Assessment of *E. coli* partitioning behavior via both culture-based and qPCR methods

Leigh-Anne Krometis, Rachel T. Noble, Gregory W. Characklis, A. Denene Blackwood and Mark D. Sobsey

ABSTRACT

Quantitative polymerase chain reaction (qPCR) offers a rapid, highly sensitive analytical alternative to the traditional culture-based techniques of microbial enumeration typically used in water quality monitoring. Before qPCR can be widely applied within surface water monitoring programs and stormwater assessment research, the relationships between microbial concentrations measured by qPCR and culture-based methods must be assessed across a range of water types. Previous studies investigating fecal indicator bacteria quantification using molecular and culture-based techniques have compared measures of total concentration, but have not examined particle-associated microorganisms, which may be more important from a transport perspective, particularly during the calibration of predictive water quality models for watershed management purposes. This study compared total, free-phase, and particle-associated *Escherichia coli* concentrations as determined by the Colilert defined substrate method and qPCR targeting the *uidA* gene in stream grab samples partitioned via a calibrated centrifugation technique. Free-phase concentrations detected through qPCR were significantly higher than those detected using Colilert although total concentrations were statistically equivalent, suggesting a source of analytical bias. Although a specimen processing complex was used to identify and correct for inhibition of the qPCR reaction, high particle concentrations may have resulted in underestimation of total cell counts, particularly at low concentrations. Regardless, qPCR-based techniques will likely have an important future role in stormwater assessment and management.

Key words | *E. coli*, partitioning, qPCR, stormwater, waterborne transport

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INTRODUCTION

Elevated concentrations of fecal indicator bacteria (FIB; including total coliforms, fecal coliforms or *Escherichia coli*, and *Enterococcus* spp.) are the most commonly identified cause of water quality impairment in the USA, and are responsible for 14% of water bodies that require total maximum daily load (TMDL) development to achieve compliance with water quality standards (USEPA 2012). The epidemiological studies historically used to establish FIB regulatory standards to identify impairments have all used culture-based methods (e.g. membrane filtration) to quantify microbial exposure (USEPA 1986; Wade *et al.* 2003). These methods identify target microorganisms via cellular metabolism of particular substrates and subsequent reproduction resulting in macroscopic endpoints (e.g. colony forming units, metabolic by-products) (Rompre *et al.* 2002). Because cell division and growth require time, results from culture-based analyses are not available for 18–24 hours or more. This time lag limits the ability of water quality managers to protect public health in recreational areas, as results identifying contamination are not available until the day after swimmers have been exposed (Leecaster & Weisberg 2001). The analytical limitations of traditional culture-based microbial detection methods may also compromise the efforts of less time-sensitive water quality monitoring programs, such as for bivalve molluscan shellfish sanitation. At present, the vast majority of US surface waters are required to meet full-body contact recreational standards for bacteria to maintain 'non-impaired' status in accordance with the national TMDL program (Keller & Cavallaro 2008). Water quality

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assessment is wholly dependent on culture-based testing which does not account for viable but non-culturable (VBNC) cells, which may still pose a threat to public health (Oliver 2000).

The relatively recent development of molecular techniques (e.g. polymerase chain reaction or PCR, microarrays) for microbial detection and quantification offers a rapid, highly sensitive alternative to culture-based testing; however, the relationships between results from the culture-based methods originally used to develop regulatory standards and results from new molecular approaches are not fully understood. Epidemiological studies in California and the Great Lakes have demonstrated relationships between bather illness and molecular indicators that are equivalent to relationships observed using culture-based methods in some cases; however, relationships remain unclear under some environmental conditions (Wade *et al.* 2006; Colford *et al.* 2012). To avoid regulatory chaos, the implementation of molecular methods in nation-wide surface water monitoring programs will require a comparison of molecular and culture-based approaches that ideally correlates observations via the new technique and historical approaches in a wide range of water types under a wide variety of environmental (climate, season, land-use, salinity, etc.) conditions (Noble & Weisberg 2005; Jofre & Blanch 2010). Predictive water quality models used to simulate microbial fate and transport to assess watershed remediation scenarios may require substantial revision or recalibration to appropriately incorporate data from monitoring programs that target molecular rather than culture-based targets.

