Microbial source tracking in shellfish harvesting waters in the Gulf of Nicoya, Costa Rica

E.M. Symonds^{a,*}, S. Young^{b,1}, M.E. Verbyla^{c,2,Δ}, S.M. McQuaig-Ulrich^{d,3}, E. Ross^{e,4}, J.A. Jimenez^{e,5}, V.J. Harwood^{b,6}, M. Breitbart^{a,7}

^a University of South Florida, College of Marine Science, 140 7th Avenue South, St. Petersburg, Florida, USA

^b University of South Florida, Department of Integrative Biology, 4202 E. Fowler Avenue, Tampa, Florida, USA

^c University of South Florida, Department of Civil & Environmental Engineering, 4202 E. Fowler Avenue, Tampa, Florida, USA

^d St. Petersburg College, Natural Sciences Department, 2465 Drew Street, Clearwater, Florida, USA

^e Fundación MarViva, Apartado 020-6151 Santa Ana, San José, CR

¹ suzanneyoung@mail.usf.edu

² verbylam@mail.usf.edu

³ shannon.mcquaig@stpetecollege.edu

⁴ erick.ross@marviva.net

⁵ jorge.jimenez@marviva.net

⁶ vharwood@usf.edu

⁷ mya@usf.edu

* Corresponding author. esymonds@mail.usf.edu. +1 (727) 553-3357

^ΔCurrent: École Polytechnique Fédérale de Lausanne, School of Architecture, Civil and Environmental Engineering, GR C2 554 (Bâtiment GR), Station 2, Lausanne, CH; matthew.verbyla@epfl.ch

ABSTRACT

Current microbial water quality monitoring is generally limited to culture-based measurements of fecal indicator bacteria (FIB). Given the many possible sources of fecal pollution within a watershed and extra-intestinal FIB reservoirs, it is important to determine source(s) of fecal pollution as a means to improve water quality and protect public health. The principal objective of this investigation was to characterize the microbial water quality of shellfish harvesting areas in the Gulf of Nicoya, Costa Rica during 2015. In order to achieve this objective, the specificity and sensitivity of 11 existing microbial source tracking (MST) PCR assays, associated with cows (BacCow), dogs (BacCan, DogBac), domestic wastewater (PMMoV), general avian (GFD), gulls (Gull2), horses (HorseBac, HoF), humans (HF183, HPyV), and pigs (PF), were evaluated using domestic wastewater and animal fecal samples collected from the region. The sensitivity of animal-associated assays ranged from 13 – 100%, while assay specificity ranged from 38 – 100%. The specificity of pepper mild mottle virus (PMMoV) and human polyomavirus (HPyV) was 100% for domestic wastewater, as compared to 94% of the HF183 *Bacteroidales* marker. PMMoV was identified as a useful human/domestic wastewater-associated marker, with concentrations as high as 1.1×10^5 copies/ml in domestic wastewater, 100% sensitivity and specificity. Monthly surface water samples collected from four shellfish harvesting areas were analyzed using culture-based methods for *Escherichia coli* as well as molecular methods for FIB and a suite of MST markers, which were selected for their specificity in the region. While culturable *E. coli* results suggested possible fecal pollution during the monitoring period, the absence of human/domestic wastewater-associated markers and low FIB concentrations determined using molecular methods indicated sufficient microbial water quality for shellfish harvesting. This is the first study to our knowledge to test the performance of MST markers in Costa Rica as well as in Central America. Given the lack of

wastewater treatment and the presence of secondary, extra-intestinal sources of FIB, this study highlights the importance of an MST toolbox approach to characterize water quality in a tropical, developing country context. Furthermore, it confirms and extends the geographic range of the suitability of PMMoV as a tool for monitoring domestic wastewater pollution.

Keywords

Bacteroidales

Enteric viruses

Enterococcus

Escherichia coli

Fecal pollution

Pepper mild mottle virus

1. Introduction

In Costa Rica, it is estimated that only 5% of domestic wastewater is treated prior to surface water discharge (Contraloría de la República, 2013). Between 70 and 80% of wastewater contamination reaching the Pacific Coast via the Gulf of Nicoya, the largest estuary in Costa Rica, stems from rivers surrounding the densely-populated San José metropolitan area (Mora Alvarado et al., 2012). The Gulf of Nicoya, a medium-sized, tropical (dry and rainy seasons) estuary located along the Pacific coast of Costa Rica, is one of the most important fishing areas in the country and home to various community fishing associations (Araya et al., 2007; Chacón

et al., 2007). The Gulf of Nicoya contains areas that have been formally designated as marine responsible fishing areas by the Costa Rican government – these are areas in which fisherman catch fish and shrimp as well as cultivate oysters using responsible practices. Since approximately 30% of Costa Rica’s domestic seafood comes from the Gulf of Nicoya (INCOPECA, 2013), domestic wastewater pollution threatens the sustainable use of this coastal ecosystem as well as the local and national economy.

The microbial water quality of the Gulf of Nicoya is influenced by a mixture of human and non-human sources that enter the Gulf in both the upper (shallow, less than 20 m) and lower (deeper, ranging from 25 to 100 m in depth at the mouth) regions. The upper region is influenced by the second largest watershed in Costa Rica, Greater Tempisque River Watershed (Palter et al., 2007), which contributes high concentrations of fecal coliforms (as much as 2821 colony forming units (cfu) 100 ml⁻¹) from the surface wastewater discharge of several cities (Mora Alvarado, 2004) as well as thousands of commercial farms that raise chicken, cattle, pigs, sheep, and goats (CR SENASA, 2012). The lower region of the Gulf of Nicoya is influenced by the poor water quality of the Barranca River (geometric mean fecal coliforms 2150 cfu/100 ml) and the Grand River of Tarcóles (geometric mean fecal coliforms 840,000 cfu/100 ml), which has the highest concentration of fecal coliforms in comparison to other rivers and receives a large portion of the wastewater generated in the San José metropolitan area (Mora Alvarado, 2004). Wild and domesticated animals also contribute to the fecal pollution in the Gulf of Nicoya and principally include large quantities of seabirds and migratory birds, horses, livestock, and dogs. To the best of our knowledge, the contribution of each of these sources of fecal pollution over time has yet to be investigated.

Currently, the Costa Rican National Water Laboratory monitors recreational surface water quality for the presence of fecal indicator bacteria (FIB; e.g. fecal coliforms) using culture-based techniques. While no legislation exists regarding the microbial water quality of shellfish harvesting areas (Corrales and Vindas, 2014), the maximum allowed fecal coliform concentrations for fish and shrimp aquaculture are 1000 cfu/100 ml and 100 cfu/100 ml, respectively (Mora Alvarado, 2004). Although the Costa Rican governmental water quality monitoring utilizes fecal coliforms as an indicator, it has been shown that *Escherichia coli* is a more effective indicator of recent contamination as well as more representative of warm-blooded animal feces (WHO, 2009). A previous study demonstrated that *E. coli* concentrations varied over time in two shellfish harvesting areas within the Gulf of Nicoya, with noticeable differences between dry and rainy seasons (Corrales and Vindas, 2014); however, mean *E. coli* concentrations were still within the bounds of the approved category requiring no depuration or relaying (i.e., ≤ 230 most probable number (MPN)/100 g per European Union Shellfish Harvesting Criteria and ≤ 43 MPN/100 ml per United States of America (USA) National Shellfish Sanitation Program; Corrales and Vindas, 2014; Lee et al., 2008; Oliveira et al., 2011).

