Attachment of Fecal Indicator Bacteria to Particles in the Neuse River Estuary, N.C.

J. Stephen Fries[1]; Gregory W. Characklis[2]; and Rachel T. Noble[3]

Abstract: Observations of microbial contamination and particle suspensions represent valuable inputs to water quality models that form the basis of regulatory decisions regarding the use of surface waters. The Neuse River Estuary in eastern North Carolina is experiencing a decline in water quality due to increasing anthropogenic inputs. Potentially serious consequences of these inputs are the introduction and persistence of bacterial pathogenic organisms from human and animal waste. A critical factor in determining human health risk is the partitioning of these organisms between particle-attached and free-living cells in the water column. Particle-associated bacteria are generally less mobile in the environment, settle faster, and may have different rates of mortality than their free phase counterparts. Surface and bottom water samples were collected during both dry weather and storm events throughout the summer of 2004 to gage changes in particle concentration, particulate organic carbon and nitrogen, and the partitioning of two indicators of fecal contamination: *Enterococcus sp.* (ENT) and *E. coli* (EC). Increases in concentrations of these indicators coincided with increases in particles in suspension following storm events. In surface waters, both ENT and EC exhibited similar patterns, controlled primarily by runoff inputs (i.e., storms). In bottom waters, resuspension of sediments was additional source of particles and both indicators. Partitioning of these indicators between particle attached and free living exhibited an overall average of 38% of bacteria associated with particles capable of settling out of the water column. This fraction compares well with previous estimates of attachment of indicators in stormwater. The ability to estimate attachment rates and characterize particle suspensions provides a powerful tool for management and assessment of water quality in estuaries.

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CE Database subject headings: Bacteria; Sedimentation; Particles; Stormwater management; Organic matter; Public health; Contamination; Estuaries.

Introduction

The importance of monitoring coastal and estuarine waters for the presence of fecal indicators has grown as increased population and agricultural development place increasing stress on the quality of receiving waters. Current water quality regulatory approaches (e.g., total maximum daily loads, waste load allocation) depend upon the ability to understand and model transport and fate of microbial contaminants (Maguire 2003; Wool et al. 2003). Measurements of indicator bacteria are used as a proxy for the potential health risk posed by microbial contaminants (Shibata et al. 2004). Measurements of indicator bacterial densities are also the basis for regulatory decisions regarding recreational and commercial uses of water bodies (USEPA 2002). Often this contamination can be linked to rain events and resulting stormwater runoff from urban and agricultural regions (Bales 2003).

The Neuse River Estuary (NRE), in eastern North Carolina, United States, is undergoing a period of intense urban and agricultural development. This expanding human presence has increased the potential for both contamination of the estuary and exposure of local populations to bacterial and viral pathogens. Microbial contamination from human sewage, domestic and feral animal waste, and livestock waste could be introduced to the NRE through sewage treatment plant effluents, on-site wastewater treatment (septic systems), open-field manure spraying systems, agricultural animal manure runoff from confined animal feeding operations (CAFOs), sanitary wastes from boats, wildlife feces, stormwater and other nonpoint source runoff.

In this study, measurements were focused on the indicator bacteria, *Enterococcus sp.* (ENT) and *E. coli* (EC), often used by regulators as proxies for the presence of fecal contamination (Dufour and Ballentine 1986). In recreational bathing waters, ENT densities have been correlated with the incidence of gastrointestinal illness (Currieo et al. 2001; Rose et al. 2001). While these indicator bacteria are not perfect predictors of the presence of pathogens, previous studies support the use of these organisms for monitoring recreational waters, including creeks, reservoirs, beaches, and estuaries (Crabill et al. 1999; Lipp et al. 2001; Desmarais et al. 2002; Boehm et al. 2004; Brookes et al. 2004). In the NRE, indicator bacteria have the potential to help predict the presence and transport of pathogens, including *Salmonella sp., Yersinia sp.,* and *Campylobacter sp*.

Once indicator bacteria and bacterial pathogens are in the environment, their ecology determines the duration and spatial extent of contamination. In particular, the fractions of viable or-

[1]Postdoctoral Associate, Institute of Marine Sciences, Univ. of North Carolina at Chapel Hill, 3431 Arendell St., Morehead City, NC 28557 (corresponding author). E-mail: jsfries@email.unc.edu

[2]Assistant Professor, Dept. of Environmental Sciences & Engineering, Univ. of North Carolina at Chapel Hill, Rosenau Hall CB# 7431, Chapel Hill, NC 27599.

[3]Assistant Professor, Institute of Marine Sciences, Univ. of North Carolina at Chapel Hill, 3431 Arendell St., Morehead City, NC 28557.

