Faecal pollution along the southeastern coast of Florida and insight into the use of pepper mild mottle virus as an indicator

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Abbreviated running headline

Coastal water quality and PMMoV

ABSTRACT

Aims: To identify faecal pollution along the southeastern Florida coast and determine the performance of a reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) method for pepper mild mottle virus (PMMoV).

Methods and Results: In 2014, bimonthly surface water samples were collected from inlets, exposed to runoff and septic seepage, and coastal sites, exposed to ocean outfalls. Analysis of culturable enterococci and a suite of microbial source tracking (MST) markers (BacHum, CowM2, DogBact, HF183, HPyV, PMMoV) revealed faecal pollution, primarily of human origin, at all sites. Since PMMoV was detected more frequently than other MST markers, the process limits of quantification (undiluted to 10^{-2} dilution) and detection (10^{-2} dilution) for the RT-qPCR method were determined by seeding untreated wastewater into the coastal waters. Simulated quantitative microbial risk assessment, employing human norovirus as a reference pathogen, calculated a 0.286 median risk of gastrointestinal illness associated with the PMMoV limit of detection.

Conclusions: All sites met the U.S. EPA recreational water criteria, despite detection of domestic wastewater associated MST markers. PMMoV correlated only with human-associated MST markers.

Significance and Impact of Study: This study demonstrated that PMMoV is an important domestic wastewater-associated marker that should be included in the MST toolbox; therefore, future studies should thoroughly investigate the health risks associated with its detection and quantification in environmental waters.

KEY WORDS

Wastewater
Ocean outfall
Enteric viruses
Water quality
Microbial source tracking
Quantitative microbial risk assessment

INTRODUCTION

The southeastern Florida coast of Miami-Dade County is a densely populated area, with approximately 2.6 million people (U.S. Census Bureau 2014). This area has a long history of faecal pollution because domestic wastewater is treated via on-site septic systems abutting inlets or by one of two wastewater treatment plants (WWTPs) that discharge secondary-treated, chlorinated effluent via ocean outfalls (Bloetscher *et al.* 2011; Bloetscher *et al.* 2014). The major source of faecal pollution to the coastal marine environment varies depending upon the surface currents, with both inlets and ocean outfall surface boils having tested positive for human and zoonotic pathogens (e.g., norovirus, *Giardia* spp.; Rosario *et al.* 2009; Bloetscher *et al.* 2011; Futch *et al.* 2011; Bloetscher *et al.* 2014). The WWTP ocean outfalls are located several kilometers offshore where the Florida Current usually prevents the onshore movement of surface boils. However, local beaches can be exposed to WWTP effluent, via surface currents, which can subsequently lead to poor water quality and beach closures, when the Florida Current meanders and other current regimes prevail (Lekien *et al.* 2005). Furthermore, inlet waters have previously been identified as a source of faecal pollution at local beaches because they receive agricultural/cattle and storm water runoff as well as untreated domestic wastewater from leaky septic systems abutting waterways (Meeroff *et al.* 2008; Futch *et al.* 2011; Carsey *et al.* 2012; Campbell *et al.* 2015).

Microbial water quality in recreational waters has been monitored through the use of faecal indicator bacteria (FIB), specific reference human pathogens, and bacteriophages (*reviewed in* Ashbolt *et al.* 2001; Harwood *et al.* 2013). Even though a positive correlation between enterococci qPCR targets and gastrointestinal (GI) illness has been demonstrated for temperate waters exposed to point-sources of wastewater pollution (Wade *et al.* 2006; Wade *et al.* 2010), such a correlation has yet to be demonstrated for non-temperate coastal waters or any coastal waters impacted by a variety of faecal pollution sources (Ashbolt *et al.* 2001; Wade *et al.* 2006; Wade *et al.* 2010; Harwood *et al.* 2013). The use of FIB and bacteriophages as indicators of faecal pollution is complicated by their presence in non-human sources as well as in secondary reservoirs (e.g., sand, soil), which can yield false-positive results. Also, FIB are generally more susceptible to chlorination than other enteric pathogens; thus, their absence in the environment may not reflect an absence of treated wastewater effluent. Consequently, it is useful to use a microbial toolbox approach that employs multiple markers to assess the presence of faecal pollution (reviewed in Harwood *et al.* 2013).

The southeastern Florida coast is exposed to mixed domestic wastewater pollution; ocean outfalls are a source of secondary-treated, chlorinated wastewater, which may contain infectious pathogens (Rosario *et al.* 2009; Bloetscher *et al.* 2011; Futch *et al.* 2011; Bloetscher *et al.* 2014) and inlets are exposed to leaking septic systems that may offer little to no treatment (Meeroff *et al.* 2008; Futch *et al.* 2011; Carsey *et al.* 2012; Campbell *et al.* 2015). The primary objective of this study was to determine the extent of domestic wastewater pollution from point and non-point sources along the southeastern Florida coast. In addition to measuring enterococci by both culturing and qPCR, the following microbial source tracking (MST) markers were analyzed using (RT-)qPCR: pepper mild mottle virus (PMMoV; 100% sensitivity and specificity ranging

from 90-92% for humans), human polyomavirus (HPyV; 100% sensitivity and specificity for domestic wastewater), Dog *Bacteroidales* (DogBact; 100% sensitivity and ≥55% specificity), CowM2 (≥98% sensitivity and 100% specificity), and two human *Bacteroidales* assays (HF183, 100% sensitivity and 60% specificity; BacHum, 100% sensitivity and 97% specificity; reviewed in Harwood *et al.* 2013; Schriewer *et al.* 2013). Among these MST markers, PMMoV is unique due to its dietary origin, consistently high concentrations in human faeces and wastewater, and lack of known secondary reservoirs (Zhang *et al.* 2006; Rosario *et al.* 2009; Colson *et al.* 2010). PMMoV has been proposed as an alternative indicator of human faecal pollution in Asia, Europe, and the Americas (Rosario *et al.* 2009; Hamza *et al.* 2011; Haramoto *et al.* 2013; Betancourt *et al.* 2014; Kitajima *et al.* 2014; Kuroda *et al.* 2015) because its high concentrations facilitate sensitive detection in contaminated environmental waters and circumvent the false-negative results typically encountered with the use of reference viral pathogens (Rosario *et al.* 2009; Hamza *et al.* 2011; Haramoto *et al.* 2013; Betancourt *et al.* 2014; Kuroda *et al.* 2015).