Previous studies comparing FIB quantification methods in recreational areas have observed quantitative PCR (qPCR) concentrations of targeted bacteria greater than those detected by culture-based methods (Haugland *et al.* 2005; Noble *et al.* 2006; Khan *et al.* 2007; Morrison *et al.* 2008; Lavender & Kinzelman 2009). This result is expected, as qPCR quantifies the DNA of metabolically active, VBNC, and lyzed cells indiscriminately. Despite differences in the magnitude of the signal resulting from different analytical endpoints, correlations in total FIB concentrations as identified by qPCR and culture-based methods have generally been significant for samples collected from estuaries or recreational areas, with R^2 values ranging from 0.68 to 0.93 (Haugland *et al.* 2005; Morrison *et al.* 2008). The relationship between measurements from contaminated upland freshwater tributaries, particularly those affected by sediment-laden stormwaters, is less clear. Analysis of multiple samples collected from an urban stream throughout a single storm event by Noble *et al.* (2006) indicated that, while qPCR measures of enterococci remained roughly constant over storm duration, culture-based measures of concentration declined as the storm progressed. A recent study by Lavender & Kinzelman (2009) of samples collected upstream and downstream of an urban stormwater outfall also failed to observe consistent relationships between culture-based and qPCR measures of enterococci. Although significant differences in concentration were observed between sampling sites and dry/storm samples when comparing concentrations determined via Enterolert, concentrations of enterococci as measured by qPCR were statistically equivalent across sites and climatic conditions. As stormwater is frequently identified as a primary contributor of microbial loadings to impaired waterways (Gaffield *et al.* 2003), further investigation of the use of molecular methods to quantify bacteria in these flows is required before these techniques can be validated and fully integrated into existing regulatory programs.

Comparisons between culture-based and molecular detection of FIB have focused solely on correlations between measurements of total concentration. While useful in monitoring for standard violations, these results do not compare concentrations of settleable and free-phase microbes, which are valuable in fate and transport predictions (Seurinck *et al.* 2005). Past investigations of FIB or pathogen partitioning behavior have quantified concentration and particle association using either culture-based methods (Characklis *et al.* 2005; Jeng *et al.* 2005; Fries *et al.* 2006) or direct microscopic enumeration (Medema *et al.* 1998; Cizek *et al.* 2008) rather than molecular approaches. The nature of the partitioning technique (e.g. filtration, centrifugation) used to separate settleable (i.e. particle-associated) and free-phase cells prior to enumeration may yield different results when molecular techniques are used to quantify concentration, thereby affecting estimates of partitioning behavior and transport in receiving waters.

The objective of this study was to identify relationships between molecular and culture-based technique observations of particle-associated and free-phase *E. coli* concentrations in samples collected from urban freshwater streams during dry weather and storm events. Although *E. coli* remains the recommended FIB for freshwater monitoring (USEPA 1986; Wade *et al.* 2003), comparisons of *E. coli* detection via culture-based and PCR analysis have generally focused on positive detection and have not included attempts to correlate concentration measurements obtained by the two techniques (Frahm & Obst 2003; Lleo *et al.* 2005). In the present study, samples were collected from four local watersheds

of varying levels of contamination, partitioned via a calibrated centrifugation technique, and analyzed concurrently for *E. coli* concentration and particle association via the Colilert defined substrate technique and an *E. coli*-specific qPCR assay. Analysis via qPCR targeted the *uidA* gene coding for the enzyme β-glucuronidase that metabolizes 4-methyl-umbelliferyl (MUG), which is also responsible for the fluorescent signal indicating *E. coli*-positive wells in the Colilert assay. Because both methods use the presence of the same enzymatic ability to identify *E. coli*, potential differences in concentration are likely due to differences in physiology rather than the presence of non-MUG strains. Quantification of both free-phase and settleable concentrations allowed for comparisons of potential methodological differences in estimating transport potential.

METHODOLOGY

Sampling regimen

Samples were collected from four separate watersheds in central North Carolina, USA: Eno River, Booker Creek, Meeting of the Waters Creek, and Northeast Creek. The basins vary in primary contributing land use, with Booker and Meeting of the Waters Creek classified as primarily commercial (high impervious) and/or residential, Eno River classified as primarily low-density residential, and Northeast Creek classified as primarily urban. In order to maximize the range of *E. coli* concentrations observed, the selected watersheds differed substantially in terms of Clean Water Act section 303(d) impairment status, which was used as a proxy for anticipated water quality. The Eno River is currently not listed as impaired, and was considered the 'cleanest' site. Both Booker Creek and Meeting of the Waters Creek in the town of Chapel Hill currently require TMDL development to address biological impairments (i.e. general ecological degradation). Northeast Creek, which drains a rapidly urbanizing section of the city of Durham, has multiple water quality impairments, including low dissolved oxygen, high turbidity, high fecal coliform concentrations, and a biological impairment. As the only one of the four study watersheds formally designated as impaired due to FIB, samples were collected from Northeast Creek at three separate locations. Site characteristics are further detailed by Characklis *et al.* (2005) and Krometis *et al.* (2010).

Two-liter samples were collected in pre-sterilized polypropylene bottles during seven dry weather periods and three storm events. Dry weather periods were defined by a minimum of three previous days of no appreciable precipitation (i.e. less than a 25% increase over the mean monthly baseflow), and storm events were defined as precipitation resulting in a fourfold or greater increase in baseflow as indicated by real-time US Geological Survey streamgages (http://waterdata.usgs.gov). In total, 47 samples were collected for partitioning and analysis, as time and weather constraints did not permit sampling at every site during every event. Following partitioning of these samples into raw and supernatant subsamples, 94 measures of *E. coli* via both Colilert and qPCR were available for statistical analysis.

