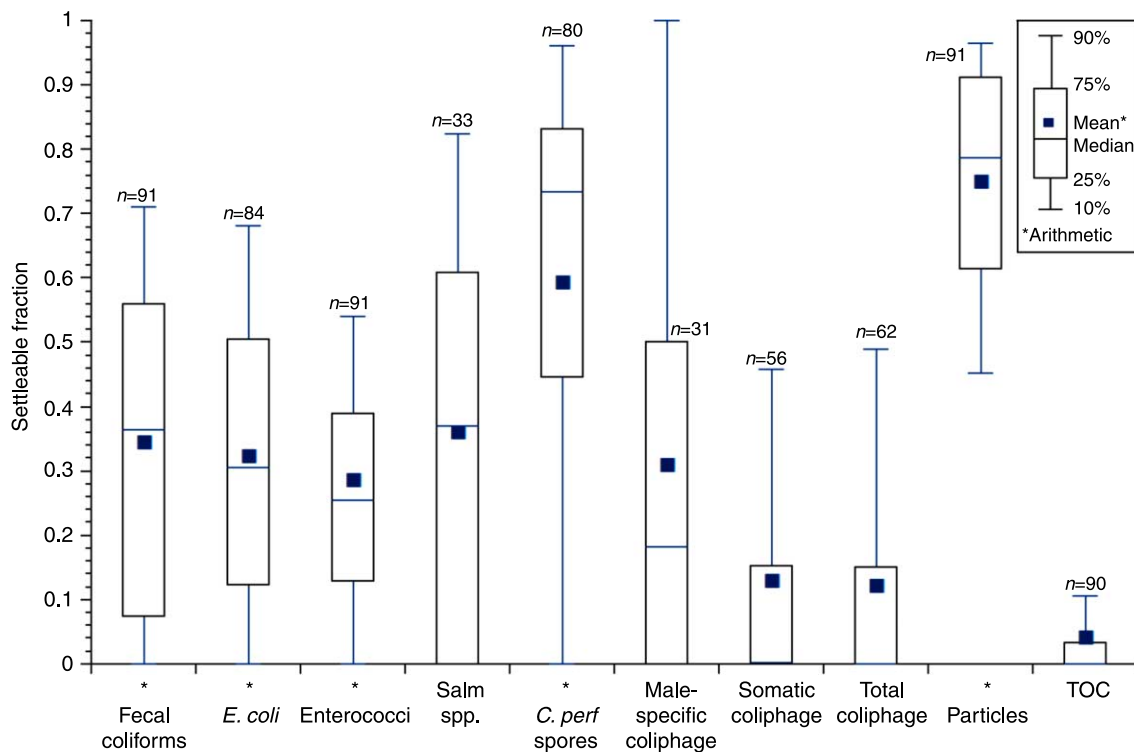
### Particle association

The centrifugation-based partitioning technique removed a high fraction of total particle number, while the vast majority of total organic carbon (TOC) remained in suspension, confirming that the procedure largely removed inorganic and associated microorganisms (Figure 3). Association with settleable particles appeared to differ by microbial type, which is in keeping with previous studies investigating indicator organism partitioning behavior (Characklis *et al.* 2005; Krometis *et al.* 2007; Cizek *et al.* 2008). The average settleable fraction of *Salmonella* bacteria was most similar to that of the traditional fecal indicator bacteria, fecal coliforms, *E. coli* and enterococci (25–35% associated). However, *Salmonella* bacteria showed a much wider range in terms of settleable fraction and measures of supernatant and total concentration were not statistically different from one another, i.e. the settleable concentration was not statistically different from zero (Table 3). This may be a result of the uncertainty inherent in current methods of

**Table 2** | Spearman coefficients ( $\rho_s$ ) correlating indicator organism and *Salmonella* spp. incidence

Indicator organism	Type	$\rho_s$	p-value	n
Fecal coliforms	Bacteria	0.52	<0.0001	83
<i>E. coli</i>	Bacteria	0.51	<0.0001	77
Enterococci	Bacteria	0.48	<0.0001	83
<i>C. perfringens</i> spores	Sporulated bacteria	0.24	0.03	83
Male-specific coliphage	Virus	0.17	0.15	74
Somatic coliphage	Virus	0.46	<0.0001	76