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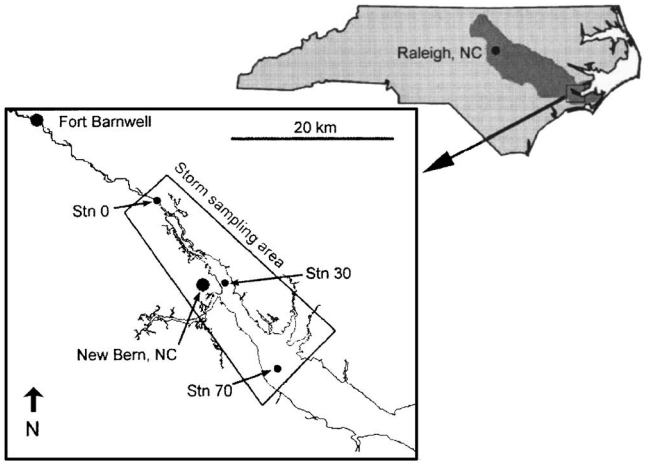

Fig. 1. Map of Neuse River Estuary, located in eastern North Carolina. Watershed, including Raleigh, shaded on state map. Particle and indicator bacteria measurements reported in this study are from samples collected at Stations 0, 30, and 70 within "storm sampling area" on map.

ganisms attached to particles could mediate the populations in the water column through increased survivability and decreased time in suspension (Faust et al. 1975; Burkhardt et al. 2000). Laboratory studies have documented reduced mortality due to predation or environmental exposure for ENT attached to particles (Davies and Bavor 2000; Jin et al. 2004). These particles also have the potential to contaminate shellfish and sediments (Burton et al. 1987; DeLuca-Abbott et al. 2000). Particle settling transports attached microbial contaminants from the water column to the sediment, retarding downstream transport of organisms in suspension. The dynamics of microbial attachment to settling material also has direct bearing on the effectiveness of detention basins, a primary means of reducing stormwater-related contaminant loads (Schillinger and Gannon 1985; Chen et al. 2004). In addition, estimates of the fractions of attached indicators will be useful for modelers interested in making predictions of microbial transport in estuaries (Steets and Holden 2003; Jamieson et al. 2004). In terms of necessary model inputs, the partitioning of bacteria helps define the removal rate of organisms from the water column and could improve models of microbial transport that consider resuspension as a source.

The overall goal of this study was to measure particle suspension characteristics and the concentrations and partitioning of selected indicators in the NRE to document changes associated with storm events. This work incorporates a suite of integrated techniques for measuring particle size distribution, organic content of suspensions, and concentrations of indicator bacteria split into fractions based on their effective settling velocity. Sampling was conducted regularly during periods of dry weather and also following a number of storms. Observations in the water column are affected by many environmental and development factors that complicate prediction of their transport (Mallin et al. 2000). The results of this study provide evidence for changes in particles and indicator populations due to rain events, with new insights into microbial partitioning and its potential role in downstream transport of microbial contaminants.

Materials and Methods

The NRE is a shallow, bar-built estuary with inputs from an extensive watershed in eastern North Carolina (Fig. 1), little tidal

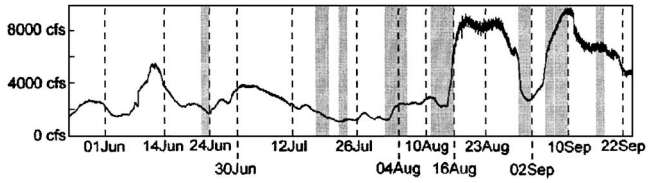

Fig. 2. Time series of inflow in cubic feet per second (cfs) into sampling reach during summer of 2004. Sampling dates are listed on time axis and marked by dashed lines. Shaded regions denote dates where 24 h rainfall at New Bern, N.C. airport exceeded 13 mm (0.5 in.).

influence, and flows into the Pamlico Sound (Paerl et al. 2001; Bowen and Hieronymus 2003). Collection of water samples occurred 13 times during the summer of 2004. Eight of these trips were conducted during dry weather as part of the NRE Modeling and Monitoring project ⟨http://www.marine.unc.edu/neuse/modmon⟩. Five samples were collected the day after storms, including two hurricanes: Alex on August 3, and Charley on August 14. Stream flow, gauged at Fort Barnwell, N.C. (USGS 02091814: ⟨http://waterdata.usgs.gov/nc⟩), increases after most rain events (Fig. 2), with an event defined as exceeding 0.5 in. (13 mm) over 24 h at New Bern, N.C. Airport (National Weather Service: ⟨http://www.erh.noaa.gov/mhx/f6.html⟩). Wind events, in this case defined as daily-averaged winds exceeding 4.5 m/s (10 mi/h), often coincided with rain events. However, early June was both dry and windy, while midsummer was wet, but calm.

