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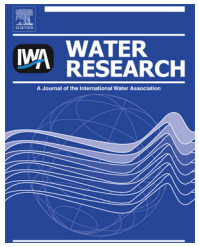

Characterization of fecal concentrations in human and other animal sources by physical, culture-based, and quantitative real-time PCR methods

Jared S. Ervin [a], *Todd L. Russell* [b], *Blythe A. Layton* [c], *Kevan M. Yamahara* [e], *Dan Wang* [b], *Lauren M. Sassoubre* [b], *Yiping Cao* [c], *Catherine A. Kelty* [d], *Mano Sivaganesan* [d], *Alexandria B. Boehm* [b], *Patricia A. Holden* [a], *Stephen B. Weisberg* [c], *Orin C. Shanks* [d,*]

[a] *Earth Research Institute and Bren School of Environmental Science & Management, University of California, Santa Barbara, CA 93106, USA*

[b] *Environmental and Water Studies, Department of Civil and Environmental Engineering, Stanford University, Stanford, CA 94305, USA*

[c] *Southern California Coastal Water Research Project, Costa Mesa, CA 92626, USA*

[d] *U.S. Environmental Protection Agency, Office of Research and Development, National Risk Management Research Laboratory, 26 West Martin Luther King Drive, Cincinnati, OH 45268, USA*

[e] *Center for Ocean Solutions, Stanford University, Stanford, CA 94305, USA*

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

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* *Corresponding author*. Tel.: +1 513 569 7314; fax: +1 513 569 7328.
E-mail address: shanks.orin@epa.gov (O.C. Shanks).

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Abbreviations

BAC-qPCR Bacteroidales by qPCR
CFU Colony forming unit
DNA-MASS DNA mass by Nanodrop
EC-DS *E. coli* by defined substrate
EC-MF *E. coli* by membrane filtration
EC-qPCR *E. coli* by qPCR
ENT-DS Enterococci by defined substrate
ENT-MF Enterococci by membrane filtration
ENT-qPCR Enterococci by qPCR
MPN Most probable number
MST Microbial source tracking
qPCR Quantitative real-time polymerase chain reaction
WET-MASS Wet weight of feces added

1. 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 (*Escherichia 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.

2. Materials and methods

2.1. 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 famililaris*; $n = 12$), 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 Table 1.

2.2. Fecal source concentration measurements

The quantity of fecal pollution source material in each composite source slurry was estimated using nine different methods of measurement including: 1) most probable number (MPN) of enterococci measured by defined substrate (ENT-DS) (Enterolert, IDEXX Laboratories, Inc.), 2) *E. coli* MPN measured by defined substrate (EC-DS)(Colilert, IDEXX Laboratories, Inc.), 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 $\log_{10}$ copies of Bacteroidales measured by GenBac3 qPCR (BAC-

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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	ENT-DS		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*	*Posterior Mean*	*BCI*	*Mean*	*StDev*	*Posterior Mean*	*BCI*	*Posterior Mean*	*BCI*	*Posterior Mean*	*BCI*
Human	31.4	ND	4.46	(4.30–4.62)	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	2.75	(2.59–2.89)	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[a]	ND	2.47	(2.29–2.64)	3.13	(2.98–3.27)	2.65	(2.46–2.82)	3.23	(3.17–3.29)	408	90	3.90	(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	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	3.90	(3.57–4.18)	5.73	(5.58–5.87)	3.56	(3.42–3.69)	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)	6.24	(6.18–6.30)	6.31	(6.12–6.48)	1027	25	5.75	(5.63–5.88)	6.50	(6.46–6.53)	7.81	(7.71–7.91)
Horse	19.0	ND	2.94	(2.84–3.03)	2.86	(2.76–2.96)	ND	ND	3.78	(3.73–3.83)	691	22	3.97	(3.87–4.07)	3.90	(3.86–3.93)	7.32	(7.22–7.42)
Pig	52.0	ND	4.05	(3.91–4.18)	6.60	(6.44–6.75)	3.40	(3.23–3.55)	6.67	(6.55–6.79)	1113	51	5.24	(5.13–5.36)	6.69	(6.65–6.73)	7.47	(7.38–7.57)
Chicken	0.30	ND	3.15	(2.89–3.36)	5.36	(5.16–5.55)	3.09	(2.84–3.31)	4.15	(4.08–4.22)	160[b]	ND	4.60	(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)	3.40	(3.23–3.55)	7.06	(6.98–7.14)	2772	87	5.60	(5.49–5.72)	8.35	(8.30–8.40)	5.46	(5.36–5.55)
Gull	1.30	ND	5.68	(5.52–5.83)	6.37	(6.21–6.52)	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[b]	ND
Pigeon	47.7	ND	6.97	(6.84–7.09)	8.34	(8.18–8.50)	6.22	(6.16–6.29)	7.70	(7.58–7.81)	197	9	6.02	(5.87–6.19)	6.90	(6.86–6.95)	2.41[b]	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/100 mL; sewage = 0.029 mL/100 mL).
b 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.