**Figure 3** | Average settleable fractions of indicator organisms and *Salmonella* spp. (\*raw concentration values were significantly greater than supernatant concentrations, Wilcoxon,  $\alpha = 0.05$ ).

microbial detection, particularly at low concentrations. Higher variability in *Salmonella* behavior would therefore be expected, as observed concentrations were generally two to four orders of magnitude less than that of the fecal indicator bacteria. Collection of a larger set of *Salmonella* samples may also resolve this variability and produce statistically significant results. Similarly, although the mean values presented in Figure 3 suggest that male-specific

coliphage may associate with particles at a slightly higher rate than somatic coliphage, it is worth noting that a smaller number of samples evaluated for male-specific coliphage met the minimum analytical threshold of 3 microbes per 100 mL for inclusion in partitioning analysis, resulting in a smaller sample size. Adding male-specific and somatic coliphage concentrations to obtain total coliphage values yielded results similar to those observed in earlier studies,

**Table 3** | Geometric means of raw (total) and supernatant (free-phase or suspended) concentrations of microorganisms used in the partitioning analysis

Microorganism	Sample size, <i>n</i>	Total concentration	Suspended (free-phase) concentration
Fecal coliforms*	91	12,115	7,301
<i>E. coli</i> *	84	663	407
Enterococci*	91	2,924	1,882
<i>C. perfringens</i> spores*	80	210	60
<i>Salmonella</i> spp.	33	13	10
Male-specific coliphage	31	20	10
Somatic coliphage	56	182	159
Total coliphage	62	182	167

\*Microorganisms indicate that the total concentration was statistically greater than the suspended concentration (Wilcoxon,  $\alpha = 0.05$ ).

with less than 10% of total coliphage identified as particle-associated (Characklis *et al.* 2005; Krometis *et al.* 2007). Additionally, total and suspended concentrations of both types of coliphage were statistically equivalent (i.e. settleable fraction equal to zero).

There remains some debate on whether fecal indicator bacteria are the best surrogates for *Salmonella* in receiving waters, mostly centering on the occasional recovery of viable *Salmonella* from waters in which coliforms or enterococci are absent (Morinigo *et al.* 1990; Polo *et al.* 1998). Results from the present study suggest that, while the average fraction of settleable bacteria is roughly equivalent for these bacteria and *Salmonella* in Northeast Creek, further work will be required to more thoroughly characterize *Salmonella* partitioning and resolve current uncertainties. The data collected do suggest that no alternative set of indicator organisms investigated appears superior as a surrogate for *Salmonella* presence and transport in this watershed. Although *C. perfringens* spores have been promoted as more conservative markers of fecal pollution due to their prolonged survival in the environment (Medema *et al.* 1997a), they were only weakly correlated with the presence of *Salmonella* in Northeast Creek. In addition, a much larger fraction of *C. perfringens* spores were particle-associated (>50%) than *Salmonella*, suggesting differences in settling behavior as *C. perfringens* would be likely to settle out of the water column more quickly. While the association between *Salmonella* and somatic coliphage was strong, a much lower fraction of viral particles were particle-associated, indicating that a larger portion of these microbes may remain in suspension for longer periods of time. Only the fecal indicator bacteria (fecal coliforms, *E. coli*, enterococci) exhibited both a strong statistical correlation with *Salmonella* presence and potentially similar partitioning behavior (i.e. presumably similar transport properties), suggesting that these bacterial indicators, while imperfect, remain the most potentially useful surrogates for *Salmonella*, whether from a monitoring or modeling perspective.