Three stations spaced 13 km apart in the upper reach of the estuary were sampled: Station 0 at Streets Ferry Bridge, Station 30 near New Bern, and Station 70 downstream of New Bern (Fig. 1). These stations have depths of approximately 6.5, 3.5, and 4 m, respectively. These stations and depths represent a range of different environments due to changing stratification within the sampling reach depending on recent weather conditions (Fig. 3). The wastewater treatment plant outfall for the city of New Bern (4.7 million gal./day capacity) is located less than 1 km upstream from Station 30. Samples were collected from boats in acid rinsed, 51 polypropylene containers. Salinity profiles were collected in situ using a YSI 6600 (Yellow Springs Instruments Inc.).

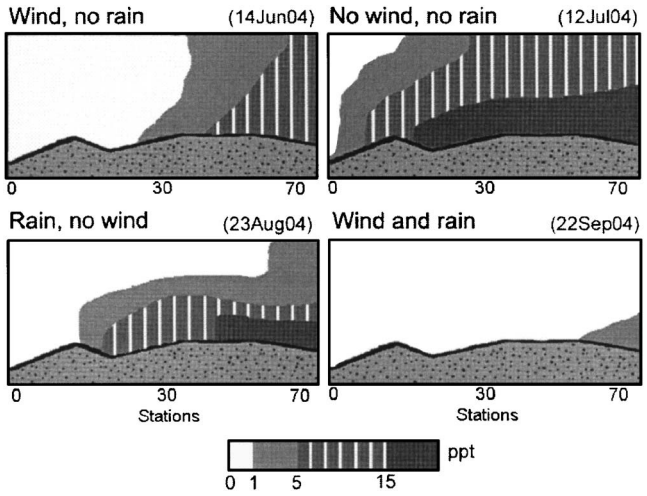

Fig. 3. Effects of rain and wind on vertical stratification in study reach. Data presented from sampling on June 14, July 12, August 23, and September 22. Salinity contours for 1, 5, and 15 ppt are plotted.

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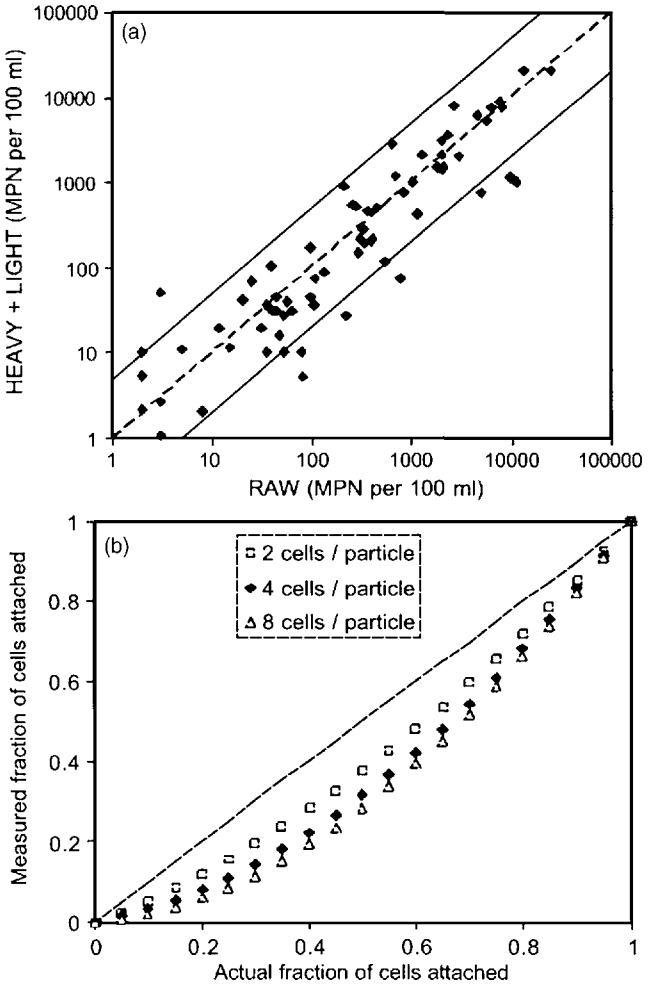

Fig. 4. Assessment of use of IDEXX for determining fractions attached to particles: (a) comparison of Raw concentration with sum of Heavy and Light plotted along with lines representing 1:1, 1:5, and 5:1 relationships; (b) calculated error due to multiple cells attached to individual particles in water sample. Dashed line represents 1:1 curve.