Partitioning method

Prior to *E. coli* quantification in parallel by Colilert and qPCR analysis, all samples were first partitioned into a settleable (i.e. likely particle associated) and free phase via a previously calibrated centrifugation technique (Cizek *et al.* 2008; Krometis *et al.* 2010). This method is similar to that presented in Characklis *et al.* (2005), although the centrifugation speed is reduced to provide a more conservative measure of the settleable fraction. Each 2-L sample was gently inverted three times to resuspend settled particles and separated into two 1-L subsamples. One subsample ('raw') was analyzed without further treatment in order to obtain total concentrations of *E. coli* and other relevant water quality parameters. The second subsample was centrifuged (Sorvall RC-3B centrifuge with a H-6000A rotor) at 500 rpm ($73 \times g$) for 10 min at 4 °C with a brake of 4 (approximately 5 min deceleration time). Following gentle removal from the centrifuge, the top 700 mL of supernatant was removed via a sterile vacuum pipette. The extracted supernatant was subsequently analyzed in parallel with the raw subsample to determine the concentration of free-phase ('non-settleable') *E. coli* and particles. Previously reported calibration of this technique using latex and glass beads as surrogates for organic and inorganic constituents indicated that, while over 90% of low density (1.05 g/cm^3) beads remained in suspension following centrifugation, over 95% of glass beads (density = 2.65 g/cm^3) were removed (Cizek *et al.* 2008). This suggests that the technique is sufficiently discriminatory to remove most inorganic material and associated cells while retaining the majority of unassociated cells and organic material in suspension. Fries *et al.* (2006) analyzed raw, suspended, and centrifuge pellet concentrations of *E. coli* following centrifugation, and confirmed that cell recoverability (i.e. die-off, loss of culturability) was not measurably affected by the regimen.

Settleable (i.e. particle-associated) *E. coli* concentrations are operationally defined by this technique as the difference between total and free-phase concentrations. It is worth noting that, while distinctions between 'settleable' and 'free-phase' bacteria are useful in estimates of microbial transport, they are simplifications of actual attachment behavior. Microorganisms may be associated with organic material retained in the centrifugation supernatant; however, because this material is of similar low density, associated microbes will likely retain significant mobility in receiving waters.

Culture-based analysis of samples

Raw ('total') and supernatant ('free-phase') concentrations of *E. coli* were evaluated in parallel via both qPCR targeting the *uidA* gene and the Colilert defined substrate method (www.idexx.com, Westbrook, Maine, USA). In order to detect only thermotolerant fecal coliforms (generally considered a more accurate predictor of health risk than total coliforms) and *E. coli*, incubation times for Colilert were modified (Yakub *et al.* 2002). Each Quantitray was incubated for 2–4 hours at 37 °C followed by 20 hours at 44.5 °C prior to examination. Wells that solely turned yellow were assumed to be positive for fecal coliforms, and wells that were both yellow and fluorescent were assumed positive for *E. coli*. Two Quantitray-2000s were used to double the number of wells used in the Colilert analysis so as to reduce the related MPN (mean probable number) confidence intervals. For example, using the Thomas equation (Hurley & Roscoe 1983), a Quanti-tray with 48 positive large wells and 15 positive small wells would yield a 95% confidence interval of 144–292 MPN/100 mL. If two Quanti-trays are used (essentially doubling the number of total potential positive well observations) the 95% confidence interval for two trays each with 48 large wells positive and 15 small wells positive is 160–264 MPN/100 mL, a 30% decrease in confidence interval range.

Pre-concentration of samples for qPCR analysis

Following partitioning into raw and supernatant samples, an aliquot from each fraction was filtered through a 47 mm, 0.45 μm polycarbonate filter (Millipore Isopore, Bedford, MA, USA), washed with 25–50 mL laboratory-grade distilled water, aseptically transferred to a Whirl-Pak bag, and stored at −20 °C until qPCR analysis. Filtered volumes ranged from 1 to 100 mL of sample, as heavy contamination by suspended sediments periodically resulted in filter clogging and an inability to filter a full 100 mL. An effort was made using previous data and site knowledge to capture a minimum of 50 *E. coli* cells per filter. Filters for qPCR analysis were prepared in duplicate with resultant concentrations averaged for analysis. Analysis of filter-captured cells via qPCR occurred within 3 months of sample collection.