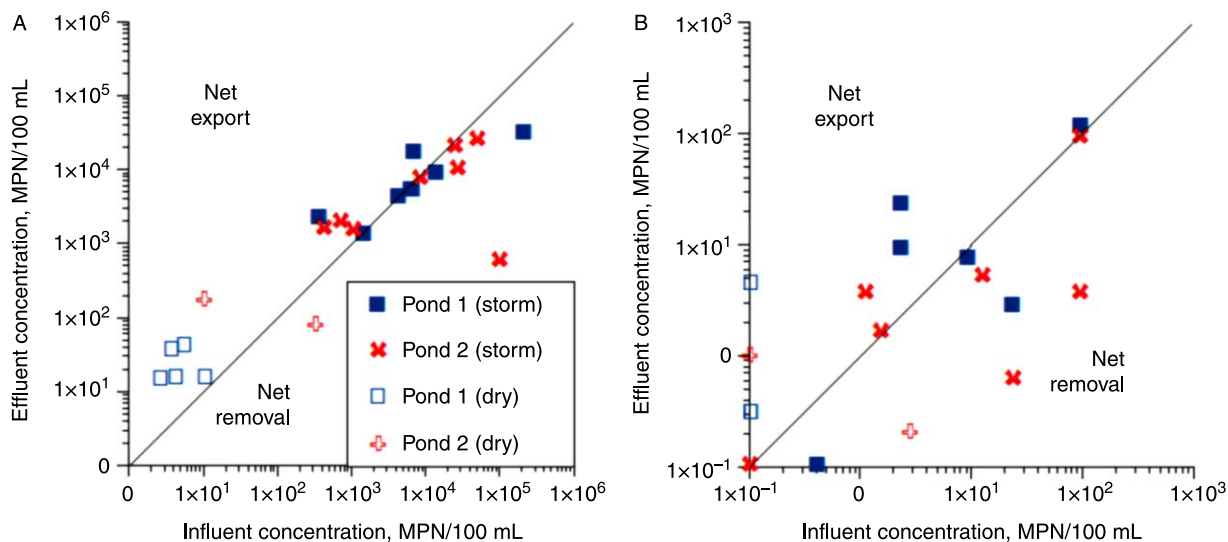
Attempts to correlate water quality parameters with observed changes in microbial partitioning behavior provided limited additional insights. Increases in the settleable fractions of fecal coliform, *E. coli* and enterococci were correlated with increases in total particle concentration

(Spearman's test,  $\alpha = 0.05$ ); however, no statistically significant relationship existed between particle concentration and attached fractions of *Salmonella*, coliphage or *C. perfringens* spores. The lack of consistency in this trend across microorganisms argues against differential settling as responsible for observed microbial reductions after centrifugation. Increases in the fecal indicator bacteria settleable fraction may instead be due to increased available sites for attachment. Changes in TOC concentration were not correlated with changes in the settleable fraction of any microorganism. Further investigation would be required to determine whether other unexplored environmental factors, such as microbial origin/strain (human, wildlife, etc.), contribute to fluctuations in microbial partitioning behavior.

### Wet pond efficiency

Samples were collected at the inflow and outflow points of two suburban wet ponds during eight storm events and six times during dry weather. Although North Carolina design regulations indicate that discharge from wet ponds should cease two to five days after significant rainfall, there was sufficient effluent to allow sampling of pond 1 outflow on all dry weather sampling trips. Additionally, effluent microbial concentrations in pond 1 frequently exceeded influent concentrations during dry weather, with the increase statistically significant for *E. coli*, enterococci and *C. perfringens* spores, suggesting that the pond might actually be a source of microorganisms. While in the case of *Salmonella* there was no statistically significant increase between influent and effluent concentrations in pond 1, *Salmonella* were recovered from the pond effluent on three occasions when influent concentrations were zero. Sufficient outflow for sampling from pond 2 was only present during three of the six dry weather sampling trips. Effluent concentrations from pond 2 exceeded influent concentrations for each bacteria type at least once over the three sampling events; however, the number of samples was insufficient for statistical analysis.

Wet pond removals of all six indicator organisms were highly variable over storm events, with no consistent trends exhibited to conclusively indicate that the ponds were either removing or exporting microbes. Figure 4 illustrates raw effluent vs. influent concentrations for *E. coli*, the



**Figure 4** | Wet pond influent vs total effluent concentrations for pairs of storm event and dry weather samples for (A) total *E. coli* and (B) total *Salmonella* spp.

primary freshwater regulatory organism, and *Salmonella*. Although the USEPA describes a “typical” microbial removal rate for detention ponds as 65% (USEPA 2007), microbial concentrations were occasionally higher in the effluent than in the influent for both ponds.