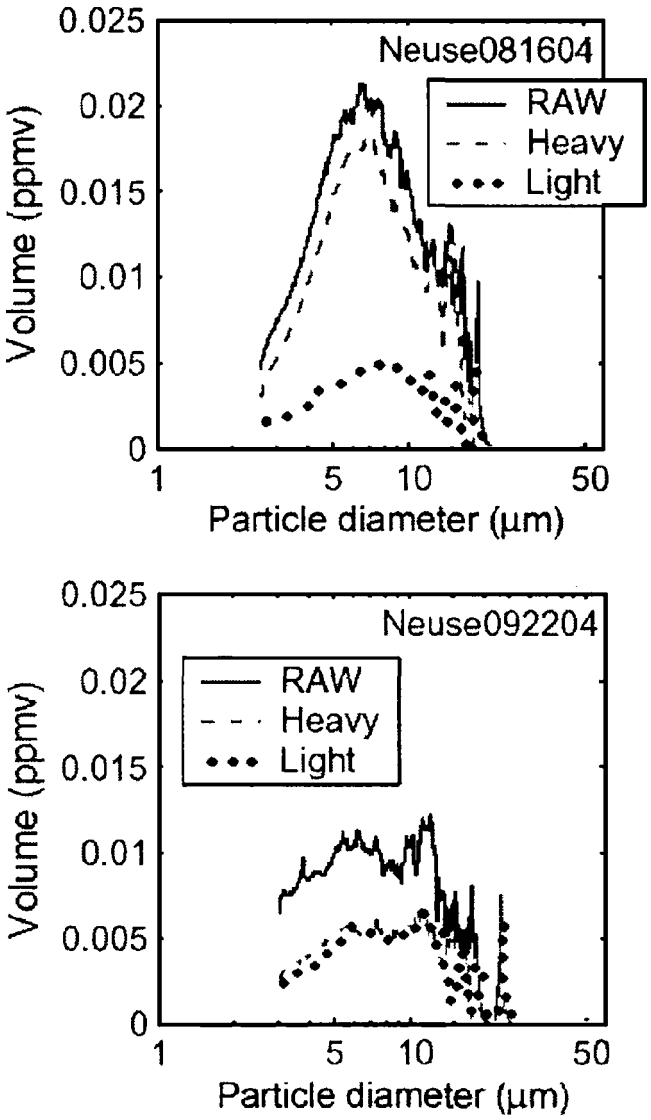

Fig. 5. Particle size distributions from surface water samples collected at Station 30. Top panel shows suspension from storm sample dominated by Heavy, likely runoff particles, and bottom panel shows mixed suspension of Heavy and Light particles from dry weather sample.

Surface samples were acquired by directly filling containers while bottom samples were collected 0.5 m above the sediment using flow-through beta water sampling bottles.

Particle size distribution $[N(dp)]$ between 3 and 60 μm was measured using a Coulter counter (Beckman Coulter MultiSizer III). Samples for total suspended solids (TSS) were determined using the mass collected on 0.7 μm glass fiber filters [Standard Method 2540D (APHA 1998)]. The same filters were used for particulate organic carbon and nitrogen measurements [Standard Method 5310B (APHA 1998)] using a CHN analyzer (Perkin Elmer 2400 Series II). The atomic ratio of carbon to nitrogen in organic matter (CN) was used as a metric for the composition and source of particles. Average particle density was computed using the ratio of TSS to total particle volume, based on the particle size distribution

$$\rho_p = \frac{\text{TSS}}{\pi/6 \sum dp^3 N(dp)} \tag{1}$$

This density was applied to the particle size distribution to determine a median particle (dp_{50}) settling velocity as determined using Stokes law

$$\text{WS} = \frac{(\rho_p - \rho) g dp_{50}^2}{18\mu} \tag{2}$$

In addition to measuring particle sizes in unmanipulated water samples (termed "Raw"), samples were centrifuged to separate more dense particles (termed "Heavy") from water containing low density particles and free living cells (termed "Light"). This method was modified from a similar technique used to separate settling particles from stormwater (Characklis et al. 2005). A refrigerated centrifuge (Beckman Coulter Allegra 25R) was used to spin 250 mL bottles at $500 \times g$ for 10 min at 20°C in a swinging bucket rotor. Gentle spinning was used to limit changes in bacterial culturability and particle size distribution due to shear and pelletization. A loose coating of particles was usually visible on the bottom of the bottle, requiring a careful and conservative separation of the sample to avoid resuspension of settled material. The top portion (175 mL) of this sample, representing the Light fraction, was carefully removed using a 50 mL pipette. The re-