While all types of fecal pollution present a risk to human health, it has been demonstrated that pig, gull, and chicken feces pose less of a health risk to humans in comparison to cow and human feces (Soller et al., 2010). Even though fecal coliforms, *E. coli*, and enterococci are the most commonly used FIB for monitoring microbial water quality in marine systems, they cannot be used to distinguish between human and animal sources nor does their presence necessarily indicate active fecal contamination given the possibility of re-suspension from sediments (Anderson et al., 2005; Davies et al., 1995; Kelsey et al., 2004; Liang et al., 2015). Furthermore, it has been demonstrated that secondary reservoirs of *E. coli* exist in tropical environments (e.g.

soil and runoff; reviewed in Winfield and Groisman, 2003) and genomic analyses have demonstrated the existence of environmentally-adapted strains of *E. coli* that are indiscernible from commensal *E. coli* using traditional culture-based assays (Luo et al., 2011). Accordingly, a microbial source tracking (MST) toolbox approach that employs multiple markers representing different sources is preferred to circumvent the aforementioned difficulties with FIB and to accurately characterize the extent of fecal pollution (Harwood et al., 2013b; Liang et al., 2015; Stoeckel and Harwood, 2007). While the establishment of MST markers has occurred primarily in developed countries to date, the usefulness of this approach in achieving the United Nations' Sustainable Development goals in all countries was recently highlighted (UN Water, 2015).

The principal objective of this investigation was to determine the surface water quality of four responsible fishing areas within the Gulf of Nicoya as well as the sources of fecal pollution contributing to any impairment during the 2015 wet and dry seasons. To achieve this objective, the sensitivity (i.e., the percentage of target host fecal samples that yield true positive results) and specificity (i.e., the percentage of non-target host fecal samples that yield true negative test results) of MST markers for cows, dogs, birds, horses, pigs, and domestic wastewater were determined through the analysis of animal fecal samples as well as domestic wastewater collected from the region. To the best of our knowledge, this is the first study to identify MST markers for use in Costa Rica and the first study of its kind in Central America. The surface water quality of four shellfish harvesting areas was then analyzed using *E. coli* MPN methods as well as molecular methods for a suite of FIB and domestic wastewater/human-associated MST markers, selected for their suitability in the region.

2. Materials and methods

2.1 Feces and wastewater collection, preservation, and nucleic acid purification

Animal feces and domestic wastewater samples were collected using standard, sterile techniques from areas within the Gulf of Nicoya drainage basin. Fecal samples were collected from the following animals identified as most abundant in the region (CR SENASA, 2012): chickens ($n = 8$), cows ($n = 8$), pigs ($n = 7$), dogs ($n = 8$), horses ($n = 8$), and sea birds ($n = 8$). Samples were maintained at 4°C and nucleic acid purification was initiated within 24 h of collection. A wet weight of 0.25 g of each fecal sample was preserved in bead beating tubes from the MoBio PowerViral™ Environmental RNA/DNA kit (MoBio Laboratories, Inc.; Carlsbad, CA, USA) with 0.5 volumes of lysis buffer and stored at -20°C. Upon arrival to the University of South Florida, samples were thawed and nucleic acid purification was resumed by adding the remaining 0.5 volumes of lysis buffer and then following manufacturer's instructions. The First Strand Synthesis Superscript III Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA) with random hexamer primers was used to synthesize cDNA. One extraction blank was processed alongside fecal samples and was negative for all MST marker PCR assays.

Eight 100-ml untreated, domestic wastewater samples were collected, approximately each month from May to December 2015, in sterile Whirl-Pak® Bags (Nasco, Fort Atkinson, WI, USA) from the domestic wastewater treatment facility in El Roble, Puntarenas. In addition to receiving treated industrial wastewater, this facility serves an estimated 29,847 permanent habitants, as well as a local hospital, prison, and hotel. Samples were maintained at 4°C and processed within 24 h of collection. For the concentration of FIB and bacterial markers, 50-ml aliquots of wastewater were filtered onto 0.45- μ m, 47-mm mixed cellulose filters (HAWP04700; EMD Millipore; Billerica, MA, USA) using sterile syringes and filter holders. For the concentration of viruses, 50-ml aliquots were acidified with 2.0 N HCl to pH 3.0 prior to

filtration as previously described (Symonds et al., 2014). All filters for bacterial and viral analyses were stored at -20°C with 0.5 volumes of lysis buffer in 5-ml bead beating tubes from the PowerWater® DNA Isolation Kit and PowerWater® RNA Isolation Kit (MoBio Laboratories, Inc.; Carlsbad, CA, USA), respectively. Upon arrival to the University of South Florida, samples were thawed and nucleic acid purification was resumed by adding the remaining 0.5 volumes of lysis buffer and then following manufacturer's instructions. Both DNA and RNA were isolated for viral analyses using the PowerWater® RNA Isolation Kit by skipping the DNase treatment. The First Strand Synthesis Superscript III Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA) with random hexamer primers was used to synthesize cDNA.

2.2 Surface water collection, preservation, and nucleic acid purification

Coastal water samples (1.1-l) were collected monthly from March through December 2015 (with the exception of June and July) from the following oyster harvesting areas, located within designated responsible fishing zones of marine protected areas, in the Gulf of Nicoya (Figure 1; $n = 8$): Costa de Pájaros (N09°59.979', W084°06.699'), Palito de Chira (N10°05.271', W085°11.236'), Punta Cuchillo (N09°49.994', W084°53.141'), and Acumamar (N09°50.678', W084°53.009'). During each sampling event, surface water was collected in sterile Whirl-Pak® Bags (Nasco, Fort Atkinson, WI, USA), maintained at 4°C, and processed within 24 h for bacterial analyses and 48 h for viral analyses. Each 1.1-l sample was divided into three volumes and analyzed for *E. coli* (100 ml), bacterial MST markers (500 ml), and viral MST markers (500 ml).

For all surface water samples intended for bacterial and viral MST analyses, 500-ml aliquots were filtered separately as previously described for raw domestic wastewater. All filters were stored, nucleic acids purified, and cDNA synthesized as described above for raw domestic

wastewater. In addition to an extraction blank, three process blanks comprised of distilled water that was processed alongside the samples from collection to analysis were also analyzed to confirm the absence of cross-contamination. All process and extraction blanks were negative for the FIB and MST markers analyzed using (RT-)qPCR.

