
Characterization of fecal concentrations in human and other animal sources by physical, culture-based, and quantitative real-time PCR methods

Jared S. Ervin¹, Todd L. Russell², Blythe A. Layton³, Kevan M. Yamahara⁵, Dan Wang², Lauren M. Sassoubre², Yiping Cao³, Catherine A. Kelty⁴, Mano Sivaganesan⁴, Alexandria B. Boehm², Patricia A. Holden¹, Stephen B. Weisberg³ and Orin C. Shanks⁴

ABSTRACT

The characteristics of fecal sources, and the ways in which they are measured, can profoundly influence the interpretation of which sources are contaminating a body of water. Although feces from various hosts are known to differ in mass and composition, it is not well understood how those differences compare across fecal sources and how differences depend on characterization methods. This study investigated how nine different fecal characterization methods provide different measures of fecal concentration in water, and how results varied across twelve different fecal pollution sources. Sources investigated included chicken, cow, deer, dog, goose, gull, horse, human, pig, pigeon, septage and sewage. A composite fecal slurry was prepared for each source by mixing feces from 6 to 22 individual samples with artificial freshwater. Fecal

concentrations were estimated by physical (wet fecal mass added and total DNA mass extracted), culture-based (*E. coli* and enterococci by membrane filtration and defined substrate), and quantitative real-time PCR (Bacteroidales, *E. coli*, and enterococci) characterization methods. The characteristics of each composite fecal slurry and the relationships between physical, culture-based and qPCR-based characteristics varied within and among different fecal sources. An *in silico* exercise was performed to assess how different characterization methods can impact identification of the dominant fecal pollution source in a mixed source sample. A comparison of simulated 10:90 mixtures based on enterococci by defined substrate predicted a source reversal in 27% of all possible combinations, while mixtures based on *E. coli* membrane filtration resulted in a reversal 29% of the time. This potential for disagreement in minor or dominant source identification based

¹University of California, Earth Research Institute and Bren School of Environmental Science & Management, Santa Barbara, CA

²Stanford University, Department of Civil and Environmental Engineering, Environmental and Water Studies, Stanford, CA

³Southern California Coastal Water Research Project, Costa Mesa, CA

⁴US Environmental Protection Agency, National Risk Management Research Laboratory, Office of Research and Development, Cincinnati, OH

⁵Stanford University, Center for Ocean Solutions, Stanford, CA

on different methods of measurement represents an important challenge for water quality managers and researchers.

INTRODUCTION

The ability to identify and estimate the concentration of fecal material in an environmental sample is the cornerstone for many water quality monitoring and management applications (Noble *et al.* 2003, Shibata *et al.* 2004). Effective management requires not only an accurate estimate of the total fecal load, but also knowledge of the dominant source to appropriately focus mitigative actions (Meays *et al.* 2004, Field and Samadpour 2007). Fecal source identification through microbial source tracking (MST) allows prioritization of water systems presenting the greatest human health risk, which may differ depending on the dominant fecal pollution source (Soller *et al.* 2010). Fecal source identification can also play an important role in quantitative microbial risk assessment activities (Ashbolt *et al.* 2010, Shibata and Solo-Gabriele 2012).

Many fecal source identification applications involve attributing the amounts of different fecal pollution sources to the entire fecal load in a water sample. This is often done by calculating a relative fraction (or percentage) for each fecal source. However, the units of measure for fecal pollution sources, and thus how to report the proportion of contamination from a given source, are not standardized. Estimated fecal concentrations from many animal sources have been reported based on measures that used either physical (fecal mass or total DNA mass), cultivation (enumeration of cells on selective media; Wright *et al.* 2009, Farnleitner *et al.* 2010), or molecular techniques (Silkie and Nelson 2009, Shanks *et al.* 2010, Unno *et al.* 2010, Dubinsky *et al.* 2012, Kelty *et al.* 2012). However, across such studies it is clear that the use of different fecal source characterization methods can strongly affect the interpretation of contamination levels from any given source, including which source is dominant. Lack of standardization and the potential for conflicting interpretations based on different fecal source characterization measurements has led some researchers to compare multiple fecal characterization measurements from the same sample (Silkie and Nelson 2009, Farnleitner *et al.* 2010, Wang *et al.* 2010). The largest study to date compared four qPCR-based characterization methods across 21 fecal sources (Kelty *et al.* 2012) and reported substantial

differences in the relative abundances of the tested molecular measurements, indicating the need for a more comprehensive comparison of qPCR-based methods alongside traditional cultivation and physical units of measure.

The goal of this study is to determine how the interpretation of fecal concentration in water can change for 12 different fecal pollution sources when using either physical (wet fecal mass added and total DNA mass extracted), culture-based (*E. coli* and enterococci by membrane filtration and defined substrate), or qPCR (Bacteroidales, *E. coli*, and enterococci) characterization methods. Results confirm the importance of fecal source characterization method selection and illustrate the importance of the unit of measure for the interpretation of water quality data.

METHODS

Sample Collection and Preparation

Individual reference fecal pollution source samples were collected from cow (*Bos taurus*; n = 12), deer (*Odocoileus* spp.; n = 12), dog (*Canis lupus familiaris*; n = 12), horse (*Equus caballus*; n = 12), human (*Homo sapiens*; n = 12), pig (*Sus scrofa*; n = 12), chicken (*Gallus gallus*; n = 12), goose (*Branta Canadensis*; n = 14), gull (*Larus* spp.; n = 22), pigeon (*Columba* spp.; n = 12), primary influent sewage (n = 9), and septage (n = 6). Each reference sample type was collected from four different geographic regions in California (CA): Northern and Central CA, Los Angeles County, Orange County, and San Diego County. All fecal samples were collected shortly after deposition, except in the case of feral deer (time unknown). Mixtures of each fecal pollution source (composite source slurries) were prepared by adding wet mass portions of individual fecal samples to artificial freshwater as described (Boehm *et al.* 2013). The average mass of fecal material added to each composite source slurry preparation is shown in Table 1. Detailed sample collection information including geographic coordinates is reported in Supplemental Information (SI) Table SI-1; ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2013AnnualReport/ar13_555_566SI.pdf.