Specimen processing complex for quantification of potential inhibition

Inhibition of the qPCR reaction by humics or other sample matrix molecules is a frequent problem in the analysis of environmental samples, which can result in false negatives or a reduction in detectable concentrations (Noble & Weisberg 2005; Jofre & Blanch 2010). Quantification of, and subsequent correction for, inhibition in an unknown sample matrix can be achieved through the addition of a known quantity of non-indigenous DNA to the samples prior to processing and analysis (i.e. specimen processing complex). In the present study, salmon (*Oncorhynchus keta*) testes DNA (Sigma-Aldrich, St Louis, MO., USA) was used as a specimen processing complex to quantify and correct for potential PCR inhibition by sample matrix components as in previous studies quantifying bacteria in natural waters (Hamilton *et al.* 2005; Morrison *et al.* 2008; Lavender & Kinzelman 2009). Approximately 10 ng of salmon testes DNA was added to the extraction buffer and co-extracted via bead beating.

DNA extraction

Filter-captured cellular DNA was extracted via a bead-beating technique similar to that described in Haugland *et al.* (2005). Sample filters were transferred to 2 mL screw-top microcentrifuge tubes containing 0.3 g of pre-sterilized 0.1 mm silica zirconium beads (BioSpec Corp., Bartlesville, OK, USA). A negative extraction control was included that consisted of a polycarbonate filter in a tube containing beads and was carried through the extraction process. Following the addition of 500 μL of Buffer AE (QIAGEN, Valencia, CA, USA) each tube was then homogenized at maximum speed for 2 min using a mini-beadbeater (Bio Spec). Cellular debris removal and sample purification was achieved via a series of centrifugation steps (12,000 × *g* for 1 and 5 min) followed by supernatant removal. The final supernatant was transferred to a new sterile 1 mL microcentrifuge tube and stored at 4 °C until qPCR analysis. Samples were analyzed within 72 hours of extraction.

qPCR analysis

Extracted samples were analyzed for the specimen processing complex signal to assess potential inhibition, diluted if necessary, and then analyzed for *E. coli* concentration. Specimen processing complex Scorpion® primer-probe complexes were designed for a segment of the ribosomal DNA gene, internal transcribed spacer region 2 of chum salmon, as similarly described in Haugland *et al.* (2005). Primer/probe complexes, lyophilized Omnimix beads containing deoxynucleotides, magnesium chloride, buffer, and *Taq* polymerase (Cepehid, Sunnydale, CA, USA) were appropriately diluted in molecular-grade water to create a master mix for salmon testes DNA detection before insertion into a Cepheid Smart Cycler II system. Thermal cycling conditions were as follows: 120 s at 95 °C followed by 45 cycles of 5 s at 95 °C and 43 s at 62 °C. Samples were considered inhibited if the salmon testes DNA threshold cycle (C_t) value was more than 1.5 cycles (0.5 log) greater than the average C_t value from a non-inhibited salmon testes DNA value (i.e. delayed qPCR amplification). Inhibited samples were subjected to a 10-fold dilution with molecular-grade water and re-analyzed via qPCR for the salmon testes control to confirm sufficient reduction of inhibitor compounds before *E. coli* analysis. Of the 94 raw and supernatant samples analyzed, 29 (31%) were identified as inhibited. Following dilution, *E. coli* was quantified via qPCR in 23 of the inhibited samples but was not detectable in the remaining six samples. These 'non-detects' were likely the result of dilution of the target signal during dilution of inhibitors, as expected *E. coli* concentrations in these six samples (as measured by Colilert) were very low (mean = 8 MPN) and all were diluted in an attempt to reduce inhibition.

Escherichia coli analysis via qPCR was conducted using Scorpion®-based chemistry via the use of lyophilized beads that contained buffer, and primer and probe (Biogx, Inc., Birmingham, AL, USA, www.biogx.com). This set of forward and reverse primers and probe targets the *uidA* gene, which codes for the enzyme β-glucuronidase which is responsible for MUG hydrolysis. Primer and probe sequences are proprietary. A qPCR master mix of lyophilized beads containing the *E. coli*-specific primer and probe set, Omnimix lyophilized beads, and 20 μL reagent-grade dilution water was combined with 5 μL of sample and analyzed via the Cepheid Smart Cycler II system, with a thermal cycling regimen of 120 s at 95 °C, followed by 45 cycles of 5 s at 95 °C, followed by 43 s at 62 °C. C_t values for each sample were determined after manually adjusting the threshold fluorescence value to 8 units, which corresponded to the point of maximum slope of the cycle–fluorescence curve. Samples were considered non-detectable if the fluorescence curve did not cross the threshold following 45 cycles.