2.3 Enumeration of *Escherichia coli*

E. coli (MPN) concentrations were determined for each surface water sample (100 ml) as well as distilled water (negative control) using Aquagenx compartment bag tests (CBT; Aquagenx, LLC., Chapel Hill, NC, USA) per manufacturer's instructions (Stauber et al., 2014). While Aquagenx was designed to analyze potable water sources, it was used in this study to accommodate the lack of a microbiology laboratory and equipment. Analyses were executed within 24 h of collection. *E. coli* were not detected in any of the negative controls ($n = 8$).

2.4 MST marker PCR analyses of fecal and wastewater samples

In order to identify MST markers appropriate for use in Costa Rica, all fecal and wastewater nucleic acid samples were analyzed using the following (RT-)PCR assays and published conditions (Table S1): cow "BacCow" (Silkie and Nelson 2009), dog "BacCan" (Silkie and Nelson, 2009), dog "DogBac" (Sinigalliano et al., 2010), general avian "GFD" (Green et al., 2012), gull "Gull2" (Riedel et al., 2014), horse "HorseBact" (Silkie and Nelson, 2009), horse "HoF" (Dick et al., 2005), human "HF183" (Riedel et al., 2014), human "human polyomavirus (HPyV)" (McQuaig et al., 2009), domestic wastewater "pepper mild mottle virus (PMMoV)" (Zhang et al., 2006), and Pig "PF" (Dick et al., 2005). All bacterial assays target different regions of the *Bacteroidales* 16S rRNA gene, with the exception of Gull2 that targets the 16S rRNA gene of *Catellibacillus marimammalius*. The HPyV assay targets the capsid protein, while the PMMoV assay targets the replication-associated protein. All fecal and

wastewater (c)DNA samples were analyzed alongside positive and negative (e.g., water) controls and had final reaction volumes of 50 μ l, which included 2 μ l (c)DNA and a final concentration of 1 μ M of each primer. For the BacCow, DogBac, GFD, HF183, HPyV, HoF, and PF assays, 1x Promega GoTaq® Green PCR Master Mix (Promega™; Madison, WI, USA) was used while the BacCan, Gull2, y HorseBac, and PMMoV assays used 1x Apex Taq buffer with 1-unit Apex Taq Polymerase (Genesee Scientific; Research Triangle Park, NC, USA), 0.25 mM per dNTPs and 1.5 mM MgCl₂.

All PCR products were visualized using gel electrophoresis with ethidium bromide. Positive PCR products were purified with the Zymo DNA Clean and Concentrator-5 Kit (Zymo Research Group, Irvine, CA, USA). Purified PCR products were sequenced and verified using the NCBI GenBank database. No amplification was observed in any of the assay negative controls. Only sequence-verified PCR products were considered when calculating the sensitivity (e.g., proportion of true positive target organism fecal or wastewater samples) and specificity (e.g., proportion of true negative non-target organism fecal or wastewater samples) of each (RT-)PCR assay (Harwood et al., 2013b). In order to calculate the confidence interval of each sensitivity and specificity, the Jeffrey's method, a Bayesian approach well-suited for small sample sizes, was used to determine the two-sided confidence interval from a beta posterior (Brown et al., 2001).

2.5 (RT-)qPCR analyses of wastewater and surface water

Viral nucleic acid wastewater samples were analyzed as previously described using the following assays (Table S2): human adenovirus (AdV) (Jothikumar et al., 2005), HPyV (McQuaig et al., 2009), norovirus (NoV) genotype I (Svraka et al., 2009; Symonds et al., 2014), rotavirus group A (RV) (Svraka et al., 2009; Symonds et al., 2014), and PMMoV (Haramoto et

al., 2013; Symonds et al., 2014). Bacterial DNA surface water samples were analyzed for the following FIB and MST markers, using published assays and conditions (Table S2): total *Enterococcus* spp. “EnterolA” (US EPA, 2013), *E. coli* (Ahmed et al., 2016b; Chern et al., 2011), and human-associated *Bacteroides* “HF183” (Haugland et al., 2010; Shanks et al., 2009). Additionally, viral nucleic acid surface water samples were analyzed for HPyV and PMMoV. All analyses were executed in 25 µl reactions using the ABI 7500 Real Time PCR system (Thermo Fisher Scientific, Grand Island, NY, USA). Reactions contained 2 µl template DNA or cDNA, assay specific concentrations of primers and probes, and the TaqMan® Environmental Master Mix 2.0 no UNG (Thermo Fisher Scientific, Grand Island, NY, USA).

For each (RT-)qPCR analysis, dilution series of purified plasmids containing the assay target were analyzed in duplicate for each qPCR run and each sample was run in duplicate alongside no template controls as well as process and nucleic acid purification controls. No inhibition was observed per third-well target spikes for the *E. coli* and PMMoV assays. All (RT-)qPCR analyses were executed in agreement with the MIQE qPCR guidelines (Bustin et al., 2009); all standard curves had efficiencies between 90 – 110% with R^2 values ≥ 0.97 , with the exception of the NoV assay (76% efficiency, which may lead to an overestimation of concentrations). The ABI 7500 Software v2.0.6 (Thermo Fisher Scientific, Grand Island, NY, USA), using default settings and manufacturer’s instructions, was used to calculate mean copies for each assay and sample by linear regression analysis of the quantification cycle (C_q) and copy number for each duplicate, purified recombinant plasmid dilution series. Sample concentrations were then back-calculated to account for all dilutions in the analysis process.

Samples for which neither replicate amplified and samples that did not successfully amplify above the limit of quantification (LOQ) in both replicate qPCR reactions (i.e., only

amplified in 1 of 2), were reported as “not detected (ND)”. If both replicate samples successfully amplified below the LOQ, then these samples were considered positive but below the LOQ. Most nucleic acid purification and process controls were ND for all (RT-)qPCR assays. However, positive but below the LOQ concentrations were detected for the PMMoV assay in the wastewater process controls. Additionally, *E. coli* and EnterolA assays were positive but below the LOQ in some of the surface water process controls. The wastewater theoretical process LOQ (the minimum concentration necessary to yield quantifiable results, assuming 100% recovery) was 500 AdV, 100 HPyV, 13 RV, 131 NoV, and 131 PMMoV copies/ml. The theoretical process LOQ for surface water samples was 50 *E. coli*, *Enterococcus* spp., and HF183; 1000 HPyV; and 1300 PMMoV copies/100 ml.

2.6 Analysis of surface water quality data in shellfish harvesting areas

Non-parametric multivariate analysis of variance (NP-MANOVA) was used to determine if significant ($\alpha = 0.05$) differences existed in FIB and MST marker concentrations among shellfish harvesting areas as well as between seasons (e.g., dry (March, April, May, December) and wet (August, September, October, November)). In order to down-weight high concentrations and meet the homogeneous within-group variance assumption, a square-root transformation was executed before NP-MANOVA. Following NP-MANOVA, the similarity percentages (SIMPER) procedure was used to identify which FIB variable contributed the most to any observed significant differences among sites or seasons. All analyses were executed in MATLAB and Statistics Toolbox 2012b (MathWorks®, Natick, MA, USA) and with the Fathom toolbox (Jones, 2015).

