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Cryptosporidium and *Giardia* in tropical recreational marine waters contaminated with domestic sewage: Estimation of bathing-associated disease risks

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ABSTRACT

Sewage is a major contributor to pollution problems involving human pathogens in tropical coastal areas. This study investigated the occurrence of intestinal protozoan parasites (*Giardia* and *Cryptosporidium*) in tropical recreational marine waters contaminated with sewage. The potential risks of *Cryptosporidium* and *Giardia* infection from recreational water exposure were estimated from the levels of viable (oo) cysts (DIC+, DAPI+, PI−) found in near-shore swimming areas using an exponential dose response model. A Monte Carlo uncertainty analysis was performed in order to determine the probability distribution of risks. Microbial indicators of recreational water quality (enterococci, *Clostridium perfringens*) and genetic markers of sewage pollution (human-specific *Bacteroidales* marker [HF183] and *Clostridium coccoides*) were simultaneously evaluated in order to estimate the extent of water quality deterioration associated with human wastes. The study revealed the potential risk of parasite infections via primary contact with tropical marine waters contaminated with sewage; higher risk estimates for *Giardia* than for *Cryptosporidium* were found. Mean risks estimated by Monte Carlo were below the U.S. EPA upper bound on recreational risk of 0.036 for cryptosporidiosis and giardiasis for both children and adults. However, 95th percentile estimates for giardiasis for children exceeded the 0.036 level. Environmental surveillance of microbial pathogens is crucial in order to control and eradicate the effects that increasing anthropogenic impacts have on marine ecosystems and human health.

The shallow coastal ocean impacted by human wastes and pollution-laden runoff may harbor numerous pathogenic microorganisms that are epidemiologically associated with diseases in the human population that lives along the coastline (Shuval, 2003). Hence, the coastal habitat that provides a source of food and a natural environment for recreation may also act as a reservoir of microbial hazards to human health (Stewart et al., 2008).

Cryptosporidium and *Giardia* are intestinal protozoan parasites frequently found in surface waters worldwide. The infectious stages (i.e., (oo) cysts) excreted in large numbers with human and animal feces (10^8–10^9 per grams of stool) are environmentally robust, which facilitates the horizontal transmission of these waterborne parasites (Caccio et al., 2005). The species *Giardia duodenalis* (assemblages A and B), *Cryptosporidium parvum* and *C. hominis* are most commonly associated with human parasitic infections. Therefore, the (oo) cysts are frequently found in untreated sewage and to a lesser extent in treated domestic sewage (Medema and Schijven, 2001; Van Dyke et al., 2012; Robertson et al., 2006; Betancourt et al., 2010). Marine waters polluted with wastewater discharges may become potential exposure pathways for transmission of parasitic diseases through recreational water contact (World Health Organization (WHO), 2005, Overstreet, 2013). The risk for disease transmission increases with exposure (e.g., direct contact while swimming) to recreational marine waters adjacent to populated coastal areas with inadequate sewerage and sanitation coverage; which commonly occurs on islands and continental countries of the Wider Caribbean Region (UNEP, 2010).

The severity of illness and therefore the public health impacts associated with bathing in marine recreational waters contaminated with domestic sewage were initially reported from studies conducted in the United Kingdom (Fleisher et al., 1998). More

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recent studies in the United States demonstrated the association between bather density and levels of *Cryptosporidium* and *Giardia* in beach waters (Graczyk et al., 2010). In addition, the risk of recreational exposure to *Cryptosporidium* has been assessed among persons with HIV/AIDS from studies conducted in the U.S. (McOliver et al., 2009). Studies have also been conducted investigating the occurrence of protozoan parasites in tropical recreational marine waters impacted by marine sewage discharge (Johnson et al., 1995) as well as in subtropical recreational marine waters impacted by non-point sources of pollution (Abhelzaher et al., 2010). Nevertheless, the risk of parasitic diseases via recreational exposure to sewage contaminated marine waters in the tropics has not been estimated.

The tropics corresponds to the climate zone of the earth surrounding the Equator that receives the highest amount of solar radiation (90%), which influences the regional hydroclimate and generates two main recurrent weather patterns of heavy rains alternating with dry seasons. Solar emissions of ultraviolet radiation with the highest microbicidal effect (UV-B wavelengths between 270 and 320 nm) occur in the tropics along with high (>20 °C) sea surface temperatures (Tiwari, 2002). These two abiotic factors (temperature and sunlight irradiation) may affect the survival of microbial pathogens in natural waters (Gerba, 2007).