Calculation of *E. coli* concentration

Serial $\log_{10}$ dilutions of a positive specimen processing complex sample (500 μL of AE buffer spiked with 10 ng salmon testes DNA) and an *E. coli*-positive control were extracted via bead beating and analyzed via qPCR to generate standard calibration curves. *E. coli* for generation of standard curves was grown at 37 °C and formalin fixed. Following enumeration via SYBR Green (Noble & Fuhrman 1998), 10^5 cells were filtered through a 0.45 μm 47 mm polycarbonate filter and stored at −80 °C. Serial dilutions ranging from 10^1 to 10^5 cells were used to generate standard calibration curves, which were used to determine final concentrations, as well as exponential amplification and efficiency of the qPCR reaction. Amplification efficiencies ranged from 86 to 98%. Analysis of the undiluted salmon testes DNA positive controls also provided uninhibited C_t values for comparison with salmon testes DNA values from potentially inhibited spiked environmental samples. A no-template control that consisted of water and master mix was included with every assay. All negative extraction and no-template controls were negative.

The delta-delta-C_t method, as described by Haugland *et al.* (2005), was used to simultaneously determine *E. coli* concentrations while correcting for inhibition (i.e. <100% specimen processing complex detection). Briefly, the *E. coli* concentration is calculated via the following equation:

$$C_{\text{observed}} = C_o \times \text{EA}^{-\Delta\Delta C_t} \quad (1)$$

where C_{observed} = sample concentration; C_o = concentration of *E. coli* in the non-diluted positive control (cell equivalents/volume); EA = exponential amplification; and $\Delta\Delta C_t$ = the difference in ΔC_t values between the observed sample values and the calibration values. Because only one *uidA* gene is assumed present per *E. coli* cell, concentration units are reported as number of cell equivalents per volume.

Physical analysis

Following partitioning, all settleable and free-phase subsamples were analyzed for particle concentration (2–60 μm), particle surface area, and particle volume by a

Coulter Multsizier I (Coulter Electronics Ltd, Luton, UK) with a 100 μm aperture tube. Total suspended solids (TSS) and total organic carbon (TOC) concentrations were determined via Standard Methods 5310B and 2540D, respectively (APHA 2009). Particle-volume data and TSS values were used to estimate average particle density.

Statistical analysis

All statistical analyses were conducted using SAS Statistical Software (SAS Inc., Cary, NC, USA) with significance defined as $p < 0.05$. Nonparametric methods were applied, as the Shapiro–Wilk test indicated a non-normal distribution. Transformation to a log-normal scale did not result in a normal distribution according to this test.

RESULTS AND DISCUSSION

Water quality across sampling sites

Differences in water quality observations between streams were most apparent through comparisons of dry weather samples (Table 1). Concentrations of every physical water

Table 1 | Geometric mean and 95% confidence intervals (CI) for water quality parameters at all sites. Mean values are shown in bold; values above and below each mean are the upper and lower limit, respectively, of the 95% CI

		Fecal coliforms (MPN/100 mL)	*E. coli*, IDEXX (MPN/100 mL)	Total particles (#/mL)	TSS (mg/L)	Density (g/cm^3)	TOC (mg/L)	Turbidity (NTU)
Booker Creek		50,599	18,203	317,258	130	3.3		124
	Storm	**8,810**	**1,916**	**197,061**	**56**	**2.9**	**12.8**	**71**
	$n = 3$	1,534	202	122,403	24	2.6		41
		8,897	1,185	63,199	16	3.8	9.6	25
	Dry	**2,933**	**388**	**40,768**	**9**	**2.3**	**7.4**	**13**
	$n = 7$	967	127	26,298	5	1.4	5.7	7
Eno River		110,339	38,731	1,097,521	538	4.6		362
	Storm	**6,008**	**1,565**	**152,569**	**57**	**3.5**	**13.2**	**89**
	$n = 3$	327	63	21,209	6	2.7		22
		1,860	144	12,993	3	3.9	5.7	5
	Dry	**792**	**76**	**8,927**	**1.3**	**1.9**	**5.1**	**4**
	$n = 7$	337	40	6,134	1	0.9	4.5	3
Meeting of the Waters Creek		90,726	18,915	536,120	324	6.2		302
	Storm	**9,472**	**1,158**	**134,591**	**63**	**4.9**	**8.4**	**92**
	$n = 3$	989	71	33,789	12	3.9		28
		3,282	558	50,780	20	5.3	5.5	18
	Dry	**1,390**	**230**	**27,145**	**7**	**2.5**	**4.3**	**9**
	$n = 7$	588	95	14,511	3	1.2	3.4	4
Northeast Creek – Site 1		54,074	9,715	552,513	216	6.2		274
	Storm	**10,922**	**1,967**	**249,328**	**82**	**4.0**	**12.2**	**144**
	$n = 3$	2,206	398	112,512	32	2.6		75
		2,645	179	77,712	17	4.0	11.9	26
	Dry	**1,542**	**92**	**58,582**	**12**	**2.8**	**10.3**	**18**
	$n = 7$	899	47	44,160	8	1.9	9.0	13
Northeast Creek – Site 2		101,482	11,584	810,107	317	7.9		313
	Storm	**9,096**	**1,542**	**234,846**	**89**	**5.5**	**12.3**	**119**
	$n = 3$	815	205	68,081	25	3.8		46
		7,922	519	43,469	20	9.5	10.3	16
	Dry	**1,845**	**162**	**31,597**	**12**	**5.1**	**9.5**	**12**
	$n = 5$	430	51	22,968	7	2.7	8.8	10
Northeast Creek – Site 3		89,701	19,087	771,270	311	7.0		327
	Storm	**10,027**	**2,293**	**436,805**	**117**	**3.5**	**14.3**	**158**
	$n = 3$	1,121	275	247,382	44	1.8		76
		6,213	301	138,032	44	4.8	10.7	38
	Dry	**3,251**	**219**	**89,797**	**24**	**3.8**	**9.7**	**27**
	$n = 6$	1,701	160	58,418	13	3.0	8.8	19