3. Results

3.1 Microbial source tracking PCR markers

The eleven (RT-)PCR assays analyzed had sensitivities (i.e., true positives for target organisms) ranging from 13 – 100% and specificities (i.e., true negatives for non-target organisms) ranging from 38 – 100% (Table 1). Of the human/domestic wastewater-associated MST markers, HF183 had higher sensitivity (100%) than HPyV and PMMoV (75%); however, HF183 had lower specificity (94%) than either of the viral markers (100%) because sequence-verified amplicons were produced from chicken feces. While both assays designed to identify dog feces cross-reacted with domestic wastewater, the BacCan assay had greater sensitivity and specificity compared to the DogBac assay, which also produced amplicons from horse and seabird feces. The GFD assay, a general assay for avian feces, was only 44% sensitive to seabird and chicken feces and was 56% specific because it cross-reacted with domestic wastewater, horse, dog, cow, and pig feces. Both assays designed for horse feces performed similarly, with 38% sensitivity and 100% specificity. The PF assay had among the greatest sensitivity (100%) and was 82% specific to pig feces, cross-reacting with domestic wastewater, chicken, horse, and seabird feces. The BacCow assay had high sensitivity (88%) to cow feces; however, it was the least specific (62%), cross-reacting with all non-target animals except seabirds. The Gull2 assay had the lowest sensitivity (13%) but 100% specificity. Assays with lower sensitivities and/or specificities (< 90%) also had a greater range in the upper and lower confidence intervals.

3.2 Viruses in domestic wastewater

Unlike RV, which was detected in only one sample, AdV, HPyV, NoV, and PMMoV were detected in all domestic wastewater samples (Figure 2). PMMoV concentrations were generally greater (10^2 – 10^5 copies/ml) than those of the other viruses (10^1 – 10^4 copies/ml) for each sample collection. While minimum PMMoV concentrations were similar to the minimum

concentrations of AdV, HPyV, and NoV (10^2 copies/ml), the median and maximum PMMoV concentrations were at least an order of magnitude greater than those of the aforementioned human viruses. Sixty-three percent and 100% of the detected quantities for AdV and RV, respectively, were below the LOQ.

3.3 Surface water quality in shellfish harvesting areas

Only FIB, using both culture-based and molecular methods, were detected in the shellfish harvesting area surface waters during 2015 (Figure 3). The human-associated HF183 bacterial and viral (HPyV and PMMoV) markers were not detected in any samples. Even though *E. coli* was always detected using MPN methods, *E. coli* and EnterolA were only detected by qPCR in 81% and 41% of the samples, respectively. Furthermore, 50% of the *E. coli* and 6% of the EnterolA concentrations reported were below the LOQ. Maximum *E. coli* MPN concentrations were ≥ 100 MPN/100 ml for all sites; however, the minimum *E. coli* MPN concentrations ranged from 4.7 MPN/100 ml at Aquamar to ≥ 100 MPN/100 ml at Palito.

No significant difference in FIB concentrations among sites was identified with 95% confidence ($\alpha = 0.05$, $p = 0.3700$) using NP-MANOVA. However, significant differences in FIB concentrations between the dry and rainy seasons were observed ($\alpha = 0.05$, $p = 0.0200$). Overall, geometric mean concentrations of FIB nearly doubled in the rainy season, increasing from 55 *E. coli* MPN/100 ml, 29 *E. coli* copies/100 ml, and 57 EnterolA copies/100 ml to 91 *E. coli* MPN/100 ml, 55 *E. coli* copies/100 ml, and 95 EnterolA copies/100 ml. Furthermore, the percentage of samples with FIB detection increased from 50% in the dry season to 72% in the rainy season. The subsequent SIMPER analysis results identified that *E. coli* concentrations determined by qPCR contributed the most to the total average dissimilarity observed among

seasons (42%), followed by EnterolA concentrations (39%), and *E. coli* MPN measurements (19%).

4. Discussion

4.1 Microbial source tracking markers identified for use in Costa Rica

To identify human health risks accurately and overcome the false-positive results associated with using FIB (Lamparelli et al., 2015), eleven MST (RT-)PCR assays, originally developed in North America, were evaluated for their use in Costa Rica. Since species-specific fecal microbial communities may vary by region, it is essential to determine the specificity and sensitivity of MST markers developed in other regions prior to their implementation in a new region (Harwood et al., 2013b). In general, the animal-associated assays tested in this study were less sensitive and less specific to their target organism(s) in Costa Rica than previously described (Ahmed et al., 2016a; Boehm et al., 2013; Harwood et al., 2013b; Schriewer et al., 2013). However, the performance of the PigBac and BacCow assays were similar to previous studies in the United States of America and Canada (Boehm et al., 2013). The BacCow, BacCan, DogBac, GFD, and PF assays cross-reacted with 75 – 100% of the domestic wastewater samples, which are primarily composed of human feces. It is possible that animal feces were also introduced to the Roble wastewater treatment system via illegal inputs of stormwater runoff and/or infiltration of surface runoff (Walski et al., 2004); alternatively, it is plausible that these animal-associated assays cross-reacted with human feces as previously demonstrated (Schriewer et al., 2013). The Gull2 assay had the lowest sensitivity, which is likely biased by the fact that only mixed seabird fecal samples were tested; thus, future research is needed to more accurately evaluate its sensitivity in Costa Rica. While future investigations should expand the sensitivity and

specificity analyses to improve confidence intervals as well as improve the human- and animal-associated MST assays evaluated, this study provides an essential first step in developing an MST toolbox for distinguishing sources of fecal pollution in Costa Rica.

Since previous studies have highlighted the advantages of using wastewater-related viruses to identify human fecal pollution (Ahmed et al., 2016b; Harwood et al., 2013a), the concentrations of the most commonly used viral MST markers and reference pathogens were determined for Costa Rican domestic wastewater to identify the most suitable, human-associated, viral MST marker. The observed median PMMoV and NoV concentrations (10^4 and 10^3 copies/ml, respectively) were within the range previously reported in Bolivia (Symonds et al., 2014), Germany (Hamza et al., 2011), Japan (Katayama et al., 2008), United States of America (Kitajima et al., 2014; Rosario et al., 2009) and Vietnam (Kuroda et al., 2015). However, in this study AdV and HPyV were detected in concentrations (e.g., 10^2 and 10^3 copies/ml, respectively) an order of magnitude less than in Australia (Ahmed et al., 2016b) and within the lower range previously reported in Japan (Katayama et al., 2008) and the United States of America (Fong et al., 2010; Harwood et al., 2013a; McQuaig et al., 2009). Interestingly, RV was not detected in quantifiable concentrations, which may be the result of low rates of infection (Simhon et al., 1985) and vaccination programs (Bucardo et al., 2011; Tate et al., 2012). PMMoV, and to a lesser extent HPyV, were the viruses most consistently observed in quantifiable concentrations; thus, they are the most promising viral MST markers analyzed in this study.