Fecal Source Concentration Measurements

The quantity of fecal pollution source material in each composite source slurry was estimated

Table 1. Average or mean fecal concentration estimates per 100 ml of composite source slurry measured by physical, culture-based, and qPCR methods.

Source	mg/100 ml	Log ₁₀ MPN/100 ml			Log ₁₀ CFU/100 ml			ng/100 ml			Log ₁₀ Copies/100 ml					
		WET-MASS		EC-DS	ENT-CFU		EC-CFU	DNA-MASS		ENT-qPCR		EC-qPCR	BAC-qPCR			
		Average	StDev	Posterior Mean	BCI	Posterior Mean	BCI	Posterior Mean	BCI	Mean	StDev	Posterior Mean	BCI	Posterior Mean	BCI	
Human	31.4	ND	7.61	(7.44-7.77)	4.14	(4.07-4.21)	7.45	(7.40-7.50)	927	88	5.02	(4.91-5.14)	7.96	(7.91-8.01)	8.18	(8.07-8.28)
Septage	ND ^a	ND	3.33	(3.18-3.48)	2.96	(2.84-3.07)	3.08	(3.00-3.15)	1454	45	5.25	(5.14-5.36)	4.59	(4.56-4.62)	6.33	(6.24-6.42)
Sewage	ND ^b	ND	2.47	(2.29-2.64)	2.65	(2.46-2.82)	3.23	(3.17-3.29)	408	90	3.9	(3.80-4.01)	3.99	(3.96-4.02)	5.38	(5.29-5.47)
Cow	175	ND	3.38	(3.21-3.54)	ND	ND	4.17	(4.10-4.24)	1890	49	4.23	(4.13-4.33)	5.52	(5.49-5.55)	7.97	(7.87-8.07)
Deer	21.8	ND	3.9	(3.57-4.18)	5.73	(5.58-5.87)	5.06	(4.98-5.13)	666	51	4.63	(4.53-4.75)	6.25	(6.21-6.29)	7.08	(6.98-7.17)
Dog	19.8	ND	5.91	(5.77-6.04)	7.55	(7.38-7.72)	6.31	(6.12-6.48)	1027	25	5.75	(5.63-5.88)	6.5	(6.46-6.53)	7.81	(7.71-7.91)
Horse	19	ND	2.94	(2.84-3.03)	2.86	(2.76-2.96)	3.78	(3.73-3.83)	691	22	3.97	(3.87-4.07)	3.9	(3.86-3.93)	7.32	(7.22-7.42)
Pig	52	ND	4.05	(3.91-4.18)	6.6	(6.44-6.75)	6.67	(6.55-6.79)	1113	51	5.24	(5.13-5.36)	6.89	(6.65-6.73)	7.47	(7.38-7.57)
Chicken	0.3	ND	3.15	(2.89-3.36)	3.09	(2.84-3.31)	4.15	(4.08-4.22)	160*	ND	4.6	(4.48-4.72)	5.94	(5.90-5.98)	5.21	(5.12-5.31)
Goose	151	ND	4.32	(4.16-4.48)	6.96	(6.85-7.06)	7.06	(6.98-7.14)	2772	87	5.6	(5.49-5.72)	8.35	(8.30-8.40)	5.46	(5.36-5.55)
Gull	1.3	ND	5.68	(5.52-5.83)	5.26	(5.20-5.32)	6.19	(6.12-6.25)	257	38	6.54	(6.37-6.73)	7.11	(7.06-7.16)	2.41*	ND
Pigeon	47.7	ND	6.97	(6.84-7.09)	6.22	(6.16-6.29)	7.7	(7.58-7.81)	197	9	6.02	(5.87-6.19)	6.9	(6.86-6.95)	2.41*	ND

ND = no data available; MPN = most probable number; CFU = colony forming units; "StDev" represents standard deviation; "BCI" indicates 95% Bayesian credible interval; a septage and sewage samples were added on a volume basis (septage = 0.516 mL/100mL, sewage = 0.029 mL/100mL); * denotes the reported value is equal to the lower limit of quantification (for DNA mass 1 ng/μl; for qPCR 10 copies/reaction). Concentrations were not normalized to a specific unit of measure in this table. Average or mean values are presented to allow easy normalization to method of interest.

using nine different methods of measurement including: 1) most probable number (MPN) of enterococci measured by defined substrate (ENT-DS; Enterolert, IDEXX Laboratories, Inc., Irvine, CA), 2) *E. coli* MPN measured by defined substrate (EC-DS; Colilert, IDEXX Laboratories, Inc., Irvine, CA), 3) colony forming units (CFU) of enterococci measured by membrane filtration (ENT-MF; EPA method 1600), 4) *E. coli* CFU measured by membrane filtration (EC-MF; EPA method 1603; American Public Health Association *et al.* 2005), 5) mean log₁₀ copies of Bacteroidales measured by GenBac3 qPCR (BAC-qPCR; Dick and Field 2004, Siefring *et al.* 2008), 6) mean log₁₀ copies of enterococci measured by Enterol qPCR (ENT-qPCR; Ludwig and Schleifer 2000, Siefring *et al.* 2008), 7) mean log₁₀ copies of *E. coli* measured by EC23S857 qPCR (EC-qPCR; Chern *et al.* 2011), 8) mass of total extracted DNA calculated from concentrations measured with a NanoDrop ND-1000 UV spectrophotometer (DNA-MASS; NanoDrop Technologies, Wilmington, DE), and 9) wet fecal mass added measured on Mettler Toledo NewClassic MS (Columbus, OH) and Ohaus Pioneer balances (Parsippany, NJ; WET-MASS). Culture-based measurements were made directly on composite source slurries, while DNA and qPCR-based measurements were made on total DNA extracted from filters of composite source slurries. Filter preparation is described by Boehm *et al.* (2013). WET-MASS was measured on all fecal samples prior to composite source slurry preparation.