The potential direct impacts of the oceans on human health have been intricately linked to climate (i.e., temperature, precipitation, and winds) and its interaction with biological, socioeconomic and cultural factors (Fleming et al., 2006). Furthermore, the interactions between oceans and human health are increasingly important in subtropical and tropical areas where significant proportions of the population lives in close proximity to the coastal ocean (Knap et al., 2002; Tibbetts, 2002). Thus, expanding human populations are altering coastal environments through increased residential, commercial, and recreational development (i.e., tourism) and through the introduction of land-based waste products (i.e., sewage and chemicals) into coastal waters (Trujillo and Thurman, 2008; Karydis and Kitsiou, 2013).

Venezuela is a tropical country situated on the northern coast of South America and therefore also integrated to the geographical area of the Wider Caribbean Region. Venezuela's central coast is intensively used for recreation but is also severely affected by improper waste management infrastructure (i.e., outdated sewerage systems) from tourism facilities and residential areas, and by communities lacking any sewage treatment that practice direct disposal of wastes into nearby coastal waters. These conditions generate significant impacts on the quality of coastal resources, including environmental degradation of marine recreational areas with the introduction of sewage-borne pathogens.

In this study several marine beaches located in Venezuela's central coast were selected for sampling for *Cryptosporidium* and *Giardia* in near-shore swimming areas in order to estimate the risks of waterborne parasitic diseases acquired while undertaking water-based recreation in tropical marine waters. Fecal indicator bacteria (FIB, enterococci and *C. perfringens*) were evaluated in order to estimate the extent of water quality deterioration based on quantitative data derived from conventional bacterial culture methods. For accurate and rapid identification of the major source of marine pollution affecting the coastal environment, genetic markers of sewage pollution (human-specific *Bacteroidales* marker [HF183] and *Clostridium coccoides*) were assayed using sequence detection methods based on SYBR Green I real time PCR followed by post-amplification melting-curve analysis.

Sampling was conducted during periods of no rain; therefore environmental parasite load corresponds primarily to point sources of pollution with little or no contribution from non-point sources, except those from the bathers themselves. The sampling survey included control sites equivalent to secluded beaches with limited access and no evidence of human sewage pollution. Table 1 lists the geographical coordinates of sampling locations and physicochemical parameters measured at the different beaches during sample collection.

Cryptosporidium and *Giardia* occurrence was evaluated by two filtration methods using ColorSeed C&G spikes (EasyStain, BTF A bioMérieux Company, Sydney, Australia) as internal quality control parameters to determine (oo) cyst recovery efficiencies in marine waters. All beach water samples (10 L) were prefiltered through 11 μm pore-size nylon membranes (EMD Millipore Corp., Billerica, MA, USA). For the primary concentration of (oo) cysts the samples were filtered separately through the Filta-Max system (IDEXX Laboratories, Inc., USA) or through 3 μm polycarbonate track-etched membrane disc filters (EMD Millipore Corporation, Billerica, MA, USA). Immunomagnetic separation (IMS) for selective separation of (oo) cysts was performed with a Dynal GC-Combo kit following the manufacturer's instructions and modifications previously described (Quintero-Betancourt et al., 2003). Briefly, IMS concentrates (50 μl) were fixed with absolute methanol and stained with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (EasyStain, BTF A bioMérieux Company, Sydney, Australia) specific for *Cryptosporidium* and *Giardia*. Confirmation of (oo) cysts was accomplished through vital dye staining (4',6'-diamidino-2-phenylindole [DAPI]/propidium iodide [PI]) along with Nomarski differential interference contrast (DIC) microscopy to look at the internal morphology of (oo) cysts. (oo) cyst positive slides plus additional IMS concentrates (50 μl) were processed for DNA extraction and genotyping of *Cryptosporidium* and *Giardia*. The protocol described by Nichols et al. (2006) was used to recover the (oo) cysts from the slides applying 40 μl of lysis buffer (LB; 50 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0, 0.5% sodium dodecyl sulfate). The scraped samples in LB and IMS concentrates were transferred to screw-cap micro centrifuge tubes and mixed with 100 μl of InstaGene Matrix (Bio-Rad, Hercules, CA, USA). (oo) cyst DNA was extracted using the freeze–thaw method that consisted of eight cycles of freezing in liquid nitrogen for 1 min and thawing at 95 °C for 1 min. After a quick spin the DNA recovered from the supernatant was used for molecular detection of parasites. The molecular assays were based on nested PCR amplification of the small-subunit (SSU) rRNA gene of *Cryptosporidium* and the triosephosphate isomerase (*tpi*) gene of *Giardia* (Xiao et al., 2001; Sulaiman et al., 2003). Randomly selected PCR products were directly sequenced with the nested forward primer to confirm species identity.