Three human-associated PCR assays, HF183, HPyV, and PMMoV, were identified for use in Costa Rica since they each had high sensitivity and specificity to domestic wastewater. Even though HPyV and PMMoV were 75% sensitive to domestic wastewater using (RT-)PCR, both viruses were consistently quantified in all samples using (RT-)qPCR (e.g., 100%

sensitivity). It is likely that this observed disparity reflects the higher detection limit of the PCR assays. Like other studies (Ahmed et al., 2016b; Harwood et al., 2013b), the HF183 marker was less than 100% specific to domestic wastewater in Costa Rica. However, unlike previous MST studies in the United States of America, PMMoV was 100% specific to domestic wastewater (Harwood et al., 2013b), which may be a consequence of sample size, differences in assay amplification of the PMMoV target, or possibly animal diets. This study suggests that the HF183, HPyV, and PMMoV assays are appropriate to utilize along the Pacific Coast of Costa Rica; however, future studies are needed to expand the sample size and geographic range of this investigation.

4.2 Limited fecal pollution observed in shellfish harvesting area surface waters

The Gulf of Nicoya is a medium-sized estuary where microbial water quality is heavily influenced by riverine input of untreated wastewater from densely populated regions (Mora Alvarado et al., 2012). Despite the lack of wastewater treatment in Costa Rica, limited fecal pollution was identified and no human fecal pollution was observed in any of the surface waters from the four shellfish harvesting areas investigated. While a similar range of FIB concentrations was detected across all sites, significant differences in FIB concentrations were observed by season. FIB concentrations were greatest during the rainy season, which may be the result of runoff and resuspension of sediments. The results of this study support the only other fecal pollution study of shellfish harvesting area waters in the region, which also identified limited fecal pollution as well as increased FIB concentrations in the rainy season and found limited evidence of fecal pollution (Corrales and Vindas, 2014).

Similar to other microbial water quality studies, various methodological biases could have affected the results and conclusions of this study. It is possible that the results of this study

were biased by the inevitable losses associated with concentrating and detecting FIB and MST markers from surface waters; however, no qPCR inhibition was observed and the most efficient nucleic acid purification kits were used (Iker et al., 2013). Additionally, just as Stauber et al. (2014) had previously shown for surface freshwater, the *E. coli* MPN methods were limited in their ability to quantify concentrations exceeding 100 MPN/100 ml. Thus, it is difficult to directly compare the *E. coli* MPN and FIB qPCR results. However, it is well-known that FIB concentrations derived from culture-based methods are typically orders of magnitude less than those estimated from qPCR methods because culture-based methods only target metabolically active cells while qPCR methods detect all cells (Haugland et al., 2005; Noble et al., 2010) and qPCR assays often target multi-copy genes (e.g., 23s rRNA; Chern et al., 2011). Consequently, the low FIB concentrations and absence of human-associated markers of fecal pollution suggest that the shellfish harvesting areas studied were exposed to low levels of fecal pollution in 2015. To ensure safe oyster cultivation and overcome possible false-positive FIB results, future studies should include these MST markers to monitor shellfish harvesting area water quality as wastewater management practices change.

5. Conclusions

- This is the first study to our knowledge to test the performance of MST markers in Central America. A suite of MST assays was identified for bird, dog, horse, pig, and cow feces as well as domestic wastewater for use in the Costa Rica.
- PMMoV is an important tool in the Costa Rican MST toolbox for monitoring domestic wastewater pollution because of its high concentrations as well as 100% sensitivity and specificity.

- All shellfish harvesting areas had sufficient water quality for shellfish production per the low FIB concentrations and absence of human/domestic wastewater-associated markers during the monitoring period.

Acknowledgments

This research was facilitated and financed by Fundación MarViva, the US Fulbright Scholar program (2014-2015; E.M.S), as well as by US National Science Foundation awards #CBET-1234237 (V.J.H) and #OCE-1566562 (M.B, E.M.S, and V.J.H). In addition to the US Fulbright Scholar program, E.M.S also received support from the University of South Florida, College of Marine Science William and Elsie Knight Fellowship. This study could not have been executed without the following individuals and institutions, who facilitated sample collection: Cecilia Soto, David Castillo Vega, Jose Armijo (INCOPESCA); Dr. Minor Cordero, Marcos Rodriguez, Yajaira Salazar Chacon, Vanessa Soto Blanco (CR SENASA); Karla Ordóñez (CR AyA); and Gloriana Chaverri, Eduardo Leitón, Irene Morales, and Alberto Quesada (Fundación MarViva). Additionally, special thanks to Juliana Galeano Pardo for creating Figure 1 and Dr. Bina Nayak as well as the entire Harwood laboratory for their guidance with the MST (q)PCR assays.

REFERENCES

- Ahmed, W., Harwood, V.J., Nguyen, K., Young, S., Hamilton, K. and Toze, S. (2016a) Utility of *Helicobacter* spp. associated GFD markers for detecting avian fecal pollution in natural waters of two continents. *Water Res.* 88, 613-622.
- Ahmed, W., Sidhu, J.P.S., Smith, K., Beale, D.J., Gyawali, P. and Toze, S. (2016b) Distributions of fecal markers in wastewater from different climatic zones for human fecal pollution tracking in Australian surface waters. *Appl. Environ. Microbiol.* 82(4), 1316-1323.

447 Anderson, K.L., Whitlock, J.E. and Harwood, V.J. (2005) Persistence and differential survival of
 448 fecal indicator bacteria in subtropical waters and sediments. *Appl. Environ. Microbiol.* 71(6),
 449 3041-3048.

450 Araya, H., Vásquez, A., Marín, B., Palacios, J., Soto, R., Mejía, F., Shimazu, Y. and Hiramatsu,
 451 K. (2007) Reporte del comité de evaluación de los recursos pesqueros No. 2/2007. Proyecto
 452 "Manejo sostenible de la pesquería para el Golfo de Nicoya, Costa Rica". Instituto Costarricense
 453 de Pesca y Acuicultura.

454 Boehm, A.B., Van De Werfhorst, L.C., Griffith, J.F., Holden, P.A., Jay, J.A., Shanks, O.C.,
 455 Wang, D. and Weisberg, S.B. (2013) Performance of forty-one microbial source tracking
 456 methods: A twenty-seven lab evaluation study. *Water Res.* 47(18), 6812-6828.

457 Brown, L.D., Cai, T.T. and DasGupta, A. (2001) Interval estimation for a binomial proportion.
 458 *Stat. Sci.* 16(2), 101-133.