While the high concentration of PMMoV in untreated and (frequently in) treated human wastewater presents an advantage for using this virus as a sensitive indicator of faecal pollution in surface waters (Rosario *et al.* 2009; Hamza *et al.* 2011; Haramoto *et al.* 2013; Betancourt *et al.* 2014; Kuroda *et al.* 2015), this same characteristic creates the possibility that PMMoV detection may overestimate the risk of GI illness. To address this concern, the analytical (A), method (M), and process (P) limits of detection and quantification (LOD and LOQ, defined in Table 1; Staley *et al.* 2012) were determined for Florida's southeastern coastal surface waters and untreated domestic wastewater. Subsequently, exploratory QMRA was used to estimate the associated risk of swimming-related GI illness related to PMMoV detection in surface waters contaminated with untreated wastewater pollution.

MATERIALS AND METHODS

Surface water collection and enterococci analysis

Surface water samples exposed to point and non-point sources of faecal pollution were collected during cruises aboard the U.S. NOAA Ship *Hildebrand* in January, March, May, July, September, and November 2014 as part of a larger study of regional water quality and coral health by the U.S. NOAA Atlantic Oceanographic and Meteorological Laboratory. Surface water samples, presumed to be exposed to human faecal pollution, were collected at the Miami-Dade North WWTP and Miami-Dade Central WWTP ocean outfall surface boils (N25.923061°, W80.089369°; N25.742817°, W80.085967°, respectively; Figure 1) as well as from the following inlets during an outgoing tide: Port Everglades (N26.093450°, W80.109722°), Port of Miami (N25.763611°, W80.132778°), and Bakers Haulover (N25.900000°, W80.121389°; Figure 1). Offshore coastal water was also collected to serve as a process control during each sampling event. All surface water samples were transported on ice and maintained at 4°C. All sites were analyzed within 24 h for viable enterococci by the membrane-Enterococcus Indoxyl-ß-D-Glucoside (mEI) agar plate count technique using U.S. Environmental Protection Agency (U.S. EPA) method 1600 (U.S. EPA 2009).

Bacterial and viral concentration from coastal waters

Within 24 h of collection, bacteria and viruses were separately concentrated from 1-l aliquots of the surface water samples. Bacteria were filtered onto sterile 0.2-µm mixed cellulose ester filters (Millipore, Billerica, MA, USA) and stored at -80°C prior to nucleic acid purification and bacterial qPCR analyses (Sinigalliano *et al.* 2010; Campbell *et al.* 2015). In order to concentrate the viruses, the 1- l aliquot was acidified with 1 mol l^{-1} acetic acid to pH ~ 3.0 and

then viruses were filtered onto a 47-mm, 0.45-µm HA negatively charged nitrocellulose filter (Millipore, Billerica, MA, USA; American Public Health Association *et al.*1998; Fong *et al.* 2005). These filters were aseptically transferred to a 50-mL conical tube and were initially stored at -80°C, transported to the lab at -20°C, and remained at -20°C for one week prior to nucleic acid extraction.

Nucleic acid extraction and reverse transcription

Bacterial DNA was extracted directly from the filters using the Fast DNA Spin Kit (MP Biomedicals LLC, Santa Ana, CA, USA) as previously described (Campbell *et al.* 2015). Viral nucleic acid was purified using the QIAmp MinElute Virus Spin Kit (Qiagen, Valencia, CA, USA) for all samples and one blank; however, a modification was made to allow for direct lysis off of the filter similar to Fuhrman *et al.* (2005). Briefly, the samples were thawed, filters were placed at the bottom of the conical tube, and 600 µl of sterile, phosphate-buffered saline (PBS) solution was added. The QIAmp MinElute Virus Spin Kit (Qiagen, Valencia, CA, USA) was modified such that sample lysate was created by adding an additional 2 volumes of protease and buffer AL prior to a 30-min incubation at 56°C. All samples were pulse-vortexed for 10 s before, after 15 min, and after 30 min of incubation. Two additional volumes of molecular-grade ethanol were added prior to lysate passage through the spin columns, after which the QIAmp MinElute Virus Spin Kit protocol resumed. cDNA was immediately synthesized using the First Strand Synthesis Superscript III Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA) with random hexamer primers.

Given the inherent variability in RNA extraction/RT efficiency, each sample was seeded with an RNA extraction /RT efficiency control. A synthetic single-stranded RNA oligonucleotide (~ 5×10^9 copies), targeted by an existing RT-qPCR assay for equine arteritis virus (EAV; Svraka

159 *et al.* 2009), was added just prior to viral nucleic acid extraction and analyzed as previously
160 described (Symonds *et al.* 2014). The absence of EAV in all un-seeded surface waters was
161 confirmed using RT-qPCR. The geometric mean RNA purification-RT efficiency per EAV
162 recovery was 1.5% for the surface water samples.

163 **(RT-)qPCR analyses for indicators and MST markers**

164 Bacterial DNA samples were analyzed for the following MST markers, using previously
165 published assays and conditions: human-associated *Bacteroides* "HF183" (Shanks *et al.* 2009;
166 Haugland *et al.* 2010) and human-associated *Bacteroidales* "BacHum-UCD" (Silkie and Nelson
167 2009), total enterococci "EPA entero1A" (Haugland *et al.* 2005), cow *Bacteroidales* faecal
168 marker "CowM2" (Shanks *et al.* 2008), and dog *Bacteroidales* faecal marker "DogBact"
169 (Sinigalliano *et al.* 2010). These analyses were executed using an ABI StepOnePlus Real-Time
170 PCR thermocycler (Thermo Fisher Scientific, Grand Island, NY, USA), with the 2X QuantiTect
171 Probe Mastermix (Qiagen, Valencia, CA, USA). Viral nucleic acids were analyzed with an ABI
172 7500 Real Time PCR system (Thermo Fisher Scientific, Grand Island, NY, USA) with the
173 TaqMan® Environmental Master Mix 2.0 no UNG (Thermo Fisher Scientific, Grand Island, NY,
174 USA) using published qPCR assays and conditions for HPyV (McQuaig *et al.* 2009) and
175 PMMoV (Zhang *et al.* 2006) with minor modifications (Haramoto *et al.* 2013; Symonds *et al.*
176 2014). All (RT-)qPCR analyses were executed in accordance with established MIQE qPCR
177 guidelines (Bustin *et al.* 2009). For each assay, dilution series of purified plasmids containing the
178 assay amplicon were analyzed in duplicate for each qPCR run and each sample was run in
179 triplicate alongside process and nucleic acid purification controls. (RT-)qPCR analyses
180 confirmed the absence of all bacterial and viral markers in all nucleic acid purification/RT
181 controls as well as process controls collected at offshore sites (with the exception of positive but