Microbial contamination at the beaches was evaluated with methods for enumeration of fecal indicator bacteria (FIB) using the Defined Substrate Technology (DST) with the Quanti-Tray/2000 method and the Enterolert test (IDEXX Laboratories, Inc., USA) for enumeration of enterococci. Membrane filtration was used for enumeration of *Clostridium perfringens* and also enterococci. Water samples (100–500 mL) were filtered through 47-mm, 0.45-μm- pore- size mixed cellulose esters MF white gridded (EMD Millipore Corp., Billerica, MA, USA) that were subsequently placed onto mCP medium with m-CP supplements (Oxoid Limited, United Kingdom) for enumeration of *Clostridium perfringens* (21 ± 3 h, 44 ± 1 °C) and onto mEI medium (Messer and Dufour, 1998) for enumeration of enterococci (24 or 48 h, 41 ± 0.5 °C).

Quantitative real-time polymerase chain reaction assays (qPCR) were applied for microbial source tracking of human sewage using SYBR Green qPCR and melting curve analysis for specific detection of 16S rRNA gene sequences of the human *Bacteroidales* marker (HF183) and members of the *Clostridium coccoides* group (Seurinck et al., 2005; Matsuki et al., 2004). Total DNA was extracted from 47 mm Supor-200 filters (Pall Corporation, USA) used to filter 100 mL of seawater followed by bead beating treatment (Noble et al., 2010). Ten-fold dilutions of DNA extracts were used to test for PCR inhibition.

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Table 1
Geographical coordinates of sampling locations and physicochemical parameters measured at the different beaches during sample collection.

Sample locations	Geographical Coordinates	Water temperature (°C)	Salinity (pss)	Turbidity (NTU)	Dissolved oxygen (mg/L)
Beach I	10°37′ 0.87″ N	27.54	39.67	1.24	8.20
	66°50′ 19.6″ W	28.19	37.88	0.81	7.30
		30.30	37.30	7.58	7.80
		28.20	37.30	0.81	7.30
Beach II	10°37′ 13.3″ N	27.87	39.80	1.29	8.68
	66°50′ 19.6″W	28.00	36.94	2.34	7.68
Beach III	10° 37′41.0″ N	28.80	35.70	1.52	7.85
	66° 27′233.1″ W				
Beach IV	10° 36′37.6″ N	27.59	38.46	0.23	7.88
	66° 52′26.5″ W	28.30	37.84	1.96	7.82
Control sites	10° 21′ 0.3″ N	29.60	35.84	0.43	6.93
	64° 24′45.9″ W	29.70	39.25	0.01	6.43
	10° 58′ 22.0″ N	29.80	36.17	0.01	7.31
	68° 14′56.9″ W	29.70	38.33	0.19	7.81

Note: Geographical coordinates of sampling locations, water temperature, and salinity (pss; practical salinity scale) were determined with the GPS Multiparameter Meter with Fast Tracker Tag Identification System (Hanna Instrument). Turbidity (NTU, Nephelometric Turbidity Unit) and dissolved oxygen (mg/L) were measured with a Hach Handheld Turbidity meter and an YSI ProODO optical dissolved oxygen instrument, respectively.

Data were analyzed using SPSS statistical software (IBM Inc., Armonk, NY). To account for skewed (non-normal) distributions typical of microbial count data, non-parametric tests were used. Kendall Tau rank correlation coefficients were used to assess associations between two continuous variables, while the Mann–Whitney rank sum test was used for comparisons between two groups. Tests were considered statistically significant at p values of 0.05 or less.

The potential risks of *Cryptosporidium* and *Giardia* infection from recreational water exposure were estimated from the levels of (oo) cysts found in near-shore swimming areas using an exponential dose response model (Haas et al., 1999): $\text{Risk} = 1 - \exp(-k * d)$, where k is the exponential dose response parameter and d is the ingested dose of viable pathogens. Point estimates of risk were calculated for each pathogen concentration measurement. In these point estimates, values of 0.026 (*Giardia*) and 0.0572 (*Cryptosporidium*) were used for k (Enger 2013a,b). For *Cryptosporidium* and *Giardia* it was assumed that 39% and 50% of infected individuals became ill, respectively (Haas et al., 1999). Dose was calculated as $\text{dose} = C * 1/R * I * V$, where C is the concentration of pathogenic microorganisms in surface water, R is the recovery efficiency of the method (based on the spike recovery for the sampling method), I is the viability, or fraction of the detected pathogens that is capable of infection (values for I of 40% for *Giardia* and 20% for *Cryptosporidium* were used based on data obtained from this study), and V is the volume of water ingested (100 mL) per swim based on National Research Council data (National Research Council, 1993).