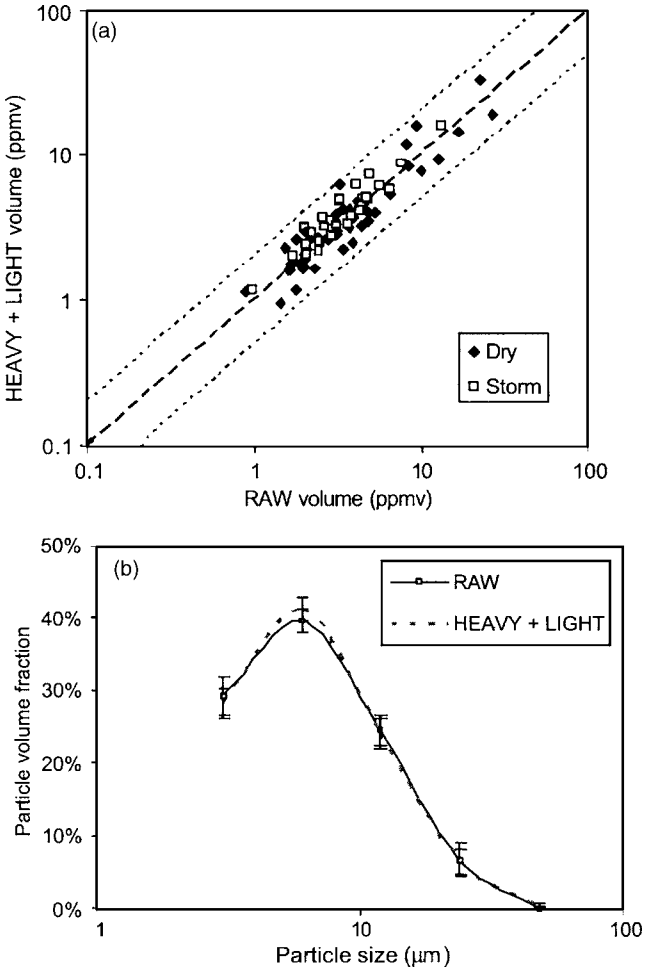

Fig. 6. Comparisons of particle suspension fractions: (a) total particle volume measurements from dry weather (diamonds) and storm (squares) samples plotted along with lines representing 1:1, 1:2, and 2:1 relationships (N=78); (b) average particle size distributions for fractions from dry weather samples (N=48): Raw (solid line) and Heavy + Light (dashed line). Error bars represent ±1 standard error.

mainder of the sample (75 mL) was a mix of Light water with Heavy particles that settled during centrifugation. This centrifugation procedure resulted in a threshold settling velocity for Heavy particles of 1.2 mm/h under quiescent conditions. This threshold is equivalent to particle size thresholds of 4 μm for 1.05 g/cm^3 particle density, 2 μm for 1.2 g/cm^3 particles, and 0.5 μm for 2.65 g/cm^3 particles. Viral particles and bacterial cells that are not attached to larger or denser particles will be in

Table 1. Laboratory Tests of Centrifugation Separation. Fractions of Test Suspensions in Terms of Particle Number Found in Heavy and Light Fractions.

Particles	Diameter μ(m)	Heavy (%)	Light (%)	Remainder (%)
Latex	5	37	**61**	2
	20	9	**74**	17
	45	23	**80**	−3
Glass	10–40	**93**	3	4

Note: Bold values indicate where majority of particles were found.

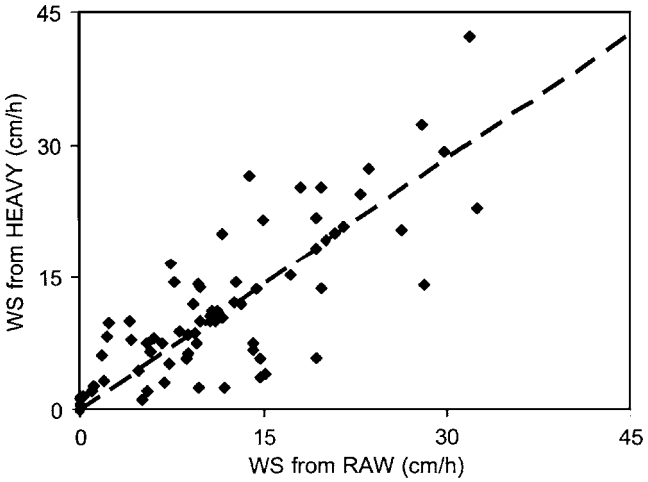

Fig. 7. Comparison of suspension settling velocities (WS) from Raw and Heavy samples. Linear regression (zero intercept with slope of 0.94±0.04, r^2=0.87) plotted with measurements.

the Light fraction due to their size (<2 μm: AWWA 1999) and low densities (1.02–1.4 g/cm^3: Bratbak and Dundas 1984; USFDA 2003).