below the limit of quantification (+BLOQ) concentrations detected in November and September process controls). Potential inhibition and DNA extraction efficiency were monitored by use of internal amplification controls and third-well target spikes for inhibition assessment (*i.e.*, all seeded-sample quantification cycle (C_q) values were within one SD of the average plasmid DNA C_q value). No PCR inhibition was observed for the qPCR analyses; however, RT-qPCR inhibition was identified in September's Port of Miami Inlet sample, which was therefore analyzed as a 1:10 dilution to overcome inhibition. Since the geometric mean RNA purification-RT efficiency was 1.5% for surface water samples, it is possible that inhibitors affected the RT step of PMMoV quantification.

Estimation of indicators and MST markers derived from (RT-)qPCR C_q values

For each bacterial indicator and MST marker assay, average gene copies were determined for each sample via linear regression analysis of the C_q and gene copy number for each duplicate, purified recombinant plasmid dilution series, using default settings in 7500 Software v2.0.6 per manufacturer's instructions (Thermo Fisher Scientific, Grand Island, NY, USA). The standard curves generated across all qPCR runs for each assay were compared to examine potential inter-plate variability. The number of gene copies for each viral MST marker was estimated using the C_q values measured for the duplicate, purified recombinant plasmid dilution series from seven and five qPCR runs for PMMoV and HPyV, respectively; thus, the *'pooled approach'* as described by Sivaganesan *et al.* (2010) was employed to estimate the number of viral gene copies in each unknown sample. This approach, which uses Markov Chain Monte Carlo simulations (50,000 iterations; burn-in period of 10,000) executed in WinBUGS software V1.4.3 (Imperial College and Medical Research Council, UK) to calculate a single standard curve, has been shown to overcome the stochastic, inter-plate variability intrinsic to qPCR analyses involving

more than four instrument runs. For all analyses, all standard curves had efficiencies between 90 and 110% and R^2 values ≥ 0.97. For all indicators and MST markers, concentrations were back-calculated to account for all dilutions in the analysis process. Back-calculations did not take into account virus concentration or RNA purification-RT efficiencies.

Regardless of the method used to estimate gene copy number, only duplicate measurements within +/- 0.5 C_q for each sample were considered quantifiable. If no florescence was observed in duplicate reactions during 40 cycles, the sample concentration was classified as 'less than the LOD' (< LOD). In the event that the target was detected in 2 out of 3 qPCR reactions with a mean C_q < 40 and C_q standard deviation (SD) > 0.5, then the concentration was considered positive but below the LOQ (+BLOQ). The concentration equivalent to the theoretical P_{LOQ} (i.e., the minimum, original concentration necessary to yield quantifiable results, assuming 100% recovery of viral cDNA) was 25 gene copies 100 ml^{-1} for enterococci and the bacterial MST markers, 500 gene copies 100 ml^{-1} for the HPyV assay, and 80 gene copies 100 ml^{-1} for the PMMoV assay.

Data analysis of indicators and MST marker concentrations in surface waters

In order to accommodate censored data, non-parametric multivariate analysis of variance (NP-MANOVA) was executed to determine if indicator and MST marker concentrations were significantly different (α = 0.05) among inlet and outfall sites using MATLAB and Statistics Toolbox 2012b (MathWorks ®, Natick, MA, USA) and the Fathom toolbox (Jones 2015). Prior to NP-MANOVA, a square-root transformation was applied to the data as a means to down-weight high concentrations and ultimately, to meet the assumption of homogeneous within-group variance required by NP-MANOVA. The similarity percentages (SIMPER) procedure was subsequently used to determine which indicator or MST marker contributed the most to the

differences observed between inlet and outfall sites. Additionally, Spearman rank order analysis was executed to identify significant cross-correlations ($\alpha = 0.05$) between the faecal indicator and MST markers (Table 2).

PMMoV analytical and method limits of detection and quantification

The PMMoV RT-qPCR assay A_{LOD} and A_{LOQ} were determined by analyzing the seven standard curves generated during the course of this study, as previously described for other MST assays (Staley *et al.* 2012). In order to determine the M_{LOD} and M_{LOQ}, untreated municipal wastewater was collected on five occasions from the South Cross Bayou Water Reclamation facility in St. Petersburg, Florida, USA. All wastewater samples were collected post-grit removal in sterile 1-l, HDPE bottles, maintained at 4°C, and processed within 24 h for $M_{LOD/LOQ}$ experiments. For each untreated wastewater sample, DNA and RNA were simultaneously extracted from triplicate 200-µl aliquots as well as a blank purification control using the QIAmp MinElute Virus Spin Kit (Qiagen, Valencia, CA, USA) per manufacturer's instructions. cDNA was immediately synthesized from the purified RNA as previously described. Nucleic acids were stored at -20ºC. cDNA was diluted with molecular grade water to create a 1:10 dilution series to 10^{-5} and analyzed via RT-qPCR (see above) to determine the $M_{LOD/LOQ}$, as previously described by Staley *et al.* (2012). PMMoV was not detected in any of the nucleic acid purification-RT blank purification controls.

PMMoV process limit of detection and quantification

In order to determine the extent to which PMMoV could be reliably quantified and detected via RT-qPCR, coastal ($n = 2$) and inlet ($n = 2$) water samples were augmented with different dilutions of untreated wastewater prior to virus concentration and subsequent processing as described above (Staley *et al.* 2012). In order to identify the P_{LOD} in the absence of

inhibitors, distilled water (n = 1) was also augmented with the same dilutions of untreated wastewater prior to virus concentration. The surface water samples were collected from two coastal locations along the southeastern Florida Coast (site 1: N26.000000°, W80.096419°; site 2: N25.590592°, W80.095419°) and the Port of Miami and Everglades inlets during the September cruise (Figure 1). Surface water samples were maintained at 4°C and processed within 48 h. The untreated wastewater dilution series was created on two separate occasions (using the same wastewater collected for $ML_{OD/LOQ}$ analyses; Table 3), in sterile buffered water (*prepared per* U.S. EPA 2009) to create a serial, ten-fold dilution series ranging from undiluted (containing 100 ml untreated wastewater) to 10^{-3} dilution (containing 100 µl untreated wastewater).