quality constituent measured were lowest for the Eno River, which is currently classified as unimpaired by North Carolina, although mean fecal coliform concentrations remained high. Interestingly, although only Northeast Creek has been officially identified by the state as impaired due to high FIB concentrations, dry weather (i.e. background) mean concentrations of *E. coli* were higher in Booker Creek and Meeting of the Waters Creek than in Northeast Creek.

During storm events, water quality was uniformly poor (Table 1), despite differing watershed impairment classifications. Mean fecal coliform and *E. coli* concentrations during storms as measured by Colilert were at least an order of magnitude greater than corresponding mean dry weather levels at all sites. Nearly every storm sample exceeded both the state of North Carolina single sample standard for freshwater of 400 fecal coliform per 100 mL (NCDWQ 2012) and the USEPA single threshold limit of 235 *E. coli* per 100 mL (USEPA 1986). Particle concentrations during storms were 5 to 20 times those measured during dry weather, and TOC, TSS, and turbidity also increased to double or more the dry weather value. Average particle density, estimated using measures of particle volume and TSS, also increased during storms, possibly as a result of the mobilization of denser inorganic material by overland flow and/or increased streamflow. No statistical associations were observed between storm intensity or duration and measures of in-stream water quality.

Molecular and culture-based detection of *E. coli*

Collection of samples from watersheds of varying state-identified impairment status provided a wide and relatively continuous range of *E. coli* concentrations across multiple orders of magnitude (Figure 1). While *E. coli* was detectable in all subsamples by Colilert, concentrations were not always detectable by qPCR. These non-detects, likely due to high inhibition in samples with high TSS, were assigned a qPCR concentration value of 0.1 for the purposes of plotting and will lie along the *x*-axis in Figure 2. Equivalent measures of concentration by the two detection methods should fall along the 1:1 line of equivalence. Differences between measures of total *E. coli* concentration by Colilert (culture) and qPCR were not statistically significant (Wilcoxon matched pairs signed rank, $p > 0.05$; $R^2 = 0.81$), but qPCR measures of free-phase *E. coli* concentration were significantly higher than corresponding concentrations determined via Colilert (Wilcoxon, $p < 0.05$; $R^2 = 0.10$). These statistical relationships did not change when the non-detect pairs were removed. Apparent differences in relative measures of concentration (free-phase vs total) could be due to differences in physiology (culturability) between the particle-associated and free phases of *E. coli*, differences in the extent of *E. coli* aggregation or particle association between the two sample types, and/or decreased quantities of inhibitors in the supernatant samples.