qPCR Amplification

Three qPCR assays were used in this study including EC23S857, GenBac3 (BAC-qPCR), and multiplex Enterol (ENT-qPCR; Sivaganesan *et al.* 2008, Haugland *et al.* 2010), for *E. coli*, Bacteroidales, and enterococci, respectively. Thermal cycling was conducted using a 7900 HT real-time sequence detector (Life Technologies, Grand Island, NY). Simplex reaction mixtures contained 1x TaqMan universal master mix, 0.2 mg/ml bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 1 μm each primer, 80 nM FAM-labeled TaqMan probe (Life Technologies, Grand Island, NY), and fecal DNA extracts containing 1 to 5 ng total DNA or 10 to 10⁵ target copies (plasmid standard) in a total reaction volume

of 25 μ l. The multiplex Enterol reaction mixtures were prepared in the same manner except that 80 nM VIC-labeled UC1P1 TaqMan probe and 50 copies of an internal amplification control (IAC) template were added to the reaction mixture. Calibration curve and IAC DNA plasmid constructs were linearized by NotI restriction digestion (New England BioLabs, Beverly, MA), quantified with a NanoDrop ND-1000 UV spectrophotometer, and diluted in 10 mM Tris and 0.1 mM EDTA (pH 8.0) to generate 10, 100, 10³, 10⁴, and 10⁵ copies per 2 μ l dilutions, corresponding to the range of quantification (ROQ) for all three assays. All reactions were performed in triplicate. The thermal cycling conditions were 2 minutes at 95°C, followed by 40 cycles of 5 seconds at 95°C and 30 seconds at 60°C. Data were initially analyzed with Sequence Detector software (version 2.3.2; Life Technologies, Grand Island, NY) at a threshold determination of 0.03. Quantification cycle (C_q) values were exported to Microsoft Excel in preparation for further statistical analysis. Amplification efficiencies (E) were based on the following equation: $E = 10^{(-1/\text{slope})} - 1$. The lower limit of quantification (LLOQ) for each assay was determined based on the average C_q value measured for the lowest concentration standard within the ROQ. To monitor for potential sources of extraneous DNA during laboratory analysis, 3 no-template and 6 extraction blank amplifications with purified water substituted for template DNA were performed for each 96-well instrument run.

DNA Isolation Efficiency and Amplification Interference

For each test sample filter, the efficiency of DNA isolation was estimated using a salmon testes DNA control and subsequent amplification with the Sketa22 qPCR assay as previously described (Haugland *et al.* 2010). The DNA isolation acceptance threshold was defined as any test sample filter DNA extract with a Sketa22 C_q that differed from a control mean $C_q \pm 3$. This threshold was determined from repeated control experiments where laboratory grade water was substituted for artificial freshwater. An IAC template designed to evaluate the suitability of isolated DNA for qPCR amplification was performed on each test sample DNA extract with the Enterol multiplex qPCR assay. Inhibition criterion was based on repeated experiments measuring the mean C_q of a 50-copy IAC spike in buffer only. The threshold for inhibition was defined as any observed

IAC C_q value in a test sample DNA extract greater than the control mean $C_q + 1$. Any DNA extract or amplification reaction failing the above criteria was discarded from the study.

Quality Assurance and Controls

Standard deviations of triplicate DNA-MASS measurements did not exceed 0.56 ng/ μ l. For qPCR data, master calibration models were high quality ($R^2 \geq 0.98$, $E \geq 0.95$), all amplification reactions exhibited no inhibition based on IAC reactions (control mean $C_q 32.75 \pm 1$), all DNA extracts yielded acceptable DNA isolation efficiencies based on Sketa22 results (control mean $C_q 25.93 \pm 3$), and no extraneous DNA contamination was detected within the range of quantification for any of the qPCR assays ($n = 36$ control reactions). The ROQ for all three qPCR assays was from 10 to 10⁵ copies per reaction, and calculated LLOQs were 7.7, 8.1, and 9.2 copies per reaction for the ENT-qPCR, BAC-qPCR, and EC-qPCR assays, respectively.

Data Normalization to WET-MASS, ENT-DS, and EC-MF

To demonstrate how fecal concentrations measured in composite source slurries varied across different fecal pollution sources and methods of measurement, the measured fecal concentrations of each composite source slurry were normalized to milligrams wet mass, enterococci as measured by ENT-DS, and *E. coli* as measured by EC-MF. Normalization was necessary because composite fecal slurries were prepared at different concentrations regardless of method of measurement across different fecal pollution sources. Normalization of data to WET-MASS was selected to illustrate how different measurement methods across fecal pollution sources compare to estimated fresh (except deer feces, which had an unknown age, as previously described) fecal loads. Septage and sewage pollution sources could not be included in WET-MASS normalizations due to their addition to the composite source slurries on a volume basis rather than a mass basis. The ENT-DS and EC-MF methods of measurement were selected for normalization because a complete data set was available for all 12 fecal pollution sources, and culture-based measurements of enterococci and *E. coli* are commonly used in regulated water quality monitoring. All data normalization calculations assumed that the fecal concentration ratio between methods of

measurement remains constant within each fecal pollution source when diluted or concentrated.

***In Silico* Simulation of Mixed Source Samples**

An *in silico* exercise was conducted to compare the relative proportions of two fecal pollution sources mixed at 10:90 ratios based on initial concentrations measured by either ENT-DS or EC-MF, two of the most common methods used for regulatory water quality monitoring worldwide. WET-MASS normalization was not included in this analysis due to: 1) the lack of a complete data set across all 12 pollution sources and 2) WET-MASS measurements are not currently used in any regulatory application. Data were generated from a computer simulation based on the raw composite source slurry concentration measurements reported in Table 1. All possible combinations of two fecal pollution sources were estimated with each source representing either a 90% (dominant source) or 10% (minor source) proportion of the total ENT-DS or EC-MF fecal concentration in a sample. For each measurement method, there were 132 possible simulated combinations of two sources. However, concentration measurements were not available for every fecal pollution source across all methods (Table 1). This resulted in a total of 950 *in silico* source combinations for the 8 methods being compared to the initial 10:90 ratios determined by ENT-DS or EC-MF.