One-l aliquots of each surface water sample and distilled water were augmented with each point of the untreated wastewater dilution series and viruses were subsequently concentrated onto a 47-mm, 0.45-µm HA nitrocellulose filter (Millipore, Billerica, MA, USA) as previously described above. Viral nucleic acid was purified and RNA reverse transcribed as described above for surface waters. Additionally, 1-l aliquots of each water type without the addition of wastewater and one process control were analyzed to determine background PMMoV concentrations and to ensure no cross-contamination during virus concentration. Without the addition of untreated wastewater, PMMoV was detected at concentrations below the LOQ in surface waters from coastal water site 1 and the Port of Miami inlet. PMMoV was not detected in any of the process or nucleic acid purification-RT controls. The geometric mean RNA purification-RT efficiency from surface and distilled water augmented with untreated wastewater averaged 1.9% and 32.4%, respectively.

Exploratory quantitative microbial risk assessment related to PMMoV detection

The objective of this QMRA simulation was to evaluate whether or not the detection of PMMoV in surface waters augmented with serially-diluted untreated wastewater posed a potential health risk to swimmers. For the health risk estimation and health benchmark comparison, norovirus (NoV) was selected as the microbial reference pathogen because human enteric viruses cause the majority of swimming-associated gastroenteritis (Sinclair *et al.* 2009) and because the risk of viral infection via water can be 10-10,000-fold higher than infection by pathogenic bacteria at similar rates of exposure (Haas *et al.* 1993; Teunis *et al.* 2008). Untreated wastewater was used in this QMRA simulation because sufficient data on NoV concentrations in treated domestic wastewater does not currently exist in the literature. It was assumed that NoV diluted similarly to PMMoV during the $P_{LOD/LOQ}$ experiments; therefore, the hypothetical NoV dose was modeled, as previously described by Staley *et al.* (2012) for other MST markers, for the untreated wastewater dilution corresponding to the PMMoV P_{LOD} identified for coastal, inlet, and distilled water samples.

Briefly, the NoV concentration in untreated wastewater and the accidental ingestion volume due to recreation in water were obtained from the literature and a Monte Carlo simulation was performed to capture the variation in these values (Teunis *et al.* 2010). NoV concentration was modeled as a log-normal distribution with parameters ($\mu = 10.8$ gene copies l^{-1}; $\sigma = 6$ gene copies l^{-1}) corresponding to a median density of 4.94×10^4 gene copies l^{-1} (Teunis *et al.* 2010). The volume of water ingested was also modeled as a log-normal distribution with parameters ($\mu = 2.92$ ml; $\sigma = 1.43$ ml) corresponding to a mean volume of 18.6 ml (U.S. EPA 2014). The dose of NoV for each untreated wastewater dilution for corresponding water types was calculated by multiplying the concentration of NoV in untreated wastewater by the ingestion volume and the wastewater-dilution factor. The wastewater dilutions were assumed to have a

ratio of total to infectious viruses equivalent to the inoculum used for the dose-response parameterization.

The 2012 U.S. EPA ambient water quality criteria of 36 GI illnesses per 1,000 exposures (i.e., 0.036), which is equivalent to the 1986 criteria, was used as the health benchmark (U.S. EPA 2012). The probability of infection (P_{inf}) was modeled using the hypergeometric function with parameters $\bar{\alpha} = 0.04$ and $ß = 0.055$ for NoV doses measured by qPCR from the work of Teunis *et al.* (2008) for non-aggregated virus suspensions. Finally, the probability of illness was estimated by multiplying the probability of infection by a constant morbidity (fraction of infections resulting in illness) of 0.6 (Soller *et al.* 2010).

RESULTS

Faecal pollution in southeastern Florida coastal waters

Five sites off the southeastern Florida coast exposed to point and non-point sources of domestic wastewater were analyzed for PMMoV, HPyV, Entero1A, HF183, BacHum, DogBact, CowM2, as well as culturable enterococci on six different occasions ($n = 30$; Figure 1). Using NP-MANOVA, the null hypothesis that there was no difference in the indicator and MST marker concentrations among inlet and outlet sites was rejected with 95% confidence ($\alpha = 0.05$, $p = 0.001$). Overall, geometric mean concentrations of MST markers were greater at the WWTP ocean outfalls than in the inlets. In order to identify which MST marker contributed the most to the differences observed between inlet and ocean outfall sites, a SIMPER analysis was executed and indicated that PMMoV concentrations contributed the most to the total average dissimilarity observed among site types (39.46%), followed by Entero1A (27.87%), HF183 (9.90%), DogBact (8.81%), BacHum (7.07%), HPyV (3.87%), and culturable enterococci (3.02%). Significant ($\alpha = 0.05$) positive cross-correlations were identified between the following FIB and MST markers,

except PMMoV and Entero1A: PMMoV, HPyV, HF183, BacHum, and Entero1A. No significant cross-correlations were identified among the animal-associated MST markers.

While concentrations of culturable enterococci were generally low (< 23 CFU 100 ml^{-1}), the qPCR for total enterococci revealed concentrations up to two-orders of magnitude higher (Figure 2). PMMoV was detected more frequently (60% of the samples) than HPyV or the MST bacterial markers, with the highest concentrations up to 8.73×10^4 gene copies 100 ml^{-1} measured at the WWTP ocean outfall sites and most inlet sites being +BLOQ (Figure 2). HPyV, BacHum, and HF183 were detected in ~40% of the samples; however, BacHum and HF183 were detected in concentrations as high as 1.00×10^3 and 2.15×10^3 gene copies 100 ml^{-1}, respectively, while the HPyV concentrations at all sites were +BLOQ. DogBact was quantifiable in 40% of the samples, which included all sites. CowM2 was the genetic marker detected in the fewest (17%) number of samples and was only quantifiable from the Port Everglades Inlet and the Miami Central outfall.