qPCR) (Dick and Field, 2004; Siefring et al., 2008), 6) mean $\log_{10}$ copies of enterococci measured by Entero1 qPCR (ENT-qPCR) (Ludwig and Schleifer, 2000; Siefring et al., 2008), 7) mean $\log_{10}$ 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.

2.3. qPCR amplification

Three qPCR assays were used in this study including EC23S857 (EC-qPCR), GenBac3 (BAC-qPCR), and multiplex Entero1 (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, United States). 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), and fecal DNA extracts containing 1–5 ng total DNA or 10 to 10^5 target copies (plasmid standard) in a total reaction volume of 25 μl. The multiplex Entero1 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, 10^3, 10^4, and 10^5 copies/2 μl dilutions, corresponding to the range of quantification (ROQ) for all three assays. All reactions were performed in triplicate. The thermal cycling conditions were 2 min at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. Data were initially analyzed with Sequence Detector software (version 2.3.2; Life Technologies) 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, three no-template and six extraction blank amplifications with purified water substituted for template DNA were performed for each 96-well instrument run.

2.4. 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

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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 Entero1 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.

2.5. Quality assurance and controls

Standard deviations of triplicate DNA-MASS measurements did not exceed 0.56 ng/μL. 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 ± 1), all DNA extracts yielded acceptable DNA isolation efficiencies based on Sketa22 results (control mean C_q 25.93 ± 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 10^5 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.

2.6. 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.

2.7. 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 eight 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.

2.8. Statistical analysis

Log_{10} 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. qPCR master calibration curves, mean log_{10} copy estimates, posterior means, and Bayesian credible intervals were also determined using a Monte Carlo Markov

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Chain approach (Sivaganesan et al., 2008). Simple statistics including linear regressions were calculated with SAS software (Cary, NC) and Microsoft Excel (Redmond, WA).

3. Results

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

3.2. 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 (Fig. 1), three methods of measurement commonly employed for water quality management applications. For the WET-MASS normalized data (Fig. 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 (Fig. 1b) and EC-MF normalized data (Fig. 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.

3.3. 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 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 2 – Correlation of fecal concentration estimates by different methods of measurement.

	ENT-DS	EC-DS	ENT-MF	EC-MF	DNA-MASS	ENT-qPCR	EC-qPCR	BAC-qPCR
WET-MASS	0.06 (10)	0.32 (9)	0.08 (8)	0.31 (10)	**0.78** (10)	0.12 (10)	0.16 (10)	0.40 (10)
ENT-DS		**0.86** (11)	**0.96** (10)	**0.82** (12)	0.26 (12)	**0.82** (12)	**0.65** (12)	0.46 (12)
EC-DS			**0.74** (10)	**0.95** (11)	0.02 (11)	**0.71** (11)	**0.89** (11)	0.16 (11)
ENT-MF				**0.63** (10)	0.25 (10)	**0.75** (10)	0.41 (10)	0.31 (10)
EC-MF					0.01 (12)	**0.69** (12)	**0.90** (12)	0.20 (12)
DNA-MASS						0.12 (12)	0.12 (12)	**0.66** (12)
ENT-qPCR							**0.68** (12)	0.55 (12)
EC-qPCR								0.16 (12)

Values represent the Pearson correlation coefficient (r) of a linear regression. Bold values are significant at a 95% confidence level (P-value < 0.05). Numbers in parentheses represent the sample size.

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(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.