To investigate the potential influence different methods of measurement can have on a particular fecal pollution source, the frequency of “dominant source reversals” and “minor source reversals” were determined for each method of measurement when 10:90 ratios were initially defined by either ENT-DS or EC-MF. A “dominant source reversal” was recorded when the source initially at the 90% proportion in a sample as measured by ENT-DS or EC-MF, “shifted” to a <50% proportion based on predicted concentrations from another method of measurement. A “minor source reversal” occurred when the initial ENT-DS or EC-MF 10% pollution source “shifted” above 50%. “Dominant” and “minor” source reversal frequencies were tabulated by summing the appropriate number of “shift” events and dividing by the respective total number of possible combinations.

Unlike the comparison of source reversals by a fecal pollution source, it was not necessary to distinguish between “dominant” and “minor” source reversals for comparisons by method of measurement. Therefore, a frequency of “source reversals”

for each method of measurement was calculated by summing the number of times the initial minor source (10%) defined by either ENT-DS or EC-MF “shifted” to a >50% proportion based on predicted concentrations from another method across all available fecal pollution source combinations, and then dividing the number of “source reversals” for each method of measurement by the respective total number of possible fecal pollution source combinations.

Statistical Analysis

Log₁₀ MPN/100 ml posterior means and Bayesian credible intervals were determined using a Monte Carlo Markov Chain approach (Sivaganesan *et al.* 2011), and CFU/100 ml posterior means and Bayesian credible intervals were determined using a similar approach. The qPCR master calibration curves, mean log₁₀ copy estimates, posterior means, and Bayesian credible intervals were also determined using a Monte Carlo Markov Chain approach (Sivaganesan *et al.* 2008). Simple statistics including linear regressions were calculated with SAS software (Cary, NC) and Microsoft Excel (Redmond, WA).

RESULTS

Comparison of Fecal Concentrations by Different Measurement Methods

Estimates of fecal pollution source concentration in composite source slurries for nine different measurement methods are reported in Table 1. Concentrations were not normalized to a specific unit of measure, instead average or mean values are presented to allow easy normalization to any method of interest. Pearson correlation coefficients (r) based on a linear regression between each pair of source characterization methods ranged from 0.01 to 0.96 (Table 2). The least correlated metrics were seen when comparing WET-MASS and DNA-MASS to culture-based and qPCR units of measure. These correlations were all below r = 0.40 except for between DNA-MASS and BAC-qPCR, which showed a significant correlation at the 5% significance level (p = 0.02; r = 0.66). WET-MASS and DNA-MASS also showed a significant correlation to each other (p = 0.01; r = 0.78). The most highly correlated metrics were between culture-based methods targeting the same bacterial group (p <0.01; r = 0.96 and 0.95 for enterococci and *E. coli*, respectively), however all the cultivation methods showed a significant correlation with each other at the 5% significance level (p <0.05; r

Table 2. Correlation of fecal concentration estimates by different methods of measurement.

Method	ENT-DS	EC-DS	ENT-MF	EC-MF	DNA-MASS	ENT-qPCR	EC-qPCR	BAC-qPCR
WET-MASS	0.06	0.32	0.08	0.31	0.78	0.12	0.16	0.4
	-10	-9	-8	-10	-10	-10	-10	-10
ENT-DS		0.86	0.96	0.82	0.26	0.82	0.65	0.46
		-11	-10	-12	-12	-12	-12	-12
EC-DS			0.74	0.95	0.02	0.71	0.89	0.16
			-10	-11	-11	-11	-11	-11
ENT-MF				0.63	0.25	0.75	0.41	0.31
				-10	-10	-10	-10	-10
EC-MF					0.01	0.69	0.9	0.2
					-12	-12	-12	-12
DNA-MASS						0.12	0.12	0.66
						-12	-12	-12
ENT-qPCR							0.68	0.55
							-12	-12
EC-qPCR								0.16
								-12

>0.63). Methods measuring enterococci and *E. coli* by qPCR also correlated significantly to culture methods targeting the same bacterial group ($p < 0.05$; $r = 0.75$ to 0.90). BAC-qPCR did not correlate significantly ($p > 0.05$) with any of the other culture or qPCR metrics.

Comparison of Fecal Pollution Source Concentrations Normalized to WET-MASS, ENT-DS, and EC-MF

Since the estimated concentration of a fecal pollution source depended greatly on method of measurement (Table 1), it is important to consider how this variability could affect interpretations of water quality. To estimate, original laboratory measured fecal concentrations in composite source slurries (Table 1) were normalized to WET-MASS, ENT-DS, and EC-MF (Figure 1), three methods of measurement commonly employed for water quality management applications. For the WET-MASS normalized data (Figure 1a), the lowest relative quantity of fecal material was observed in pigeon as measured by BAC-qPCR ($0.7 \log_{10}$ copies/mg), while the highest relative quantity was observed in gull as measured by EC-qPCR ($7.0 \log_{10}$ copies/mg), representing a six order of magnitude shift in estimated mass. For both ENT-DS normalized data (Figure 1b) and EC-MF normalized data (Figure 1c), the lowest relative quantity of fecal material was observed in pigeon as measured by BAC-qPCR ($-4.6 \log_{10}$ copies/MPN enterococci; $-5.3 \log_{10}$ copies/CFU *E. coli*), while the highest relative quantity was observed in

cow, also with the BAC-qPCR measurement ($4.6 \log_{10}$ copies/MPN enterococci; $3.8 \log_{10}$ copies per CFU *E. coli*). This represents a nine order of magnitude difference in estimated DNA target copies of Bacteroidales across fecal sources when normalized with either ENT-DS or EC-MF.

In order to further investigate the variability of each measurement method across different fecal pollution sources, the \log_{10} standard deviation for each method was calculated based on the data normalized to WET-MASS, ENT-DS, and EC-MF. For the WET-MASS normalized data, standard deviations ranged from $0.6 \log_{10}$ ng/mg (DNA-MASS) to $2.0 \log_{10}$ copies/mg (BAC-qPCR). Standard deviations ranged from $0.9 \log_{10}$ copies/MPN enterococci (ENT-qPCR) to $2.9 \log_{10}$ copies/MPN enterococci (BAC-qPCR) for the ENT-DS normalized data. For the EC-MF normalized data, standard deviations ranged from $0.8 \log_{10}$ copies/CFU *E. coli* (EC-qPCR) to $2.9 \log_{10}$ copies/CFU *E. coli* (BAC-qPCR). Under all three data normalization schemes investigated, the largest standard deviation was observed for the BAC-qPCR method of measurement.