PMMoV analytical and method limits of quantification and detection

Of the seven standard curves generated from duplicate analyses of six-point, purified recombinant plasmid dilution series, the average ± standard deviation (SD) regression coefficient (R^2) and qPCR efficiencies were 0.990 ± 0.010 and 99.38 ± 6.58%, respectively. The lowest point analyzed in the PMMoV recombinant plasmid dilution series (10 copies) was quantified in all of the standard curves and had an average +/- SD C_q value of 35.576 +/- 1.605. For the second lowest point (100 copies), the average +/- SD C_q value was 31.547 +/- 0.318. Given the large (> 0.5) C_q SD observed for 10 copies, it is not possible to reliably quantify as few as 10 copies per RT-qPCR reaction; thus, the actual A_{LOQ} is somewhere between 10 and 100 copies per qPCR

reaction. The A_{LOD} was assumed to be less than the A_{LOQ} but greater than the lowest possible copy number detected (3 copies; Bustin *et al.* 2009).

All untreated wastewater samples (n = 3) contained an estimated average of $2.06 \times 10^7 \pm 2.83 \times 10^6$ SD PMMoV copies 100 ml^{-1} (Table 3). The M_{LOQ}, representing the extent to which wastewater cDNA could be serially-diluted with PMMoV still quantifiable, was consistently the 10^{-1} wastewater cDNA dilution. The M_{LOD}, which describes the extent to which wastewater cDNA could be serially-diluted with PMMoV still detectable, varied between the 10^{-2} (n = 2) and 10^{-3} (n = 1) wastewater cDNA dilutions.

PMMoV process limit of quantification and detection

In order to identify the extent to which PMMoV could be detected and quantified from surface waters, the $P_{LOQ/LOD}$ experiment was executed using two, untreated wastewater dilution series created from the same untreated wastewater used in the $M_{LOQ/LOD}$ experiments. The undiluted untreated wastewater had an average PMMoV concentration of $2.24 \times 10^7 \pm 7.73 \times 10^6$ SD and $1.73 \times 10^7 \pm 2.39 \times 10^6$ SD copies 100 ml^{-1} for the surface and distilled water experiments, respectively (Table 3). The PMMoV P_{LOD} was consistently 10^{-2} for all surface water samples; however, the P_{LOQ} varied from 10^0 to 10^{-2} (Table 4). When distilled water was augmented with serial-dilutions of untreated wastewater, PMMoV was detectable to the 10^{-4} and quantifiable to the 10^{-2} wastewater dilution.

Exploratory quantitative microbial risk assessment related to PMMoV detection

The probability of GI illness due to NoV infection from recreating in waters exposed to untreated wastewater was estimated for the PMMoV P_{LOD} for the two coastal, two inlet, and distilled waters previously discussed using QMRA. The median probability of illness associated with the PMMoV P_{LODs} for both coastal and inlet waters was 0.286, which is almost an order of

magnitude greater than the 0.036 U.S. EPA illness benchmark (i.e., 36 GI illnesses per 1000 exposures; Figure 3). For coastal and inlet waters, the 25th and 75th percentile probabilities of GI illness were 0.03 and 0.33, respectively. The median probability of GI illness associated with the PMMoV P_{LOD} for distilled water was 0.022, with 0.0004 and 0.28 25th and 75th percentile probabilities, respectively.

DISCUSSION

Faecal pollution identified along the southeastern Florida coast

According to the culturable enterococci analyses and qPCR-derived HF183 concentrations, all of the sites meet the U.S. EPA criteria for recreational waters (U.S. EPA 2012; Boehm *et al.* 2015). However, the molecular analyses for enterococci as well as human wastewater associated markers, such as PMMoV and BacHum, revealed poorer water quality at all sites. The discrepancy between the culturable and the qPCR-based enterococci detection methods is unsurprising since they discriminate two different, although related, FIB populations. The culture-based methods only enumerate live cells that are metabolically and reproductively active, while the molecular methods detect live, dead, and metabolically dormant cells; thus, estimates of enterococci using qPCR are typically higher than estimates from culture-based methods. Nevertheless, a positive correlation has been shown between culturable enterococci and qPCR results for enterococci for environmental water samples (Haugland *et al.* 2005). Such a correlation was not observed in this study, which may be the result of the relatively small sample size. Since enterococci gene copies were one to two orders of magnitude greater at ocean outfall surface boils compared to the inlets, it is possible that these waters posed a greater risk to public health (Wade *et al.* 2006; Sinigalliano *et al.* 2010; Wymer *et al.* 2013). Even though a correlation

was not observed between culturable and qPCR-derived enterococci concentrations, positive cross-correlations existed between enterococci and human/domestic wastewater-associated MST markers, with the exception of PMMoV and Entero1A.

A significant difference was observed between the water quality at inlet and ocean outfall sites. In accordance with results published by Campbell *et al.* (2015), this study demonstrated lower concentrations of human and domestic wastewater-associated markers (HF183, BacHum, and PMMoV) in the inlet waters compared to the WWTP ocean outfall surface boils. While methodological differences make it difficult to directly compare studies, these results contradict previous work showing higher concentrations of faecal pollution indicators and markers in Port Everglades Inlet water compared to another local WWTP ocean outfall not examined in the present study (Futch *et al.* 2011). This discrepancy may be due to the changes in water quality management over the last few years. Although no correlation between nearby beach water quality and inlet water quality has been previously identified for the Port Everglades Inlet (Meeroff *et al.* 2008; Futch *et al.* 2011) or the other inlets studied, it is possible that surrounding beaches may be impacted if inlet waters are moved alongshore. Although DogBact was detected at all sites, it is possible that the assay cross-reacted with predominately human sources of faecal pollution (e.g., domestic wastewater; Schriewer *et al.* 2013). Therefore, since non-human associated MST markers were quantified infrequently across all sites and did not cross-correlate with FIB, it is likely that the main source of faecal pollution is from human sources. Finally, it is important to note that the reported MST marker concentrations may have underestimated actual concentrations since the concentration efficiency of the methods employed has been previously reported <78% (Ahmed *et al.* 2015).