Concentrations of indicator bacteria were measured in the Raw, Heavy, and some of the Light fractions. Methods approved by the US EPA for ambient water quality testing were used to measure EC (Colilert-18) and ENT (Enterolert) based upon directed substrate technology (IDEXX Laboratories, Inc.). These methods detect metabolically active bacteria via a most probable number (MPN) approach. Both tests used the Quanti-Tray/2000 for enumeration of cells and the number of positive wells was converted to MPN (Hurley and Roscoe 1983). Duplicate trays were used and well counts were considered together, effectively doubling the tray size to reduce the confidence intervals associated with the MPN.

Results from environmental samples used to verify a budget of Heavy, Light, and Raw revealed that the centrifugation procedure does not alter the population sizes [Fig. 4(a)]. Another concern was the potential bias from particle attachment in the quantification of organisms. Consider a suspension with an average of α cells attached to each particle. Using well counts, each particle will be detected as a single cell, therefore reducing the number of positive wells (measured=$f+n$ when true concentration is $f+\alpha n$, where f is free-living cell count and n is number of particles with attached bacteria). Fortunately, this bias exists in both the Raw and Heavy measurements, reducing the bias in the ratio of Heavy to Raw [measured=$n/(f+n)$ and true ratio=$\alpha n/(f+\alpha n)$]. The calculated differences between measured and true ratios were largest at intermediate attachment fractions [Fig. 4(b)]. These estimates of bias apply to any method for separation that is not designed to detach cells from particles (e.g., filtration). Detachment of cells was avoided in this study due to the possibility that cells placed under mechanical or sonic stresses may change their ability to grow in media. For measurements of attachment where the true value is low, the difference is of the same order magnitude. Fortunately, these results reflect a low fraction attached in either case and were not expected to significantly alter the overall effect on bacterial transport. However, the potential biases in the concentration measurements alone should not be dismissed and the concentrations reported in this study are lower bounds.

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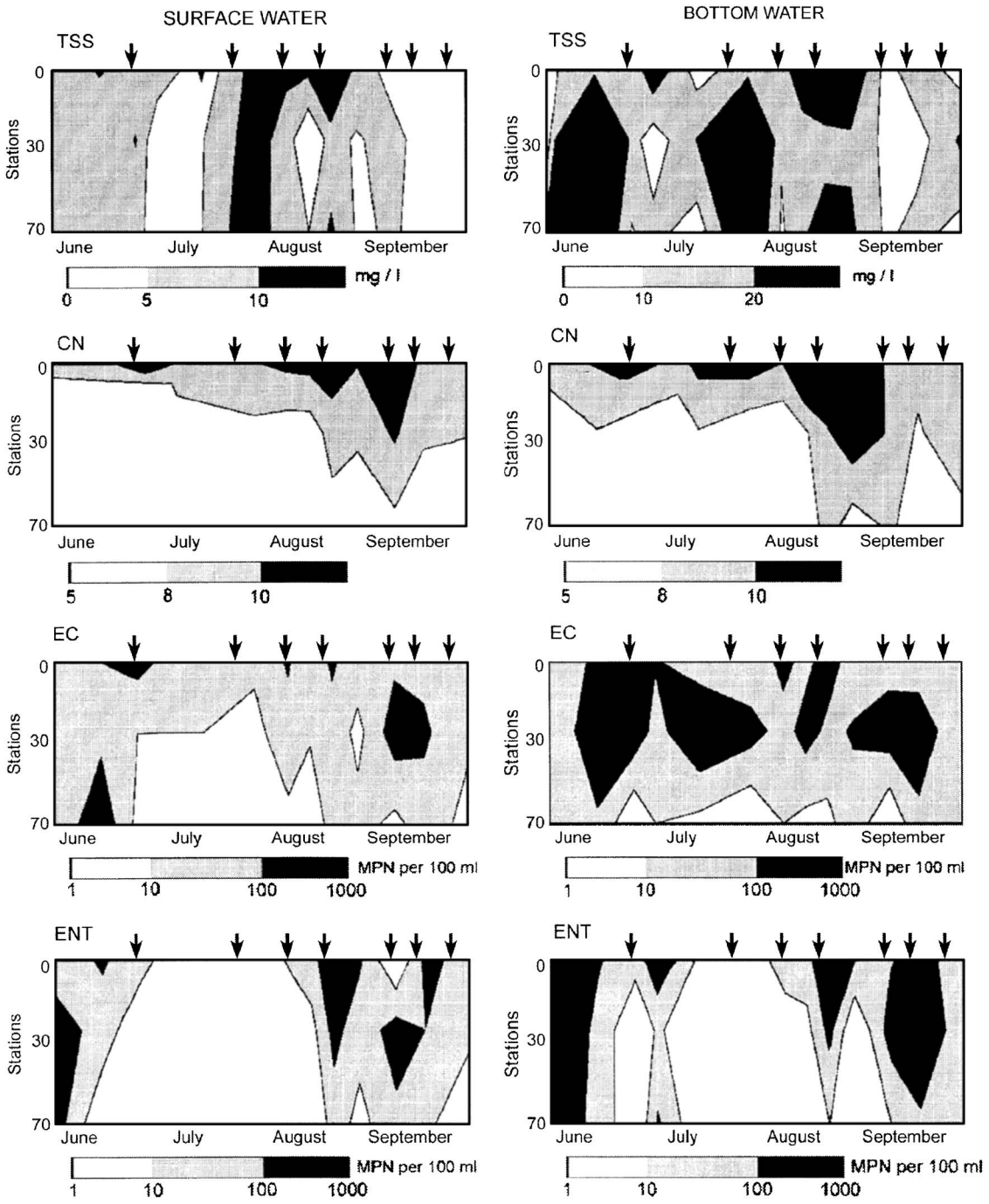