Single sample microbial removals for each microorganism in each wet pond were calculated for each storm event using an approach commonly employed by the EPA to assess BMP performance (USEPA 2007):

$$SSR = 1 - [C_e/C_i] \quad (1)$$

where SSR = single sample removal,  $C_e$  = effluent concentration and  $C_i$  = influent concentration. Single sample removals were not entirely consistent across microorganism types for a given storm event, i.e. no storm produced simultaneous increases or decreases in pond effluent concentration for all microbes. Particle-associated microbes would be expected to exhibit faster sedimentation velocities; therefore higher fractions of settleable microbes in the influent would be expected to result in higher removal rates. The Spearman rank correlation coefficient was used to investigate potential correlations between the “settleable” (particle-associated) fraction of microbes in the influent and the observed single sample removal. There was a statistically significant positive correlation between the particle-associated fraction and removal of *C. perfringens* spores in pond 2 ( $\alpha < 0.05$ ) and a positive correlation

between the particle-associated fraction of *E. coli* and *E. coli* removal was almost significant for both pond 1 and pond 2 ( $\alpha = 0.09$  and  $\alpha = 0.06$ , respectively). No statistically significant relationships were identified for any of the other microorganisms.

Mean concentration removals are frequently used to assess the performance of stormwater BMPs (USEPA/ASCE 1999) and other water treatment structures (Karpiscak *et al.* 2001); and the geometric mean is commonly used to average microbial concentrations (Davies & Bavor 2000; Hill & Sobsey 2001; Mallin *et al.* 2002). Geometric mean concentration removals were therefore used to calculate overall, or “average”, pond performance using the following equation:

$$MCR_{geo} = 1 - [C_{e,geo}/C_{i,geo}] \quad (2)$$

where  $MCR_{geo}$  = geometric mean concentration removal,  $C_{e,geo}$  = the geometric mean of all effluent concentrations and  $C_{i,geo}$  = the geometric mean of all influent concentrations. Mean concentration removals of settleable concentrations were determined in addition to removals of total (overall) concentration in order to determine whether particle association leads to greater rates of removal. Both total and settleable mean concentration removals are given in Table 4.

Removal of both the total and the settleable concentration of microorganisms appeared to differ between the

**Table 4** | Wet pond geometric mean removal of total and settleable microorganisms ( $n$  = number of storms). Positive values indicate a net removal of microorganisms, while negative values indicate a net export

		Pond 1	Pond 2
Fecal coliforms	Total	-0.34	0.29
	Settleable	1.0	0.24
$n = 8$	Total	0.00	0.41
	Settleable	0.99	0.67
<i>E. coli</i>	Total	-0.84	0.4
	Settleable	-13	-6.6
Enterococci	Total	0.85	0.61
	Settleable	-1.3	0.90
<i>Salmonella</i> spp.	Total	-1.7	0.64
	Settleable	0.57	1.0
$n = 6/7$ (pond 1/2)	Total	0.84	0.98
	Settleable	0.94	0.98
<i>C. perfringens</i> spores	Total	-8.0	0.61
	Settleable	N/A*	0.86
$n = 7$	Total		
	Settleable		
Male-specific coliphage	Total		
	Settleable		
$n = 7$	Total		
	Settleable		
Somatic coliphage	Total		
	Settleable		
$n = 7$	Total		
	Settleable		

\*Average concentration of viral particles in influent = 0.

two detention ponds. While pond 2 exhibited some overall removal of all targeted microorganisms, mean overall removals for pond 1 were either negative (indicating net export) or zero for all microbes except *Salmonella* and male-specific coliphage. Removal of *Salmonella*, *C. perfringens* spores and both types of coliphage was greater than 60% for pond 2, and removal of *Salmonella* and male-specific coliphage was greater than 80% in pond 1. Despite these promising average removal rates, it is worth noting that effluent fecal indicator bacteria concentrations from both ponds remained several orders of magnitude greater than typical water quality standards, and *Salmonella* spp. generally remained detectable (Table 1). A two to three log removal (99–99.9%) of microbial influent loadings, much higher than the 65% removal rate described as typical by the USEPA, would likely be required for the pond effluent to meet current water quality standards.

With the exception of enterococci in both ponds and *Salmonella* in pond 1, mean concentration removals of settleable microorganisms were equal to or exceeded the total mean concentration removals computed based on single storm samples. This suggests that particle-associated cells are more likely to be removed by detention ponds than their free-phase counterparts, and that sedimentation may

be an important microbial removal mechanism. In the case of enterococci, however, fairly dramatic increases in settleable concentrations were observed in pond effluents relative to influent concentrations. While there may be many reasons for this increase, previous studies have noted that gram-positive enterococci survive longer in the environment than gram-negative bacteria (fecal coliform, *E. coli*), and that much higher concentrations can be present in bottom sediments (Davies et al. 1995; Jeng et al. 2005). While somewhat speculative, it is possible that this increased persistence could lead to higher levels of particle-associated enterococci becoming resuspended from underlying pond sediment during high flows.