PMMoV as an indicator of faecal pollution

The performance of the PMMoV RT-qPCR assay used in this study followed the MIQE criteria set forth by Bustin *et al.* (2009), with nearly ideal PCR efficiencies and R^2 values. Albeit difficult to compare given differences in methodologies, the estimated PMMoV concentrations in wastewater observed in this study (10^8 copies l^{-1}) are within in the range (e.g. 10^6 to 10^{10} copies l^{-1}) previously described in Bolivia, Germany, Japan, United States of America, and Vietnam (Rosario *et al.* 2009; Hamza *et al.* 2011; Kitajima *et al.* 2014; Symonds *et al.* 2014; Kuroda *et al.* 2015). In comparison to previously reported M_{LOQ} and M_{LOD} for wastewater collected from Florida, PMMoV was more sensitive (M_{LOQ} 10^{-1}, M_{LOD} 10^{-3}) than HPyV (M_{LOQ} 10^{-0}, M_{LOD} 10^{-1}) but less sensitive than HF183 (M_{LOQ} 10^{-2}, M_{LOD} 10^{-3}; Staley *et al.* 2012). With respect to the quantification and detection in surface waters augmented with wastewater, the PMMoV RT-qPCR performed similarly to the previously reported assays analyzed by Staley *et al.* (2012). The PMMoV P_{LOQ} varied for three of the four water types from 10^0 to 10^{-2} and the P_{LOD} was consistently 10^{-2} for both coastal and inlet waters.

A variety of factors can influence the PMMoV $M_{LOD/Q}$ and $P_{LOD/Q}$ described in this study, including the range of PMMoV concentrations in wastewater and the efficiency of virus concentration, nucleic acid extraction, and reverse transcription. The efficiency of virus concentration methods was not measured during this study; however, the concentration method employed has previously been 31 to 78% effective for the concentration of human adenovirus and HPyV (Ahmed *et al.* 2015). Direct nucleic acid purification from the filters of acidified surface water samples has been shown to reduce the co-concentration of inhibitors (Ahmed *et al.* 2015) and no PCR inhibition was noted in the PMMoV RT-qPCR assay performed in this study. However, it is evident that inhibitors were present in extracted nucleic acids from surface water samples given the low recovery of the RNA purification-RT control. It is possible that the co-

purification of inhibitors could be reduced in future studies through the use of other commercial nucleic acid extraction kits (Iker *et al.* 2013). In addition, although the percent recoveries obtained in this study are similar to previously reported recoveries from surface water samples processed in a similar way (e.g. virus adsorption to filter and subsequent nucleic acid purification off the filter; Gentry-Shields and Stewart 2013), these recoveries are discouragingly low. Therefore, while the theoretical concentration of PMMoV associated with the P_{LOQ} assuming 100% process efficiency was 80 PMMoV copies 100 ml^{-1}, the actual PMMoV concentration equivalent to the P_{LOQ} is likely as high as 4,190 PMMoV copies 100 ml^{-1} once the geometric mean RNA purification-RT efficiency (1.9%) is taken into account. These methodological limitations highlight the well-known need for improved virus concentration and nucleic acid purification methods (Harwood *et al.* 2013).

Similar to previous studies, the results of this investigation suggest that PMMoV is a useful indicator of faecal pollution because its high concentrations in human wastewater circumvent the likelihood of false-negative results associated with the inefficiency of virus concentration and genetic purification methodologies (Rosario *et al.* 2009; Hamza *et al.* 2011; Haramoto *et al.* 2013; Betancourt *et al.* 2014; Kuroda *et al.* 2015). Additionally, SIMPER analyses demonstrated that PMMoV concentrations contributed the most to the significant differences observed in water quality among inlet and ocean outfall sites, which suggests its usefulness as a domestic wastewater MST marker in comparison to the other faecal indicators and MST markers analyzed. Furthermore, significant cross-correlations were observed between PMMoV and all human-associated MST markers in southeastern Florida coastal waters exposed to point and non-point sources of faecal pollution.

Health risks related to PMMoV detection in surface waters exposed to untreated wastewater

PMMoV was detected more frequently and/or at higher concentrations than other human-specific MST markers across all sites and contributed the most to the dissimilarity observed between inlet and outfall sites. Previous studies demonstrating the higher concentrations and persistence of PMMoV compared to enteric pathogens of interest have led to the speculation that using PMMoV as an indicator of faecal pollution may overestimate risk (Rosario *et al.* 2009; Hamza *et al.* 2011; Haramoto *et al.* 2013; Betancourt *et al.* 2014; Kuroda *et al.* 2015). However, the results of the exploratory QMRA related to the PMMoV P_{LOD} demonstrated that PMMoV detection in marine surface waters contaminated with untreated wastewater could represent a human health risk to swimmers equivalent to approximately one order of magnitude greater than the 0.036 U.S. EPA health benchmark. The correlation between PMMoV detection and potential risk of illness is supported by a previous study executed by Hamza *et al.* (2011), which found a significant correlation between PMMoV and culturable human adenovirus concentrations in river waters.

The QMRA performed in this study only estimated health risks from swimming in water contaminated with untreated wastewater and therefore may overestimate risk if wastewater receives treatment prior to surface water discharge. This study used untreated, instead of treated, domestic wastewater to test the PMMoV performance for two main reasons. First, untreated wastewater was used to allow for comparisons to previous studies; all similar studies to date have used untreated wastewater to test the performance of other assays, such as HPyV and human-associated markers HF183 and BacHum (Staley *et al.* 2012; Boehm *et al.* 2015). Second, insufficient data on NoV concentrations in treated domestic wastewater inhibits such an

exploratory QMRA. Additionally, it is important to remember that the actual risk of GI illness will vary based on pathogen concentrations in untreated wastewater, wastewater treatment disinfection efficiency, and the extent of contamination present in the environment. Furthermore, if the efficiency of virus concentrations, RNA purification, and RT methods improve, PMMoV detection could overestimate risk of illness since the risk of illness 25th and 75th percentile probabilities related to the PMMoV P_{LOD} in distilled water currently overlap the U.S. EPA health benchmark. While exploratory QMRA is an alternative and affordable tool for investigating the link between the presence of MST markers in environmental waters and risk of swimming-related gastrointestinal illness (Staley *et al.* 2012; Boehm *et al.* 2015), epidemiological studies may be needed to determine the risk of illness related to PMMoV detection in coastal waters exposed to untreated, treated, and mixed wastewater sources.

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CONFLICT OF INTEREST

No conflict of interest declared.