459 Bucardo, F., Lindgren, P.-E., Svensson, L. and Nordgren, J. (2011) Low prevalence of rotavirus
 460 and high prevalence of norovirus in hospital and community wastewater after introduction of
 461 rotavirus vaccine in Nicaragua. *PLoS ONE* 6(10), e25962.

462 Bustin, S.A., Benes, V., Garson, J.A., Hellems, J., Huggett, J., Kubista, M., Mueller, R.,
 463 Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. and Wittwer, C.T. (2009) The MIQE
 464 guidelines: Minimum information for publication of quantitative real-time PCR experiments.
 465 *Clin. Chem.* 55(4), 611-622.

466 Chacón, A., Araya, H., Vázquez, R., Brenes, R., Marín, B., Palacios, J., Soto, R., Mejía-Arana, F.,
 467 Shimazu, Y. and Hiramatsu, K. (2007) Estadísticas pesqueras del Golfo de Nicoya, Costa Rica,
 468 1994-2005, Instituto Costarricense de Pesca y Acuicultura-Universidad Nacional de Costa Rica-
 469 Japanese International Cooperation Agency.

470 Chern, E., Siefring, S., Paar, J., Doolittle, M. and Haugland, R. (2011) Comparison of
 471 quantitative PCR assays for *Escherichia coli* targeting ribosomal RNA and single copy genes.
 472 *Lett. Appl. Microbiol.* 52(3), 298-306.

473 Contraloría de la República (2013) Informe acerca de la eficacia del estado para garantizar la
 474 calidad del agua en sus diferentes usos, Informe Nro. DFOE-AE-IF-01-2013, San José, Costa
 475 Rica.

476 Corrales, L.A.V. and Vindas, C.M. (2014) Evaluación de las concentraciones de *Escherichia coli*
 477 en *Crassostrea gigas* y agua de mar en dos zonas de cultivo de ostras ubicadas en el Golfo de
 478 Nicoya, Costa Rica. *Revista Ciencias Marinas y Costeras* 6, 155-166.

479 Davies, C., Long, J., Donald, M. and Ashbolt, N. (1995) Survival of fecal microorganisms in
 480 marine and freshwater sediments. *Appl. Environ. Microbiol.* 61(5), 1888-1896.

481 Dick, L.K., Bernhard, A.E., Brodeur, T.J., Santo Domingo, J.W., Simpson, J.M., Walters, S.P.
 482 and Field, K.G. (2005) Host distributions of uncultivated fecal *Bacteroidales* bacteria reveal
 483 genetic markers for fecal source identification. *Appl. Environ. Microbiol.* 71(6), 3184-3191.

484 Fong, T.-T., Phanikumar, M.S., Xagorarakis, I. and Rose, J.B. (2010) Quantitative detection of
 485 human adenoviruses in wastewater and combined sewer overflows influencing a Michigan river.
 486 Appl. Environ. Microbiol. 76(3), 715-723.

487 Green, H.C., Dick, L.K., Gilpin, B., Samadpour, M. and Field, K.G. (2012) Genetic markers for
 488 rapid PCR-based identification of gull, Canada goose, duck, and chicken fecal contamination in
 489 water. Appl. Environ. Microbiol. 78(2), 503-510.

490 Hamza, I.A., Jurzik, L., Überla, K. and Wilhelm, M. (2011) Evaluation of pepper mild mottle
 491 virus, human picobirnavirus and torque teno virus as indicators of fecal contamination in river
 492 water. Water Res. 45(3), 1358-1368.

493 Haramoto, E., Kitajima, M., Kishida, N., Konno, Y., Katayama, H., Asami, M. and Akiba, M.
 494 (2013) Occurrence of pepper mild mottle virus in drinking water sources in Japan. Appl.
 495 Environ. Microbiol. 79(23), 7413-7418.

496 Harwood, V.J., Boehm, A.B., Sassoubre, L.M., Vijayavel, K., Stewart, J.R., Fong, T.-T.,
 497 Caprais, M.-P., Converse, R.R., Diston, D., Ebdon, J., Fuhrman, J.A., Gourmelon, M., Gentry-
 498 Shields, J., Griffith, J.F., Kashian, D.R., Noble, R.T., Taylor, H. and Wicki, M. (2013a)
 499 Performance of viruses and bacteriophages for fecal source determination in a multi-laboratory,
 500 comparative study. Water Res. 47(18), 6929-6943.

501 Harwood, V.J., Staley, C., Badgley, B.D., Borges, K. and Korajkic, A. (2013b) Microbial source
 502 tracking markers for detection of fecal contamination in environmental waters: Relationships to
 503 pathogens and human health outcomes. FEMS Microbiol. Rev. 38(1), 1-40.

504 Haugland, R.A., Sieftring, S.C., Wymer, L.J., Brenner, K.P. and Dufour, A.P. (2005) Comparison
 505 of Enterococcus measurements in freshwater at two recreational beaches by quantitative
 506 polymerase chain reaction and membrane filter culture analysis. Water Res. 39(4), 559-568.

507 Haugland, R.A., Varma, M., Sivaganesan, M., Kelty, C., Peed, L. and Shanks, O.C. (2010)
 508 Evaluation of genetic markers from the 16S rRNA gene V2 region for use in quantitative
 509 detection of selected *Bacteroidales* species and human fecal waste by qPCR. Syst. Appl.
 510 Microbiol. 33(6), 348-357.

511 Iker, B.C., Bright, K.R., Pepper, I.L., Gerba, C.P. and Kitajima, M. (2013) Evaluation of
 512 commercial kits for the extraction and purification of viral nucleic acids from environmental and
 513 fecal samples. J. Virol. Methods 191(1), 24-30.

514 Instituto Costarricense de Pesca y Acuicultura (INCOPECA) (2013) Estadísticas pesqueras de
 515 Costa Rica, Departamento de Estadística Pesquera, Puntarenas, Costa Rica.

516 Jones, D.L. (2015) Fathom Toolbox for Matlab: software for multivariate ecological and
 517 oceanographic data analysis, College of Marine Science, University of South Florida. Available
 518 from: <http://www.marine.usf.edu/user/djones/> St. Petersburg, FL, USA.

519 Jothikumar, N., Cromeans, T.L., Hill, V.R., Lu, X., Sobsey, M.D. and Erdman, D.D. (2005)
 520 Quantitative real-time PCR assays for detection of human adenoviruses and identification of
 521 serotypes 40 and 41. Appl. Environ. Microbiol. 71(6), 3131-3136.

522 Katayama, H., Haramoto, E., Oguma, K., Yamashita, H., Tajima, A., Nakajima, H. and Ohgaki,
523 S. (2008) One-year monthly quantitative survey of noroviruses, enteroviruses, and adenoviruses
524 in wastewater collected from six plants in Japan. *Water Res.* 42(6–7), 1441-1448.