Influence of Method of Measurement on Identification of the Dominant Source of Fecal Pollution in a Mixed Source Sample

An *in silico* analysis of samples containing simulated mixtures of two different fecal pollution sources combined at a 10:90 ratio based on both

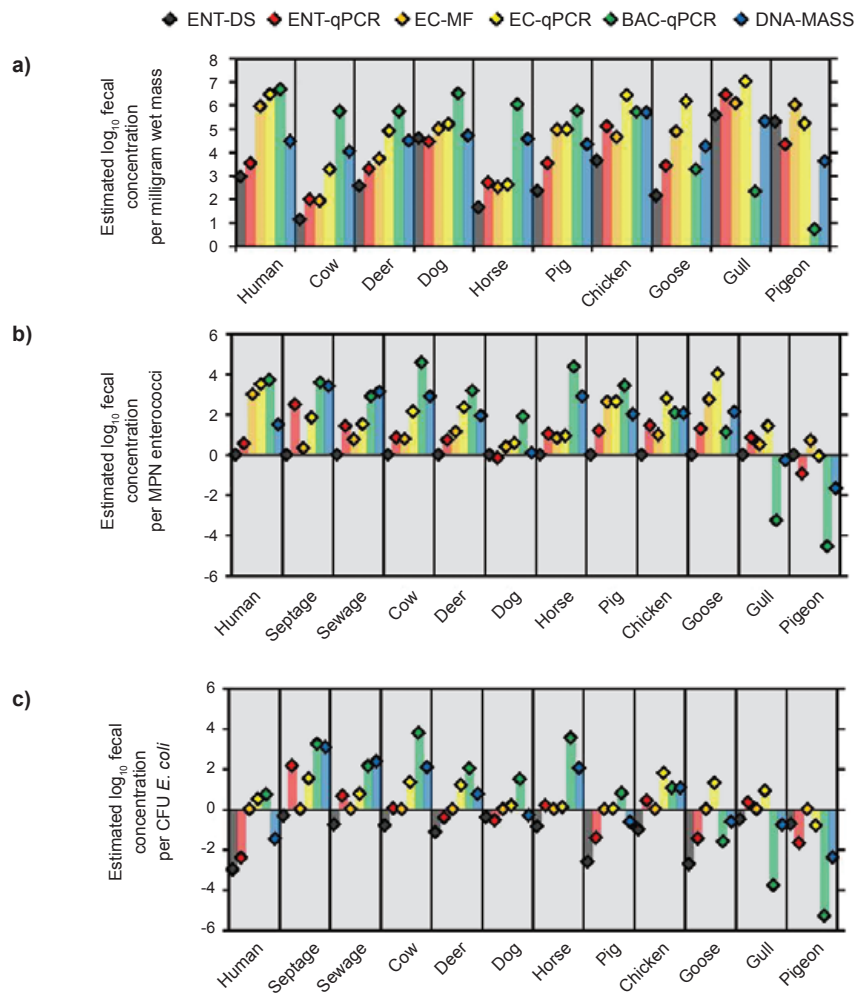


Figure 1. Fecal pollution source concentrations normalized to milligrams wet mass (WET-MASS; a), MPN enterococci (ENT-DS; b), and CFU *E. coli* (EC-MF; c). Each colored bar corresponds to a different method of measuring fecal concentration, and columns show the estimated concentrations for each fecal pollution source

ENT-DS and EC-MF measurements was completed for all available pollution source combinations and methods of measurement. A total of 950 source combinations were compared for each analysis resulting in 252 (26.5%) instances of source reversal for ENT-DS and 280 (29.5%) for EC-MF (Table 3). The frequency of source reversals was most prevalent when compared to BAC-qPCR for both ENT-DS (36.4%) and EC-MF (40.9%). As expected, the lowest occurrences of source reversal were observed between culture methods measuring the same bacterial groups, ENT-MF (6.7%) and EC-DS (17.3%) for the ENT-DS and EC-MF analyses, respectively.

The *in silico* analysis also allowed comparisons of the frequency of minor and dominant source reversals for each fecal pollution source (Table 4). Dominant source reversals occurred when the initial ENT-DS or EC-MF 90% pollution source “shifted” below 50% based on simulated estimates from another method

of measurement. A minor source reversal occurred when the initial ENT-DS or EC-MF 10% pollution source “shifted” above 50%. For the ENT-DS based simulations, pigeon exhibited the largest percentage (68.7%) of dominant source reversal. In other words, a mixed sample with pigeon making up 90% of the fecal concentration as measured by ENT-DS represented less than 50% of the fecal concentration in 68.7% of simulations across all other methods of measurement. For the EC-MF based analysis, the proportion of samples expected to result in a minor source reversal was greatest for the septage (59.5%) fecal pollution source.

DISCUSSION

Dependence of Fecal Pollution Source Concentration Estimates on Method Selection

Estimated fecal pollution source concentrations measured by nine different methods for a collection

Table 3. Percentages of fecal pollution source reversal predictions across all pollution sources by method of measurement.