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668 **TABLES**

669 Table 1. Definition of limits of quantification and detection for the performance evaluation of a

670 RT-qPCR method to detect pepper mild mottle virus (PMMoV).

671

Limit Type	Limit of Quantification (LOQ)		Limit of Detection (LOD)	
	Definition	Abbreviation	Definition	Abbreviation
Analytical	The lowest PMMoV gene copy number reliably quantified[1] by qPCR	A_{LOQ}	The lowest PMMoV gene copy number reliably detected[2] by qPCR	A_{LOD}
Method	The extent to which PMMoV cDNA can be reliably quantified[1] via qPCR from wastewater serially diluted in molecular grade water	M_{LOQ}	The extent to which PMMoV cDNA can be reliably detected[2] via qPCR from wastewater serially diluted in molecular grade water	M_{LOD}
Process	The extent to PMMoV cDNA can be reliably quantified[1] via qPCR from 1 l of concentrated environmental water augmented with wastewater serially diluted in buffered water	P_{LOQ}	The extent to PMMoV cDNA can be reliably detected[2] via qPCR from 1 l of concentrated environmental water augmented with wastewater serially diluted in buffered water	P_{LOD}

672 [1]Reliably quantified when 2/3 qPCR reactions were within +/-0.5 C_q and within the dynamic range of the assay
673 [2]Reliably detected when 2/3 qPCR reactions were positive and duplicate C_q measurements exceeded 0.5 C_q

674 Table 2. Spearman rank order coefficient (*r*) with a two-tailed p-value for cross-correlations
675 between the following faecal indicator and microbial source tracking marker concentrations in 30
676 surface water samples exposed to faecal pollution along the southeastern Florida coast:
677 culturable enterococci (Entero), total enterococci (Entero1A), human polyomavirus (HPyV),
678 pepper mild mottle virus (PMMoV), human-associated *Bacteroides* sp. (HF183), human
679 *Bacteroidales* (BacHum), dog *Bacteroidales* (DogBact), and cow *Bacteroidales* (CowM2).
680 Significant correlations ($p < 0.05$) are in bold.

	Entero	Entero1A	HPyV	PMMoV	HF183	BacHum	DogBact	CowM2
Entero		-0.25	-0.05	-0.17	-0.23	0.04	-0.09	0.10
p-value		0.1764	0.8046	0.3832	0.2173	0.8167	0.6310	0.6024
Entero1A			0.39	-0.17	0.83	0.70	0.18	0.05
p-value			**0.0325**	0.3833	**< 0.0001**	**< 0.0001**	0.3526	0.8001
HPyV				0.41	0.44	0.52	-0.27	-0.14
p-value				**0.0250**	**0.0159**	**0.0029**	0.1570	0.4507
PMMoV					0.56	0.47	-0.29	0.06
p-value					**0.0013**	**0.0093**	0.1248	0.7675
HF183						0.82	-0.13	0.12
p-value						**< 0.0001**	0.4919	0.5440
BacHum							-0.04	0.22
p-value							0.8304	0.2376
DogBact								-0.03
p-value								0.8726
CowM2								
p-value								

681

Table 3. Pepper mild mottle virus (PMMoV) qPCR method limit of quantification (M_{LOQ}) and detection (M_{LOD}) from cDNA derived from untreated wastewater and serially diluted in molecular grade water. The mean +/- standard deviation (n = 3) of PMMoV gene copies detected per qPCR reaction (2 µl cDNA) are listed for each cDNA dilution.

	Mean PMMoV gene copies detected per qPCR reaction (2 µl cDNA) +/- standard deviation			
Sample Date	Undiluted (10^0)	10^{-1} dilution	M_{LOD}[a]	Estimated mean gene copies 100 ml^{-1} in wastewater[b]
06/02/2014	672 +/- 35	18 +/- 11	10^{-2}	2.21×10^7
09/25/2014[c]	682 +/- 23	66 +/-8	10^{-2}	2.24×10^7
10/08/2014[d]	528 +/- 73	42 +/- 17	10^{-2}	1.73×10^7

[a] The extent to which PMMoV cDNA can be detected but not quantified in 2/3 qPCR reactions for at least two of the triplicate samples from wastewater serially-diluted in buffered water

[b] Gene copies ml^{-1} was calculated based upon the mean concentration of undiluted wastewater

[c] Wastewater used to determine the process limit of detection of coastal water

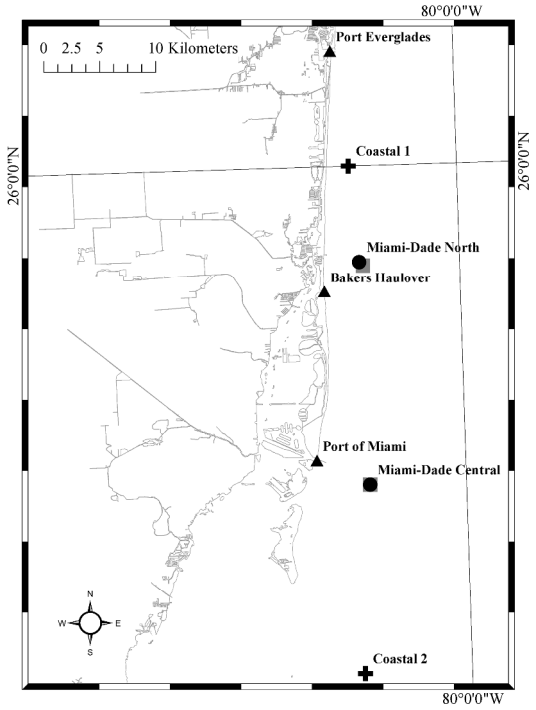
[d] Wastewater used to determine the process limit of detection of distilled water

692 Table 4. The process limit of quantification (P_{LOQ}) and detection (P_{LOD}) of pepper mild mottle
693 virus (PMMoV) in coastal and distilled water augmented with serially-diluted untreated
694 wastewater.