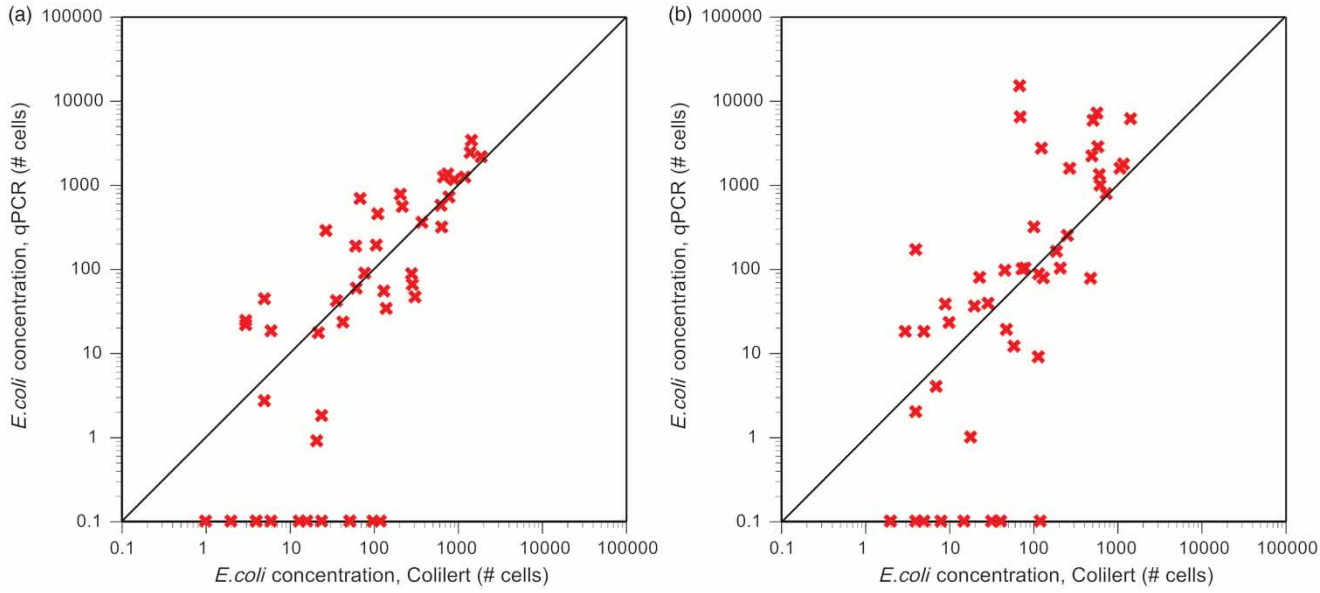

Figure 1 | Comparison of (a) total (raw) and (b) free-phase (supernatant) *E. coli* concentrations ($n = 47$ pairs) as measured by Colilert and qPCR.

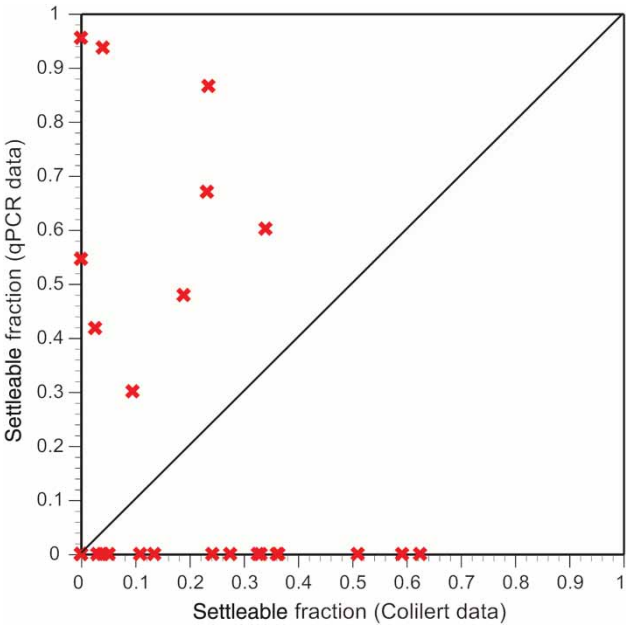

Figure 2 | Comparison of settleable fraction estimates using culture-based (Colilert) and qPCR observations of total and suspended *E. coli* concentrations.

Culture-based methods, including Colilert, only account for metabolically active cells capable of producing enough growth to detect hydrolysis of the fluorogenic defined substrate as measured by visible fluorescence upon long wavelength UV irradiation. There is evidence that cells in the natural environment frequently enter a VBNC state to maximize survival (Oliver 2000). Previous studies also suggest that particle-associated bacteria may persist longer in the environment, as measured by extended periods of detection via culture-based methods (Fish & Pettibone 1995; Howell *et al.* 1996; Jamieson *et al.* 2004). The free-phase counterparts of these more persistent particle-associated microorganisms may be in a VBNC state rather than actually inactivated. The significantly higher *E. coli* supernatant concentrations observed by qPCR may indicate that a higher proportion of these free-phase cells relative to the total (raw) number are in a VBNC state not detectable by Colilert, or have recently lyzed and released their nucleic acids. Measures of total microbial concentration may be closer between the two methods as the total population includes particle-associated cells which are more likely to remain metabolically active.

Inhibition was evaluated and corrected for in this study via simultaneous amplification and detection of a specimen processing complex (salmon sperm DNA); however, this control was present in undiluted samples at a relatively high concentration ($\sim 5 \times 10^3$ target sequences), which was generally several orders of magnitude greater than the expected *E. coli* concentrations (10–100 cells) targeted for detection. Higher concentrations of the specimen processing complex may have resulted in more success in overcoming inhibitors as compared to the relatively low copy number *E. coli* genomic target. It is critical to note that the use of salmon sperm as a specimen processing complex has recently been criticized as an imperfect predictor of PCR inhibition in water quality assessments and should be regarded with caution (Cao *et al.* 2012). Cao *et al.* observed that low concentration internal controls did not always successfully predict target inhibition; it is likely that exposed DNA added to a sample will not suffer from the same mechanisms of inhibition observed for intact FIB cells that contain cell-associated, membrane-packaged complex DNA (multiple forms) and/or may be protected by particles.