525 Kelsey, H., Porter, D.E., Scott, G., Neet, M. and White, D. (2004) Using geographic information
526 systems and regression analysis to evaluate relationships between land use and fecal coliform
527 bacterial pollution. *J. Exp. Mar. Biol. Ecol.* 298(2), 197-209.

528 Kitajima, M., Iker, B.C., Pepper, I.L. and Gerba, C.P. (2014) Relative abundance and treatment
529 reduction of viruses during wastewater treatment processes — Identification of potential viral
530 indicators. *Sci. Total Environ.* 488–489, 290-296.

531 Kuroda, K., Nakada, N., Hanamoto, S., Inaba, M., Katayama, H., Do, A.T., Nga, T.T.V., Oguma,
532 K., Hayashi, T. and Takizawa, S. (2015) Pepper mild mottle virus as an indicator and a tracer of
533 fecal pollution in water environments: Comparative evaluation with wastewater-tracer
534 pharmaceuticals in Hanoi, Vietnam. *Sci. Total Environ.* 506–507, 287-298.

535 Lamparelli, C.C., Pogreba-Brown, K., Verhougstraete, M., Sato, M.I.Z., de Castro Bruni, A.,
536 Wade, T.J. and Eisenberg, J.N.S. (2015) Are fecal indicator bacteria appropriate measures of
537 recreational water risks in the tropics: A cohort study of beach goers in Brazil? *Water Res.* 87,
538 59-68.

539 Lee, R., Lovatelli, A. and Ababouch, L. (2008) Bivalve depuration: fundamental and practical
540 aspects, *FAO Fisheries Technical Paper* 511, Rome, Italy.

541 Liang, L., Goh, S.G., Vergara, G.G.R.V., Fang, H.M., Rezaeinejad, S., Chang, S.Y., Bayen, S.,
542 Lee, W.A., Sobsey, M.D., Rose, J.B. and Gin, K.Y.H. (2015) Alternative fecal indicators and
543 their empirical relationships with enteric viruses, *Salmonella enterica*, and *Pseudomonas*
544 *aeruginosa* in surface waters of a tropical urban catchment. *Appl. Environ. Microbiol.* 81(3),
545 850-860.

546 Luo, C., Walk, S.T., Gordon, D.M., Feldgarden, M., Tiedje, J.M. and Konstantinidis, K.T.
547 (2011) Genome sequencing of environmental *Escherichia coli* expands understanding of the
548 ecology and speciation of the model bacterial species. *P. Natl. Acad. Sci. USA* 108(17), 7200-
549 7205.

550 McQuaig, S.M., Scott, T.M., Lukasik, J.O., Paul, J.H. and Harwood, V.J. (2009) Quantification
551 of human polyomaviruses JC virus and BK virus by TaqMan quantitative PCR and comparison
552 to other water quality indicators in water and fecal samples. *Appl. Environ. Microbiol.* 75(11),
553 3379-3388.

554 Mora Alvarado, D. (2004) Calidad microbiológica de las aguas superficiales en Costa Rica.
555 *Revista Costarricense de Salud Pública* 13, 15-31.

556 Mora Alvarado, D., Chávez Aguilar, A. and Portuguese, C.F. (2012) Calidad sanitaria de las
557 aguas de playa de Costa Rica período 1996-2011, Instituto Costarricense de Acueductos y
558 Alcantarillados (AyA), San José, Costa Rica.

559 Noble, R.T., Blackwood, A.D., Griffith, J.F., McGee, C.D. and Weisberg, S.B. (2010)
 560 Comparison of rapid quantitative PCR-based and conventional culture-based methods for
 561 enumeration of *Enterococcus* spp. and *Escherichia coli* in recreational waters. Appl. Environ.
 562 Microbiol. 76(22), 7437-7443.

563 Oliveira, J., Cunha, A., Castilho, F., Romalde, J. and Pereira, M. (2011) Microbial contamination
 564 and purification of bivalve shellfish: crucial aspects in monitoring and future perspectives—a
 565 mini-review. Food Control 22(6), 805-816.

566 Palter, J., León Coto, S. and Ballesteros, D. (2007) The distribution of nutrients, dissolved oxygen
 567 and chlorophyll a in the upper Gulf of Nicoya, Costa Rica, a tropical estuary. Rev. Biol. Trop.
 568 55, 427-436.

569 Riedel, T.E., Zimmer-Faust, A.G., Thulsiraj, V., Madi, T., Hanley, K.T., Ebentier, D.L.,
 570 Byappanahalli, M., Layton, B., Raith, M., Boehm, A.B., Griffith, J.F., Holden, P.A., Shanks,
 571 O.C., Weisberg, S.B. and Jay, J.A. (2014) Detection limits and cost comparisons of human- and
 572 gull-associated conventional and quantitative PCR assays in artificial and environmental waters.
 573 J. Environ. Manage. 136, 112-120.

574 Rosario, K., Symonds, E.M., Sinigalliano, C., Stewart, J. and Breitbart, M. (2009) Pepper mild
 575 mottle virus as an indicator of fecal pollution. Appl. Environ. Microbiol. 75(22), 7261-7267.

576 Schriewer, A., Goodwin, K.D., Sinigalliano, C.D., Cox, A.M., Wanless, D., Bartkowiak, J.,
 577 Ebentier, D.L., Hanley, K.T., Ervin, J. and Deering, L.A. (2013) Performance evaluation of
 578 canine-associated Bacteroidales assays in a multi-laboratory comparison study. Water Res.
 579 47(18), 6909-6920.

580 Servicio Nacional de Salud Animal de Costa Rica (CR SENASA) (2012) Informe sobre la
 581 situación sanitaria de Costa Rica, San José, Costa Rica.

582 Shanks, O.C., Kelty, C.A., Sivaganesan, M., Varma, M. and Haugland, R.A. (2009) Quantitative
 583 PCR for genetic markers of human fecal pollution. Appl. Environ. Microbiol. 75(17), 5507-5513.

584 Silkie, S.S. and Nelson, K.L. (2009) Concentrations of host-specific and generic fecal markers
 585 measured by quantitative PCR in raw sewage and fresh animal feces. Water Res. 43(19), 4860-
 586 4871.

587 Simhon, A., Mata, L., Vives, M., Rivera, L., Vargas, S., Ramírez, G., Lizano, L., Catarinella, G.
 588 and Azofeifa, J. (1985) Low endemicity and low pathogenicity of rotaviruses among rural
 589 children in Costa Rica. J Infect Dis 152(6), 1134-1142.

590 Sinigalliano, C.D., Fleisher, J.M., Gidley, M.L., Solo-Gabriele, H.M., Shibata, T., Plano, L.R.,
 591 Elmir, S.M., Wanless, D., Bartkowiak, J. and Boiteau, R. (2010) Traditional and molecular
 592 analyses for fecal indicator bacteria in non-point source subtropical recreational marine waters.
 593 Water Res. 44(13), 3763-3772.