Method	% Source Reversal	
	10:90 ENT-DS	10:90 EC-MF
ENT-DS		20.5
ENT-MF	6.7	28.9
ENT-qPCR	20.5	31.8
EC-DS	28.2	
EC-MF	20.5	
EC-qPCR	29.5	20.5
BAC-qPCR	36.4	40.9
DNA-MASS	32.6	38.6
WET-MASS	34.4	37.8
Total	26.5	29.5

10:90 ENT-DS indicates initial minor (10%) and dominant (90%) pollution sources based on enterococci by defined substrate measurements. 10:90 EC-MF refers to minor (10%) and dominant (90%) pollution sources based on *E. coli* by membrane filtration measurements. A " " denotes no data available because measurement method used for normalization.

of 12 reference fecal pollution sources indicates that measured quantities can vary by more than five orders of magnitude in the same sample depending on the measurement method selected, regardless of the normalization method used. While the quantities of different groups of indicator bacteria are expected to differ in a given fecal pollution source, the magnitude and variability of these differences across fecal pollution sources and methods of measurement were immense. Differences between cultivation and qPCR methods targeting the same bacterial group were expected and well documented based on the physical and biological properties of cells compared to nucleic acids (Converse *et al.* 2012a, Kelty *et al.* 2012). In addition to physical and biological properties, the protocols that are used to isolate cells and nucleic acids are vastly different for each method of measurement. Culture-based methods rely on enrichment in selective media that may allow growth of non-target organisms or lead to cell aggregation (Sercu *et al.* 2011), while DNA-based methods rely on cell lysis and DNA purification to isolate DNA that are rarely 100% efficient (Mumy and Findlay 2004). The comparison of enterococci and *E. coli* measurements by culture-based (defined substrate and membrane filtration) and qPCR-based (entero1 and EC23S857, respectively) methods further confirms the disparity between these methods of measurement.

Other strategies commonly used to standardize fecal concentration measurements include physical

Table 4. Percentages of minor and dominant source reversal predictions across methods of measurement by fecal pollution source.

Fecal Pollution Source	% Source Reversal			
	10:90 ENT-DS		10:90 EC-MF	
	Minor	Dominant	Minor	Dominant
Human	43.40%	8.40%	6.00%	57.80%
Septage	37.80%	16.20%	59.50%	2.70%
Sewage	21.60%	21.60%	36.50%	9.50%
Cow	42.20%	9.40%	56.30%	3.10%
Deer	24.10%	16.90%	41.00%	15.70%
Dog	13.30%	50.60%	31.30%	24.10%
Horse	28.40%	23.00%	43.20%	18.90%
Pig	39.80%	9.60%	9.60%	53.00%
Chicken	21.70%	21.70%	43.40%	16.90%
Goose	43.40%	16.90%	13.30%	51.80%
Gull	6.00%	49.40%	16.90%	31.30%
Pigeon	1.20%	68.70%	8.40%	56.60%

10:90 ENT-DS indicates initial minor (10%) and dominant (90%) pollution sources based on enterococci by defined substrate measurements. 10:90 EC-MF refers to minor (10%) and dominant (90%) pollution sources based on *E. coli* by membrane filtration measurements. "Minor" indicates initial fecal pollution source of 10% shifted to >50% and "Dominant" refers to initial fecal pollution source of 90% shifted to <50%.

WET-MASS of the sample (Anderson *et al.* 1997, Okabe *et al.* 2007, Silkie and Nelson 2009) and DNA-MASS isolated from a sample (Kelty *et al.* 2012). While WET-MASS and DNA-MASS measurements correlated well (Table 2), it was evident that these measurements can vary dramatically from one animal source to another. These differences are most likely due to differences in the diet (presence of undigested materials) and digestive physiology of the animal sources tested in this study. In addition, WET-MASS will vary from one animal to another depending on animal age, disease state, and fecal moisture content, as well as laboratory sample collection procedures such as fecal sample handling and storage conditions. Less is known about the variability in DNA-MASS across different fecal pollution sources. DNA-MASS measured by NanoDrop reflects all DNA and RNA molecules from not only bacteria, but also from any eukaryotic cells that slough off during defecation or that survive the digestive process. Regardless of cause, the dramatic differences observed in fecal pollution source concentration estimates emphasize a need to critically evaluate the influence that different methods of measurement have on water quality applications and subsequent management decisions.

Trends across Different Fecal Pollution Source Types

A number of trends emerged from the *in silico* analysis designed to characterize the influence of methods of measure on determination of dominant and minor sources of fecal pollution in samples containing 10:90 ratios of two fecal pollution sources initially defined by either ENT-DS or EC-MF. First, it is important to note that defined substrate and membrane filtration culture-based measurements for enterococci ($r = 0.96$), as well as *E. coli* ($r = 0.95$) were highly correlated strongly suggesting that patterns observed with defined substrate approaches will be similar with membrane filtration measurements for indicator bacteria within the same genus. Perhaps the most significant trend was observed when the human fecal pollution source was represented in the initial dominant source defined by a culture-based measurement of enterococci. In this scenario, alternative methods of measure rarely underestimated the human contribution (8.4%), but when the human contribution was initially defined by *E. coli* culture methods, there was a much higher proportion (57.8%) of simulated samples that underestimated human contribution by other measurement methods (Table 4). The apparent contradiction between enterococci and *E. coli* cultivation methods when human is either the minor or dominant fecal source of pollution in a mixed sample suggests that water quality managers or researchers seeking to link current culture-based water quality standards and alternative methods of measurement must not only consider the different types of fecal pollution sources present, but also the expected relative concentration of the human fecal pollution source contribution. In addition, discrepancies in estimated human fecal source contribution and method of measurement selection could lead municipalities to invest resources into remediation efforts focused on the wrong fecal pollution source.

Another potentially important trend was observed when the cow fecal pollution source was the initial minor source. Regardless if enterococci or *E. coli* cultivation methods were used to establish the initial cattle fecal pollution contribution, there was a greater than 42% occurrence of minor source reversal (Table 4) suggesting that alternative methods of measurement may frequently lead to overestimations of cattle fecal pollution, especially when the relative contribution of cow fecal pollution is low.

Finally, birds such as gulls and pigeons, which are commonly associated with fecal contamination

at many beaches (Kitts *et al.* 2010, Converse *et al.* 2012b), may also present challenges to managers and researchers hoping to combine culturable enterococci with other methods of measurement. When either of these sources represented the dominant proportion, the frequency of dominant source reversal based on other methods of measurement ranged from approximately 50 to 69% (Table 4). This same trend was also observed when dog was the initial dominant source (50.6%), consistent with other studies that reported higher concentrations of enterococci in dogs compared to many other animal fecal sources (Wright *et al.* 2009, Kely *et al.* 2012). Interestingly, this trend was not detected in chickens or geese, two avian species not commonly associated with marine waters.