	PMMoV P_{LOQ}			
Water	Gene copies 2 μl^{-1}, [a]	Gene copies l^{-1}	Wastewater dilution	P_{LOD} dilution[b]
Port Everglades Inlet	12	7.73×10^2	10^{-2}	10^{-2}
Port of Miami Inlet*	10	6.41×10^2	10^{-1}	10^{-2}
Coastal Water Site 1*	1.0	6.44×10^2	10^0	10^{-2}
Coastal Water Site 2	186	1.22×10^4	10^0	10^{-2}
Distilled Water	174	1.14×10^4	10^{-2}	10^{-4}

695
696 [a] Gene copies per qPCR reaction at the lowest wastewater dilution quantifiable (C_q SD <0.5)
697 [b]The most dilute wastewater dilution in which 2/3 qPCR analyses were positive but not quantifiable
698 * Unspiked water contained PMMoV at concentrations +BP_{LOQ} (1.0 copies per reaction)
699

700 **FIGURES**



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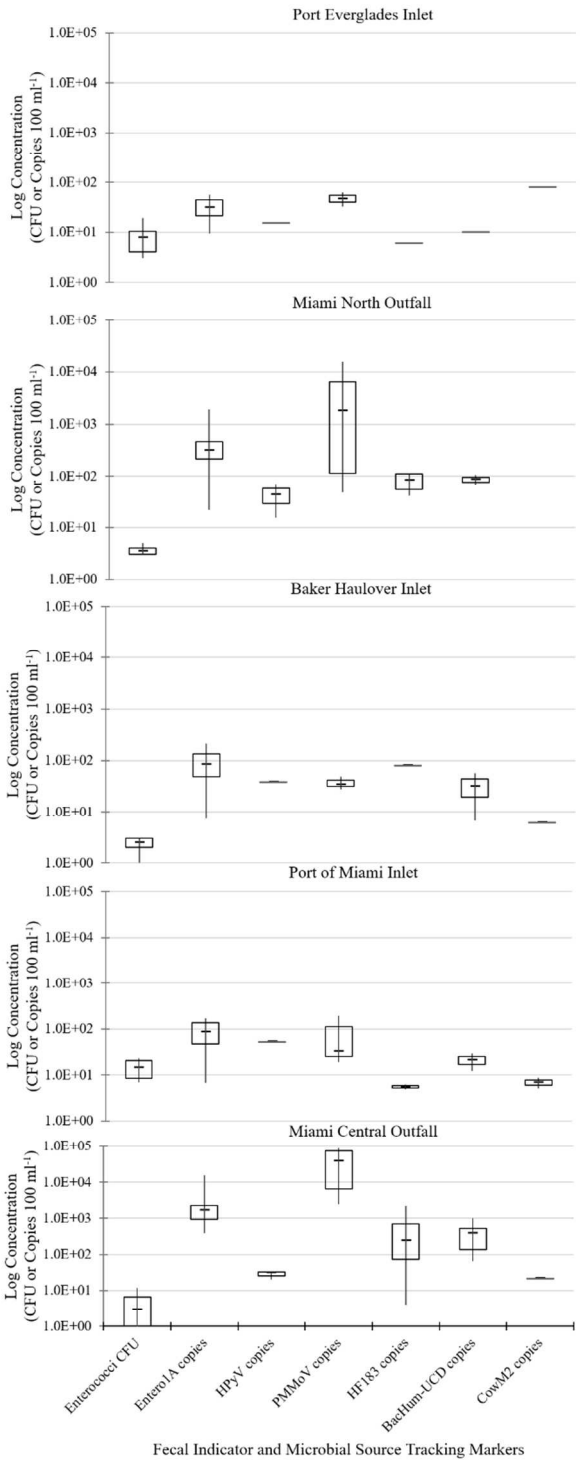
702 Figure 1. Map of the southeastern coast of Florida, USA that indicates the location of coastal

703 (cross), surface boil (circle), and inlet (triangle) sample collection sites, as well as the location of

704 the ocean outfalls (square) investigated.



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706 Figure 2. Concentrations of the following faecal indicator and microbial source tracking marker
707 concentrations in surface waters exposed to faecal pollution along the southeastern Florida coast:
708 culturable enterococci, total enterococci (Entero1A), human polyomavirus (HPyV), pepper mild

mottle virus (PMMoV), human-associated *Bacteroides* sp. (HF183), human *Bacteroidales* (BacHum), dog *Bacteroidales* (DogBact), and cow *Bacteroidales* (CowM2). Sample sites are listed from north to south (top to bottom). The lower and upper boxes represent the 25^{th} and 75^{th} percentiles, respectively. The minimum and maximum values are represented by the lower and upper bars, respectively. Concentrations below the limit of quantification (LOQ) are depicted; thus, the LOQ of each assay should be noted: 25 copies 100 ml^{-1} for EPA entero1A, HF183, BacHum, DogBact, and CowM2; 500 copies 100 ml^{-1} for HPyV; and 80 copies 100 ml^{-1} for PMMoV. Twenty-seven percent of samples were below the limit of detection (BLOD) for Entero1A, 53% for HPyV, 37% for PMMoV, 53% for HF183, 57% for BacHum, 60% for DogBact, and 83% for CowM2. Note the logarithmic scale on the vertical axis.

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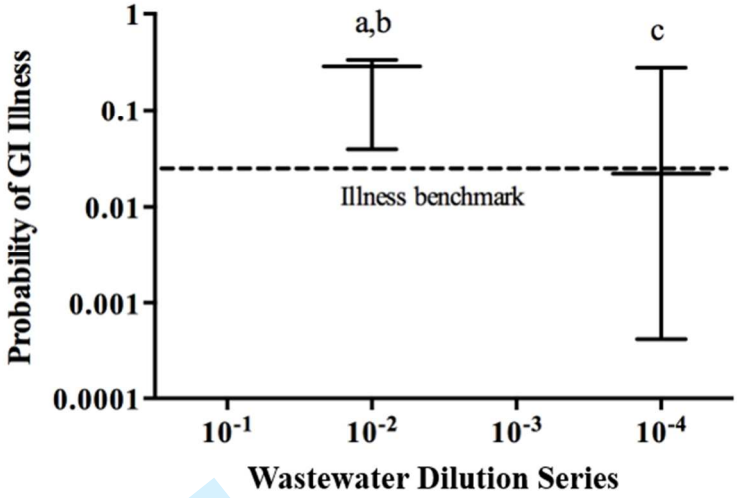

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725 Figure 3. Pepper mild mottle virus (PMMoV) process limits of detection (P_{LOD}) juxtaposed with
726 the estimated gastrointestinal (GI) illness rates due to norovirus for serially-diluted untreated
727 wastewater. The 25th, median, and 75th probabilities of GI illness are plotted for the accidental
728 ingestion due to recreation in water contaminated with each wastewater-dilution that corresponds
729 to the PMMoV P_{LOD} of coastal water (a), inlet water (b), and distilled water (c).