The centrifugation procedure used to partition samples prior to *E. coli* analysis resulted in average removals of 35% of total particle number, 55% of particle volume, 45% of TSS, and 7% of TOC. Removal of this material during centrifugation would be expected to reduce inhibitor concentration in supernatant samples, allowing for a more accurate measure of the number of *E. coli* cells present. Concentrations of particles (by number and by volume) and measures of TSS were significantly greater for samples in which the qPCR reaction was designated as inhibited compared to those in which qPCR was not inhibited (Table 2).

Table 2 | Particle, suspended sediment, and total organic carbon measures for 'inhibited' vs 'not inhibited' samples

		# Particles per 100 mL	Particle volume μm^3 per 100 mL	TSS, mg/L	TOC, mg/L
	95% CI – upper limit	2.94×10^6	2.15×10^8	8.3	2.6
Inhibited ($n = 29$)	Mean	2.42×10^6	1.53×10^8	5.8	**1.5**
	95% CI – lower limit	2.00×10^6	1.09×10^8	4.0	0.9
	95% CI – upper limit	6.81×10^5	4.83×10^7	1.8	1.3
Not inhibited ($n = 65$)	Mean	5.14×10^5	3.56×10^7	1.3	1.0
	95% CI – lower limit	3.88×10^5	2.63×10^7	1.0	0.7

The removal of matched pairs of total and free-phase concentrations including at least one qPCR non-detect yielded 32 pairs for comparison of settleable fraction estimates via Colilert and qPCR. Interestingly, the average settleable fraction (15–20%) was statistically equivalent (Wilcoxon, $p < 0.05$) for values observed via Colilert or qPCR, and within ranges of particle association observed by previous studies (Cizek *et al.* 2008; Krometis *et al.* 2010). While this may appear to indicate that bacterial transport predictions would be equivalent regardless of the monitoring method used, it is important to note that the range of settleable fractions indicated by the qPCR data was much larger than the range reported via the Colilert data; very few of the paired data points lie along the 1:1 line of equivalency in Figure 2. In particular, in two-thirds of the samples, the qPCR-observed free-phase concentration actually exceeded the total concentration (i.e. zero percent settleable; will lie along the *x*-axis). This suggests that further investigations to distinguish between the relative impact of different sources of bias between culture-based and molecular observations of FIB are critical to better inform regulatory monitoring programs that often rely on predictive water quality models in planning watershed remediation efforts.

CONCLUSIONS

Measures of total *E. coli* concentration were statistically equivalent regardless of analytical technique, suggesting that water quality monitoring programs may reach similar impairment status decisions when using either Colilert culture or qPCR targeting the *uidA* gene. However, a small number of samples were not detectable via qPCR, likely as a result of inhibition by large quantities of sediment and organic material. Difficulties associated with concentrating sufficient volumes to meaningfully assess water quality with respect to regulatory limits without simultaneously concentrating inhibitors has been cited as a major technical challenge limiting the application of molecular methods to surface water matrices and stormwater assessment approaches (Noble & Weisberg 2005; Dorevitch *et al.* 2010; Jofre & Blanch 2010). Improvements in sample pre-concentration strategies and removal of sediment and particles, along with novel approaches to assess inhibition, will be required to ensure consistent and accurate microbial quantification.

Comparisons of *E. coli* concentrations in centrifuge-partitioned samples suggest the existence of a persistent source of analytical bias in the evaluation of free-phase numbers of *E. coli*. Efforts to determine whether differences in observations of free-phase *E. coli* concentrations stem from differences in cellular growth stage influencing culturability or persistent inhibitory compounds would be aided by new and developing methods to enhance detection (Green & Field 2012), quantify inhibition (Cao *et al.* 2012), and distinguish between culturable, VBNC, and lyzed cells (Malorny *et al.* 2004). Future studies investigating microbial partitioning behavior would benefit from the inclusion of separate controls to account for extraction efficiency and inhibition; the use of *O. keta* as a specimen processing complex in this study is potentially affected by both decreases in extraction by bead beating and inhibition by components of the sample matrix. Further investigation of detection bias due to inhibition could also be achieved through the spiking of environmental samples with the intended target (e.g. *E. coli*) to determine target-specific recovery, or through the addition of a post-extraction 'washing' step that includes treatment with chemicals to reduce inhibitor concentrations.

Even though the potentially multiple and complex sources of bias between qPCR and culture-based measures of FIB in surface water samples remain unresolved, rapid molecular methods may have an immediate place in stormwater assessment and regulatory programs. Specifically, rapid qPCR-based methods can be used during storms to sample upstream and locate 'hot spots' of fecal contamination in a sanitary survey approach. Furthermore, qPCR-based approaches paired with culture-based methods may play an important role in assessment of stormwater best management practices, particularly in determining the relationships between pathogen and FIB occurrence and removal by partitioning.

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