A Monte Carlo analysis (i.e., risk estimation) was performed in order to determine the probability distribution of risk. Table 2 summarizes the statistical distributions used to characterize uncertainty in the model and input variables along with the sources used to develop these distributions. Viable fraction and probability of illness given infection were treated as constants in the analysis, and the values used for the point estimates (see above) were used in the Monte Carlo analysis. Distributions for the pathogen concentrations were fit to observations from the four beaches sampled in this study without regard to the beach from

Table 2
Input distribution for Monte Carlo uncertainty analysis.

Input parameter	Distribution	Parameters	Source
Viable *Giardia* by membrane filtration	Lognormal	Log mean = −0.44 Log standard deviation = 3.0	This study (adjusted levels in Table 3 multiplied by viable fraction of 0.4)
Viable *Giardia* by Filta-Max	Lognormal	Log mean = 0.64 Log standard deviation = 2.5	This study (adjusted levels in Table 3 multiplied by viable fraction of 0.4)
Viable *Cryptosporidium* by membrane filtration	Lognormal	Log mean = −2.9 Log standard deviation = 1.6	This study (adjusted levels in Table 3 multiplied by viable fraction of 0.2)
Viable *Cryptosporidium* by Filta-Max	Lognormal	Log mean = −1.3 Log standard deviation = 1.6	This study (adjusted levels in Table 3 multiplied by viable fraction of 0.2)
Ingestion by adults	Lognormal	Log mean = −4.1 Log standard deviation = 0.64	U.S. EPA (2011)
Ingestion by children	Lognormal	Log mean = −3.3 Log standard deviation = 0.47	U.S. EPA (2011)
Exponential dose response parameter for *Giardia*	Lognormal	Log mean = −3.9 Log standard deviation = 0.26	Enger (2013a)
Exponential dose response parameter for *Cryptosporidium*	Lognormal	Log mean = −2.9 Log standard deviation = 1.5 (k values truncated at 1)	Enger (2013b)

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Table 3
Distribution of protozoan parasites, fecal indicator bacteria and genetic markers of sewage pollution in tropical recreational marine waters from Venezuela's central coast.

	Protozoan parasites[a]				Fecal indicator bacteria[b]		Sewage markers[c]	
					CFU/100 mL (MPN/100 mL)			
	Giardia cysts/L (Recovery efficiency,%) [Adjusted level/L]		*Cryptosporidium* oocysts/L (Recovery efficiency,%) [Adjusted level/L]		Enterococci	*C. perfringens*	HF183	*C. cocc*
	MF	FM	MF	FM				
Beach I	17(13)[131]	13(7)[186]	2(56)[4]	2(15)[13]	19(>2419)	22	+	+
	<0.2	<0.2	<0.2	<0.2	35(>2419)	54	+	+
	0.1(19)[0.5]	<0.2(13)	0.2(58)[0.34]	0.1(31)[0.32]	155(1870)	45	+	+
	<0.2	0.2	<0.2	<0.2	ND	ND	+	+
Beach II	4(30)[13]	1.6(7)[23]	<0.2(43)	<0.2(9)	27(>2419)	59	+	+
	0.2	2.2	<0.2	<0.2	108(2359)	34	+	+
Beach III	<0.2(24)	<0.2(4)	<0.2(42)	<0.2(11)	104(279)	13	+	+
Beach IV	<0.2(15)	1.4(48)[3]	<0.2(35)	0.6(14)[4.28]	67(1986.3)	19	+	+
	<0.2	<0.2	<0.2	<0.2	120(1732.9)	42	+	+
Control sites	<0.2	<0.2	<0.2	<0.2	0.2(14.6)	<0.2	–	–
	<0.2	<0.2	<0.2	<0.2	0.2(100.5)	<0.1	–	–
	<0.2	<0.2	<0.2	<0.2	0.4(115.2)	<0.1	–	–
	<0.2	<0.2	<0.2	<0.2	<0.1(138.0)	<0.2	–	–

[a] Levels of (oo) cysts detected are expressed by liter of sample. Adjusted level of (oo) cysts detected based on recoveries obtained from experiments performed the same day of sample collection. Numbers preceeded by the < symbol indicate samples with no detected (oo) cysts. The latter is also applicable to FIB. MF, membrane filtration. FM, Filta-Max.
[b] CFU, colony forming units per 100 mL. MPN, most probable number per 100 mL. ND, not done.
[c] +, indicates positive qPCR signal. –, indicates negative qPCR signals.