594 Soller, J.A., Schoen, M.E., Bartrand, T., Ravenscroft, J.E. and Ashbolt, N.J. (2010) Estimated
 595 human health risks from exposure to recreational waters impacted by human and non-human
 596 sources of faecal contamination. Water Res. 44(16), 4674-4691.

597 Stauber, C., Miller, C., Cantrell, B. and Kroell, K. (2014) Evaluation of the compartment bag test
 598 for the detection of *Escherichia coli* in water. J. Microbiol. Meth. 99, 66-70.

599 Stoeckel, D.M. and Harwood, V.J. (2007) Performance, design, and analysis in microbial source
 600 tracking studies. Appl. Environ. Microbiol. 73(8), 2405-2415.

601 Svraaka, S., van der Veer, B., Duizer, E., Dekkers, J., Koopmans, M. and Vennema, H. (2009)
 602 Novel approach for detection of enteric viruses to enable syndrome surveillance of acute viral
 603 gastroenteritis. J. Clin. Microbiol. 47(6), 1674-1679.

604 Symonds, E.M., Verbyla, M.E., Lukasik, J.O., Kafle, R.C., Breitbart, M. and Mihelcic, J.R.
 605 (2014) A case study of enteric virus removal and insights into the associated risk of water reuse
 606 for two wastewater treatment pond systems in Bolivia. Water Res. 65, 257-270.

607 Tate, J.E., Burton, A.H., Boschi-Pinto, C., Steele, A.D., Duque, J. and Parashar, U.D. (2012)
 608 2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years
 609 before the introduction of universal rotavirus vaccination programmes: A systematic review and
 610 meta-analysis. The Lancet Infectious Diseases 12(2), 136-141.

611 US Environmental Protection Agency (2013) Method 1609: Enterococci in Water by TaqMan®
 612 Quantitative Polymerase Chain Reaction (qPCR), Publication No. EPA 820-R-13-005, Office of
 613 Water, Washington, D.C.

614 UN Water (2015) Wastewater management: A UN-Water Analytical Brief, United Nations, New
 615 York, NY, USA.

616 Walski, T.M., Barnard, T.E., Harold, E., Merritt, L.B., Walker, N., Whitman, B.E., Hill, C.,
 617 McKay, G., Plante, S. and Schmitz, B.A. (2004) Wastewater collection system modeling and
 618 design, Haestad Press, Exton, PA, USA.

619 Winfield, M.D. and Groisman, E.A. (2003) Role of nonhost environments in the lifestyles of
 620 *Salmonella* and *Escherichia coli*. Appl. Environ. Microbiol. 69(7), 3687-3694.

621 World Health Organization (2009) Addendum to the WHO guidelines for safe recreational water
 622 environments, volume 1, coastal and fresh waters, Geneva, Switzerland.

623 Zhang, T., Breitbart, M., Lee, W.H., Run, J.Q., Wei, C.L., Soh, S.W.L., Hibberd, M.L., Liu,
 624 E.T., Rohwer, F. and Ruan, Y.J. (2006) RNA viral community in human feces: Prevalence of
 625 plant pathogenic viruses. PLoS Biol. 4(1), 108-118.

626 **TABLES**

627 Table 1. The sensitivity (%) and specificity (%) of microbial source tracking (RT-)PCR assays in
 628 Costa Rica from a total of eight samples from each fecal source, with the exception of seven pig
 629 feces samples. The upper (U) and lower (L) Jeffrey's confidence interval (CI) is reported for all
 630 sensitivities and specificities.

(RT-)PCR Assay	Fecal Source	Sensitivity			Specificity			Non-Target Fecal Source (number positive)
		LCI (%)	(%)	UCI (%)	LCI (%)	(%)	UCI (%)	
BacCow	Cow	60.3	88	99.9	25.1	38	52.2	Chicken (5), Dog (3), Domestic wastewater (8), Horse (6), Pig (7)
BacCan	Dog	60.3	88	99.9	71.5	83	92.3	Domestic wastewater (8)
DogBac	Dog	44.9	75	96.7	56.8	70	82.3	Domestic wastewater (8), Horse (2), Seabird (4)
GFD	Chicken & Seabird	21.7	44	66.9	41.1	56	71.3	Cow (1), Dog (3), Domestic wastewater (5), Horse (3), Pig (5)
Gull2	Seabird	0.1	13	39.7	96.0	100	100.0	n/a
HorseBac	Horse	10.4	38	68.6	96.0	100	100.0	n/a
HoF	Horse	10.4	38	68.6	96.0	100	100.0	n/a
HF183	Domestic wastewater	79.2	100	100.0	85.4	94	98.9	Chicken (3)
HPyV	Domestic wastewater	44.9	75	96.7	96.0	100	100.0	n/a
PMMoV	Domestic wastewater	44.9	75	96.7	96.0	100	100.0	n/a
PF	Pig	76.8	100	100.0	69.5	81	91.0	Chicken (1), Domestic wastewater (6), Horse (1), Seabird (1)

631

632 **FIGURES**

633
 634 Figure 1. Map of the Gulf of Nicoya, Costa Rica with municipalities, rivers, marine protected
 635 areas, and the locations of the investigated shellfish harvesting area (1 – 4) depicted.

636

637

638 Figure 2. Concentration of human adenovirus (AdV; $n = 8$), human polyomavirus (HPyV; $n = 8$),
639 norovirus genotype 1 (NoV; $n = 7$), pepper mild mottle virus (PMMoV; $n = 8$), and rotavirus
640 (RV; $n = 7$) concentrations (copies/ml) in untreated domestic wastewater from El Roble,
641 Puntarenas, Costa Rica. The lower and upper boxes represent the 25th and 75th percentiles,
642 respectively. The lower and upper bars denote the minimum and maximum values, respectively.
643 Concentrations below the limit of quantification (LOQ) are depicted in gray. It should be noted
644 that five samples had AdV concentrations below the LOQ and that RV was only detected in
645 concentrations below the LOQ in one sample. Note the logarithmic scale on the vertical axis.

Figure 3. Concentrations (MPN or copies/100 ml) of fecal indicator bacteria (*E. coli* and *Enterococcus* spp. (EnterolA) at four sites in the Gulf of Nicoya ($n = 8$ samples per site). The MST markers (human-associated *Bacteroides* (HF183), human polyomavirus (HPyV), pepper mild mottle virus (PMMoV)) were not detected in any of the samples. Sites are listed from the most internal (top panel) to closest to the estuary mouth (bottom panel). The lower and upper

652 boxes represent the 25th and 75th percentiles, respectively. The lower and upper bars denote the
653 minimum and maximum value, respectively. Concentrations below the limit of quantification
654 (LOQ) are depicted in gray; note that the *E. coli* MPN concentrations are right-censored because
655 measurements ≥ 100 MPN/100 ml cannot be quantified. Note the logarithmic scale on the
656 vertical axis.

Figure 1

Figure 2

Figure 3

Safe water quality observed in shellfish harvesting areas in the Gulf of Nicoya, Costa Rica