Fig. 8. Particle suspension and bacteria observations from all stations and dates. Surface water data presented to left, bottom water to right. Scales for each concentration or ratio below panels. Rain events (from Fig. 2) denoted by arrows on each panel.

Results and Discussion

Particle suspensions in the NRE exhibited a diverse variety of particle types and size distributions. Focusing on surface water samples taken at Station 30, samples were often found to be dominated by Heavy runoff inputs following rain or containing a mixture of Heavy and Light particles during dry weather conditions (Fig. 5). Comparing Raw and Heavy–Light splits of environmental samples, total particle volume and size distribution changed minimally (Fig. 6), supporting the use of gentle centrifugation for separating particles without destroying particles, creating aggregates, or loss of particles in the processing. These results from environmental samples compared well with laboratory tests using artificial particles. Using test suspensions, a majority of latex particles (1.05 g/cm^3) remained in the Light fraction after centrifugation, while glass beads (2.65 g/cm^3) were Heavy particles (Table 1). These results also revealed some loss of latex beads, possibly due to adhesion to sample containers often observed for artificial particles. This loss did not change the result that a large majority of latex beads were found in the Light fraction. Glass bead recovery was nearly complete (97%).

Settling velocities computed for both Raw and Heavy samples were comparable, supporting the assertion that a majority of the settling material was in fact isolated in the Heavy fraction (Fig.

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Table 2. Correlation Tables of Particle and Bacterial Measurements Presented in Fig. 8. Concentrations of *E. coli* and *Enterococci* Were Log-Transformed Prior to Calculations.

Samples		EC	ENT	TSS	CN
Surface	EC	1	—	—	—
	ENT	**0.31**	1	—	—
	TSS	0.18	**0.36**	1	—
	CN	**0.38**	**0.39**	0.20	1
Bottom	EC	1	—	—	—
	ENT	**0.25**	1	—	—
	TSS	**0.23**	0.05	1	—
	CN	0.15	0.12	0.17	1
All data	EC	1	—	—	—
	ENT	**0.28**	1	—	—
	TSS	**0.28**	0.09	1	—
	CN	**0.27**	**0.21**	0.20	1

Note: Data from surface and bottom water treated separately and combined. Bold values highlight the largest correlations found.

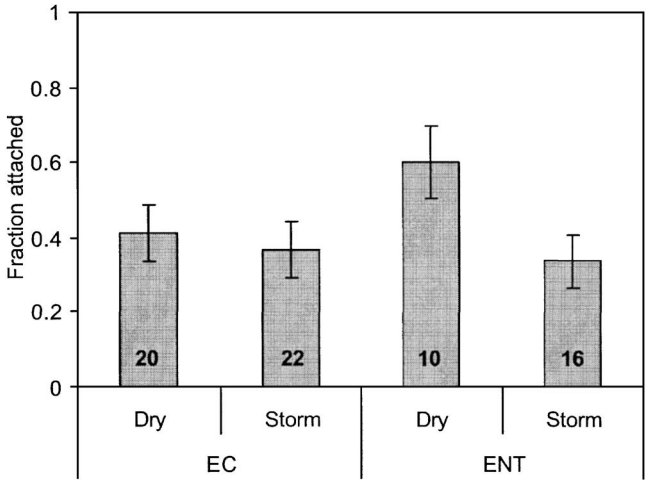

Fig. 9. Fractions of *E. coli* (EC) and *Enterococci* (ENT) attached to particles for dry and storm conditions. Each column represents average attachment for samples from all stations and depths with error bars for ±1 standard error (number of observations on each column).