Implications for Watershed Management

Study findings may have serious implications for watershed management practices that rely on the estimation of fecal pollution concentrations. For example, regulated water quality monitoring for recreational or aquaculture use is conventionally employed using cultivation measurements of *E. coli* or enterococci. While there was a high correlation between culture-based measurements (Table 2), the concentration of these indicators varied dramatically across different fecal pollution sources (Figure 1). These large differences suggest that waters impacted by different pollution sources or mixtures of sources will exceed water quality criteria at different rates. Poor correlations between culture-based measurements and fecal WET-MASS also presents challenges for managers seeking to link regulated fecal indicator criteria to fecal load mass estimates, especially for enterococci culture-based measurements ($r \leq 0.08$).

Water quality managers and researchers using the methods of fecal characterization highlighted in this study can address these issues in several ways. First, careful attention to method of measurement and potential fecal pollution sources in a watershed or beach of interest is paramount. Useful clues about these types of information can often be obtained via a thorough sanitary survey. By knowing which fecal pollution sources may be present, it may be possible to select methods with the smallest disparity between units of measure and potential fecal pollution sources, thus reducing potential bias in interpretations. Secondly, caution is recommended for fecal source apportionment with MST, fecal indicator transport modeling, or other applications where it

is critical to link data generated from two or more different measurement methods. Data in this study suggest that there is often a poor correlation between different methods of measurement used on the same samples and that estimated fecal source pollution estimates can vary by as much as five orders of magnitude. Third, it is important to clearly define the intended use of a particular method of measurement. For example, measurements of fecal concentration such as cultivation of enterococci or *E. coli* are commonly used to predict the associated public health risk due to exposure during water recreational activities. For this application, it is not important how one method of measurement compares to another in terms of physical, culture-based, or genetic concentration estimates, but how different methods of measure compare to predicted health risk. Finally, it will be important for researchers that introduce new methods of measurement for fecal concentration characterization to not only clearly define the intended use, but also report the range of variability associated with other measurement methods and fecal pollution sources of interest.

Summary

There are many methods available to estimate the concentration of fecal material in a water sample, including those that rely on physical, culture-based, and genetic measurements of fecal microorganisms. In this study, nine different methods for measuring fecal concentrations were compared for 12 different sources of fecal pollution. Important implications from the results are listed below:

- Fecal concentrations can vary dramatically across pollution sources and methods of measurement ranging up to nine orders of magnitude.
- Regardless of normalization method (WET-MASS, ENT-DS, or EC-MF), the largest differences across pollution sources and methods of measurement were observed between culture-based enterococci (ENT-DS and ENT-MF) and qPCR-based Bacteroidales (BAC-qPCR) methods, both of which are commonly used in water quality diagnosis.
- Method of measurement used to estimate fecal concentrations can greatly influence the interpretation of the minor and dominant pollution sources when more than one fecal pollution source is present.

- Managers and researchers must carefully consider the impact that a measurement method will have on a particular application, for example fecal pollution source allocation with microbial source tracking, when multiple sources of fecal pollution are present.

Future research is needed to determine if the trends observed in this study are consistent for fecal samples collected from regions outside of California, as well as how other fecal pollution sources of interest compare. It is also important to note that fresh fecal material was used in this study. Additional research is needed to characterize how the fate and transport of fecal pollution sources once discharged into the environment affect the relationship between different methods of measurement and fecal pollution sources.

LITERATURE CITED

- American Public Health Association, American Water Works Association, and Water Environment Federation. 2005. Standard Methods for the Examination of Water and Wastewater. American Public Health Association. Washington, DC.
- Anderson, S.A., S.J. Turner and G.D. Lewis. 1997. Enterococci in the New Zealand environment: Implications for water quality monitoring. *Water Science and Technology* 35:325-331.
- Ashbolt, N.J., M.E. Schoen, J.A. Soller and D.J. Roser. 2010. Predicting pathogen risks to aid beach management: the real value of quantitative microbial risk assessment (QMRA). *Water Research* 44:4692-703.
- Boehm, A.B., L.C. Van De Werfhorst, J.F. Griffith, P.A. Holden, J.A. Jay, O.C. Shanks, D. Wang and S.B. Weisberg. 2013. Performance of forty-one microbial source tracking methods: a twenty-seven lab evaluation study. *Water Research* 47:6812-6828.
- Chern, E.C., S. Siefring, J. Paar, M. Doolittle and R.A. Haugland. 2011. Comparison of quantitative PCR assays for *Escherichia coli* targeting ribosomal RNA and single copy genes. *Letters in Applied Microbiology* 52:298-306.
- Converse, R.R., J.F. Griffith, R.T. Noble, R.A. Haugland, K.C. Schiff and S.B. Weisberg. 2012a. Correlation between quantitative PCR and culture-based methods for measuring *Enterococcus* spp. over