4. Discussion

4.1. *Dependence of fecal pollution source concentration estimates on method selection*

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

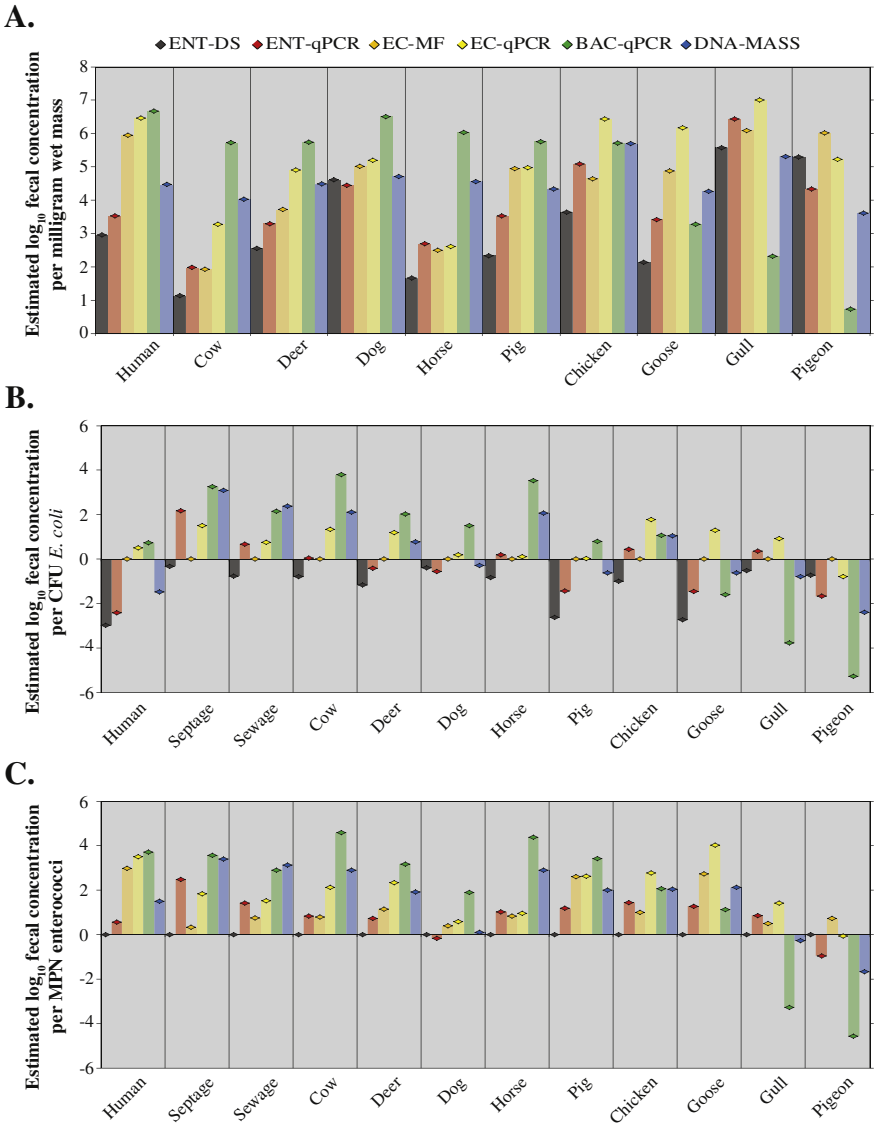

Fig. 1 – Fecal pollution source concentrations normalized to (A) milligrams wet mass (WET-MASS), (B) MPN enterococci (ENT-DS), and (C) CFU *E. coli* (EC-MF). Each colored bar corresponds to a different method of measuring fecal concentration, and columns show the estimated concentrations for each fecal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

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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, b; 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 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.

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

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%	17.3%
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.

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	43.4%	8.4%	6.0%	57.8%
Septage	37.8%	16.2%	59.5%	2.7%
Sewage	21.6%	21.6%	36.5%	9.5%
Cow	42.2%	9.4%	56.3%	3.1%
Deer	24.1%	16.9%	41.0%	15.7%
Dog	13.3%	50.6%	31.3%	24.1%
Horse	28.4%	23.0%	43.2%	18.9%
Pig	39.8%	9.6%	9.6%	53.0%
Chicken	21.7%	21.7%	43.4%	16.9%
Goose	43.4%	16.9%	13.3%	51.8%
Gull	6.0%	49.4%	16.9%	31.3%
Pigeon	1.2%	68.7%	8.4%	56.6%

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%.

4.2. *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

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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., 2012a, b), 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 50–69% (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; Kelty et al., 2012). Interestingly, this trend was not detected in chickens or geese, two avian species not commonly associated with marine waters.

4.3. *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 (Fig. 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 fiver 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.

5. Conclusions

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 MST, 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

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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.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2013.02.060.

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