Differences in performance between the two ponds may be attributable to differences in design. Although located less than a kilometer apart, the pond structures differ considerably. The length:width ratio of pond 2 is greater than 2:1, resulting in a more consistent hydraulic residence time, while pond 1 is nearly circular and likely more susceptible to short-circuiting. Also, the effluent pipe of pond 1 is roughly 1 m in diameter, while pond 2 has a small diameter effluent pipe that likely results in a longer hydraulic residence time. It should be noted, however, that while on average pond 2, which more closely adhered to the county regulations for stormwater BMPs, did reduce influent microbial concentrations, these reductions were relatively modest. Only reductions in male-specific coliphage exceeded 1 log, and the geometric mean concentration removals of fecal indicator bacteria (fecal coliforms, *E. coli*, enterococci) were roughly equal to or slightly less than the 65% typical removal value given by USEPA for stormwater BMPs (USEPA 2007). Nonetheless, effluent concentrations often exceeded water quality standards by several orders of magnitude, suggesting that detention ponds alone may be insufficient for remediating microbially impaired stormwaters.

Net exports of microorganisms by both ponds were observed several times during dry weather and storm events. Several studies have noted that, under favorable conditions, indicator bacteria and *Salmonella* may actually reproduce in the environment (LaLiberte & Grimes 1982; Burton et al. 1987; Fish & Pettibone 1995; Lee et al. 2006). As warm temperatures, ponded or low-flow areas, and association with nutrient-rich protective sediments have

been identified as conditions particularly conducive to microbial regrowth (He *et al.* 2007), accumulation and reproduction of microorganisms in these detention ponds between storm inputs seem quite possible. Later resuspension of these microorganisms by high stormflows or wildlife could therefore be responsible for observations of microbial export. Under these conditions, the ponds might actually serve as a source for additional microbial loadings eventually discharged to Northeast Creek. Similar findings for an area in San Francisco were recently presented by Shellenbarger *et al.* (2008), who noted that wetlands managed as wildlife habitat could serve as source of fecal indicator bacteria loadings during the winter months. While the ponds investigated in this study were designed as stormwater treatment structures rather than wildlife habitat, very similar environmental conditions (shallow, nutrient-rich bottom sediments, etc) are likely encouraging microbial accumulation and later release in effluent flows.

## CONCLUSIONS

Results suggest that fecal indicator bacteria (fecal coliforms, *E. coli*, enterococci) are promising surrogates for modeling *Salmonella* spp. bacteria fate and transport in urban stormwaters; however, further research will be required to resolve observed variability in *Salmonella* partitioning behavior. These indicator microorganisms exhibited both a strong statistical correlation with *Salmonella* spp. presence (Spearman's coefficient  $\alpha_s = 0.48-0.52$ ,  $p < 0.0001$ ) and similar partitioning behavior, with similar averages of 25–35% of bacteria associated with settleable particles. While other potential indicators (*C. perfringens* spores, somatic coliphage) were also statistically correlated with *Salmonella* presence, they appeared to associate with particles at different rates (>50% and <10%, respectively). Although the fecal indicator bacteria did show some promise as reasonable surrogates, it is important to note that *Salmonella* bacteria were still readily recoverable from samples with very low indicator bacteria concentrations (e.g. *E. coli* = 10 MPN per 100 mL).

Removal of microbial stormwater loadings by the suburban detention ponds was highly variable, with large

fluctuations in performance between the two ponds and between storm events. In general, pond 2 more consistently provided some level of reduction in influent microbial concentrations, with mean concentration removals near USEPA's typical removal rate of 65%. While this could be construed as satisfactory, it is important to note that fecal indicator bacteria concentrations in pond 2's effluent still remained several orders of magnitude above regulatory standards and *Salmonella* remained detectable. Although rates of microbial-particle association and pond removal rates were only significantly correlated for *C. perfringens*, higher removal rates were observed for settleable microorganisms relative to total concentrations in both ponds, suggesting that sedimentation is an important removal mechanism. Further research is required to quantify the effects of indicator organism and *Salmonella* regrowth, and resuspension from pond sediments, which may be responsible for observations of microbial export from both ponds during dry weather and storm events.

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