- various temporal scales at three California marine beaches. *Applied and Environmental Microbiology* 78:1237-42.
- Converse, R.R., J.L. Kinzelman, E.A. Sams, E. Hudgens, A.P. Dufour, H. Ryu, J.W. Santo Domingo, C.A. Kelty, O.C., Shanks, S.D. Siefring, R.A. Haugland and T.J. Wade. 2012b. Dramatic improvements in beach water quality following gull removal. *Environmental Science & Technology* 46:10206-10213.
- Dick, L. and K. Field. 2004. Rapid estimation of numbers of fecal Bacteroidetes by use of a quantitative PCR assay for 16S rRNA genes. *Applied and Environmental Microbiology* 70:5695-5697.
- Dubinsky, E.A., L. Esmaili, J.R. Hulls, Y. Cao, J.F. Griffith and G.L. Andersen. 2012. Application of phylogenetic microarray analysis to discriminate sources of fecal pollution. *Environmental Science & Technology* 46:4340-4347.
- Farnleitner, A.H., G. Ryzinska-Paier, G.H. Reischer, M.M. Burtscher, S. Knetsch, A.K.T. Kirschner, T. Dirnböck, G. Kuschnig, R.L. Mach and R. Sommer. 2010. *Escherichia coli* and enterococci are sensitive and reliable indicators for human, livestock and wildlife faecal pollution in alpine mountainous water resources. *Journal of Applied Microbiology* 109:1599-1608.
- Field, K.G. and M. Samadpour. 2007. Fecal source tracking, the indicator paradigm, and managing water quality. *Water Research* 41:3517-38.
- Haugland, R.A., M. Varma, M. Sivaganesan, C. Kelty, L. Peed and O.C. Shanks. 2010. Evaluation of genetic markers from the 16S rRNA gene V2 region for use in quantitative detection of selected Bacteroidales species and human fecal waste by qPCR. *Systematic and Applied Microbiology* 33:348-57.
- Kelty, C.A., M. Varma, M. Sivaganesan, R.A. Haugland and O.C. Shanks. 2012. Distribution of genetic marker concentrations for fecal indicator bacteria in sewage and animal feces. *Applied and Environmental Microbiology* 78:4225-32.
- Kitts, C.L., M.W. Black, M.M. Moline, M. Yeung, A.K. Hamrick, I.C. Robbins, A.A. Schaffner and N.I. Boutet. 2010. Pismo Beach Fecal Contamination Source Identification Study: Final Report. Cal Poly. San Luis Obispo, CA.
- Ludwig, W. and K. Schleifer. 2000. How quantitative is quantitative PCR with respect to cell counts? *Systematic and Applied Microbiology* 23:556-562.
- Meays, C.L., K. Broersma, R. Nordin and A. Mazumder. 2004. Source tracking fecal bacteria in water: A critical review of current methods. *Journal of Environmental Management* 73:71-9.
- Mumy, K.L. and R.H. Findlay. 2004. Convenient determination of DNA extraction efficiency using an external DNA recovery standard and quantitative-competitive PCR. *Journal of Microbiological Methods* 57:259-68.
- Noble, R.T., D.F. Moore, M.K. Leecaster, C.D. McGee and S.B. Weisberg. 2003. Comparison of total coliform, fecal coliform, and *enterococcus* bacterial indicator response for ocean recreational water quality testing. *Water Research* 37:1637-43.
- Okabe, S., N. Okayama, O. Savichtcheva and T. Ito. 2007. Quantification of host-specific Bacteroides-Prevotella 16S rRNA genetic markers for assessment of fecal pollution in freshwater. *Applied Microbiology and Biotechnology* 74:890-901.
- Sercu, B., L.C. Van De Werfhorst, J.L.S. Murray and P.A. Holden. 2011. Cultivation-independent analysis of bacteria in IDEXX Quanti-Tray/2000 fecal indicator assays. *Applied and Environmental Microbiology* 77:627-33.
- Shanks, O.C., K. White, C.A. Kelty, M. Sivaganesan, J. Blannon, M. Meckes, M. Varma and R.A. Haugland. 2010. Performance of PCR-based assays targeting Bacteroidales genetic markers of human fecal pollution in sewage and fecal samples. *Environmental Science & Technology* 44:6281-8.
- Shibata, T. and H.M. Solo-Gabriele. 2012. Quantitative microbial risk assessment of human illness from exposure to marine beach sand. *Environmental Science & Technology* 46:2799-805.
- Shibata, T., H.M. Solo-Gabriele, L.E. Fleming and S. Elmir. 2004. Monitoring marine recreational water quality using multiple microbial indicators in an urban tropical environment. *Water Research* 38:3119-31.

Siefring, S., M. Varma, E. Atikovic, L. Wymer and R.A. Haugland. 2008. Improved real-time PCR assays for the detection of fecal indicator bacteria in surface waters with different instrument and reagent systems. *Journal of Water and Health* 6:225-37.

Silkie, S.S. and K.L. Nelson. 2009. Concentrations of host-specific and generic fecal markers measured by quantitative PCR in raw sewage and fresh animal feces. *Water Research* 43:4860-71.

Sivaganesan, M., S. Seifring, M. Varma, R.A. Haugland and O.C. Shanks. 2008. A Bayesian method for calculating real-time quantitative PCR calibration curves using absolute plasmid DNA standards. *BMC Bioinformatics* 9:120.

Sivaganesan, M., S. Siefring, M. Varma and R.A. Haugland. 2011. MPN estimation of qPCR target sequence recoveries from whole cell calibrator samples. *Journal of Microbiological Methods* 87:343-9.

Soller, J.A., M.E. Schoen, T. Bartrand, J.E. Ravenscroft and N.J. Ashbolt. 2010. Estimated human health risks from exposure to recreational waters impacted by human and non-human sources of faecal contamination. *Water Research* 44:4674-4691.

Unno, T., J. Jang, D. Han, J.H. Kim, M.J. Sadowsky, O.-S. Kim, J. Chun and H.-G. Hur. 2010. Use of barcoded pyrosequencing and shared OTUs to determine sources of fecal bacteria in watersheds. *Environmental Science & Technology* 44:7777-7782.

Wang, D., S.S. Silkie, K.L. Nelson and S. Wuertz. 2010. Estimating true human and animal host source contribution in quantitative microbial source tracking using the Monte Carlo method. *Water Research* 44:4760-4775.

Wright, M.E., H.M. Solo-Gabriele, S. Elmir and L.E. Fleming. 2009. Microbial load from animal feces at a recreational beach. *Marine Pollution Bulletin* 58:1649-56.

ACKNOWLEDGEMENTS

Funding for this project has been provided in part through an agreement with the California State Water Resources Control Board. The contents of this document do not necessarily reflect the views and policies of the California State Water Resources Control Board, nor does mention of trade names

or commercial products constitute endorsement or recommendation for use. The authors would also like to acknowledge all of those involved in the Source Identification Protocol Project (SIPP). Information has been subjected to the US Environmental Protection Agency (USEPA) peer and administrative review and has been approved for external publication. Any opinions expressed in this paper are those of the author(s) and do not necessarily reflect the official positions and policies of the USEPA. Any mention of trade names or commercial products does not constitute endorsement or recommendation for use.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2013AnnualReport/ar13_555_566SI.pdf.