7). If differences in the settling velocities were found, the difference would have been due to some settling particles remaining in the Light fraction or some change in particle sizes or density due to centrifugation. Using the range of settling velocities found for suspensions (2–35 cm/h), approximate times for 90% clearance of the suspensions from surface waters in a 3 m water column ranged from 1 day to 2 weeks.

TSS in surface water increased following rain events, although changes in bottom water were not clearly linked to weather (Fig. 8). Differences in bottom water may have been difficult to discern due to the large dilution of stormwater in the estuary and possible influence of sediment resuspension. CN increased consistently with each rain event and spread downstream in both surface and bottom waters. Therefore, CN may be a more robust indicator of runoff than TSS.

Both EC and ENT increased in response to events as well, with the largest increases towards Station 0, where runoff inputs from the watershed were expected to have the greatest impact (Fig. 8). Both indicators compared well for the entire summer, however, EC in bottom water exhibited a different pattern due to persistent concentrations at Station 30, near the location of the sewage treatment outfall. Correlations between CN and both indicator bacteria in surface water supported the idea that EC and ENT were introduced along with particles in runoff (Table 2). Bacterial levels at most stations exceeded the NC water quality recommendations for single samples more frequently towards Station 0 and in bottom water (Table 3). This pattern was consistent with runoff and resuspended sediment representing the principle sources of indicator bacteria in the NRE.

Partitioning data from surface and bottom water samples taken during both storm and dry conditions showed little variation with one exception: larger fraction of ENT attached to particles during dry weather (Fig. 9). These samples also exhibited relatively low ENT concentrations and may have been residual populations from previous storms or those attached to resuspended sediments (Pettibone et al. 1996). For all other groups, the fraction attached was approximately the same (38±4%). Similar attachment for both indicators following storms was consistent with the correlation between their concentrations in surface water (Table 2). These results suggest that similar rates of removal (mortality and settlement) could be used for both bacterial groups, and potentially for pathogens of interest, following introduction to the estuary.

Physiological differences between EC and ENT (e.g., hydrophobicity and surface charge) have been used to explain observed differences in cell–particle attachment fractions in other studies. Stenstrom (1989) found differences between EC (21–29%) and ENT (56–77%) attachment to inorganic particles. Characklis et al. (2005) found similar differences in attachment to principally inorganic particles in stormwater for EC (25±9%) and ENT (45±7%). Small differences in partitioning between EC and ENT in this study may be indicative of attachment to organic, rather than inorganic, particles in the contamination source (e.g., stormwater) or following introduction to the estuary (e.g., resuspended sediments). The potential for changes in partitioning due to attachment to organic particles is especially important in eutrophying estuaries like the NRE. The change in available organic particles for attachment, from runoff to algal cells, as bacteria transports downstream provides an interesting and unique gradient of conditions to consider in modeling contamination of estuaries.

Table 3. Number of Instances Where Measured Bacterial Levels Exceeded North Carolina Recreational Water Quality Recommendations for Single Measurement (N.C. Dept of Environment and Natural Resources)

	North Carolina recommendation		Station 0	Station 30	Station 70
E. coli	320 MPN/100 mL	Surface	0/13 (0%)	1/13 (8%)	1/13 (8%)
		Bottom	0/13 (0%)	3/13 (23%)	0/13(0%)
Enterococcus sp.	104 MPN/100 mL	Surface	4/13 (31%)	3/13 (23%)	1/13 (8%)
		Bottom	6/13 (46%)	4/13 (31%)	2/13 (15%)

Note: MPN=most probable number.

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Conclusions

The observations described in this study are an important step toward evaluating the consequences of microbial attachment to particles in estuarine waters. Introduction of indicator bacteria and particulate matter associated with stormwater runoff and resuspended sediments were coincidently detected following rain events in the NRE. Transport and removal of this microbial contamination is mediated in part by sedimentation of attached organisms. Methods for determination of the fraction of bacterial population attached to particles are tested in an estuarine setting and provide a tool for future assessments of water quality and microbial transport models. The fraction attached was relatively stable throughout the summer ($38 \pm 4\%$), providing an important input for predictive models of transport and fate of the indicator bacteria and pathogens in estuarine environments.

Acknowledgments

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Notation

The following symbols are used in this paper:

- WS = particle settling velocity ($m\ s^{-1}$);
- dp = particle diameter (m);
- dp_{50} = median particle diameter (m);
- f = number of free-living cells;
- g = gravitational acceleration ($m\ s^{-2}$);
- N = particle size distribution in terms of number;
- n = number of particles with attached cells per 100 mL;
- α = number of cells per particle with attached cells;
- μ = water viscosity ($kg\ m^{-1}\ s^{-1}$);
- ρ = water density ($kg\ m^{-3}$); and
- ρ_p = particle density ($kg\ m^{-3}$).

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