which the samples were taken. As such the results of the Monte Carlo simulation are not specific to any single beach but reflect variability in risk across different beaches. Data from the control sites were not included in the data used to fit the distributions as it was not considered representative of recreational beaches. Log-normal distributions were used to fit the input variable distributions, as all variables were right-skewed and constrained to be positive. One thousand samples were taken from all input distributions. The dose–response parameters are constrained to be no greater than 1. None of the simulated values from the distribution for *Giardia* exceeded 1. However, some of the simulated values for *Cryptosporidium* did exceed 1, so these values were replaced by 1 in the Monte Carlo simulation. Eight different scenarios were considered. All scenarios consisted of exposure either to *Giardia* or *Cryptosporidium* during a single swim. The results of the membrane filtration method and the Filta-Max were treated as separate scenarios. Exposures of adults and children were also considered as separate scenarios with children having higher exposures due to a tendency to ingest more water while swimming (U.S. EPA, 2011). The same dose–response model was used for both adults and children, which may not capture the additional risk to young children with immature immune systems.

Giardia cysts were detected in 9 (35%) of the 26 samples collected from the different beaches, while *Cryptosporidium* oocysts were detected in 5 (14%) of these samples. Overall, the two filtration methods performed similarly in (oo) cyst recovery from marine waters; however membrane filtration provided higher recoveries of spiked (oo) cysts (Table 1). Mann–Whitney tests indicated that the improvement in recovery was significant for *Cryptosporidium* ($p = 0.016$) but not for *Giardia* ($p = 0.19$).

The levels of (oo) cysts detected at the beaches varied according to the extent of sewage pollution present and bather density during sample collection. Beaches I and II had the highest level of (oo) cysts (2 oocysts/L and 17 cysts/L) detected; these were also high bather density beaches that were characterized by poor water quality based on relative levels of FIB and the molecular detection of genetic markers of sewage pollution (Table 3). DIC and epifluorescence microscopy indicated that DIC+, DAPI + PI– *Giardia* and *Cryptosporidium* (oo) cysts (i.e., potentially infectious (oo) cysts) recovered from swimming recreational areas accounted for 40% and 20% of the total count obtained from microscopic-based detection, respectively. Moreover, sequence analysis of *tpi* gene identified only the anthropogenic enteric species (*G. duodenalis*), thereby indicating the potential risk of *Giardia* infections via primary contact with tropical recreational marine waters contaminated with domestic sewage. *Cryptosporidium* genotyping was unsuccessful due to insufficient PCR product for sequencing; however, the most common human-pathogenic genotypes (*C. hominis* and *C. parvum*) would be more likely found in sewage-polluted coastal waters. The higher concentration and frequency of detection of *Giardia* over *Cryptosporidium* has been previously observed in surface waters contaminated with human sewage in Venezuela (Betancourt and Mena, 2012). Moreover, additional studies have demonstrated that surface waters heavily contaminated with sewage may harbor concentrations of *Giardia* from 10,400 to 62,000 cysts/liter (Betancourt et al., 2010) and concentrations of *Cryptosporidium* from 620 to 1700 oocysts/liter (unpublished data). *Giardia* is also the most common protozoan parasite found in human fecal specimens submitted to clinical laboratories for parasitic examination, which might explain the widespread occurrence of this protozoan in sewage polluted surface waters in Venezuela.

In regard to fecal indicators, the combination of multiple culture-based methods (mEI, Enterolert, mCP) and microbial source tracking tools was able to demonstrate the extent of water quality deterioration associated with human sewage impacts. Statistically significant correlations were found between *Giardia* and *Cryptosporidium* (oo) cyst occurrence in marine waters using the membrane filtration method ($r = 0.732$, $p = 0.025$) but not further significant correlations were observed between pathogen occurrence and FIB. Although levels of *C. perfringens* were not correlated with (oo) cyst counts, positive results of this bacterium plus the *C. coccoides* group marker and the HF183 marker provided more conclusive information on the occurrence of sewage pollution which leads to the introduction of enteric pathogens into swimming recreational areas. *C. perfringens* is a conservative indicator for fecal excreta from human-associated sewage in both tropical and temperate waters, therefore an excellent indicator for point sources of pollution (Fujioka 2001; Vierheilig et al., 2013). The levels of *C. perfringens* in tropical coastal waters have been correlated with infection risks from recreational activities (Viau et al., 2011).

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Table 4
Point estimates of daily risk of *Giardia* and *Cryptosporidium* infections from levels of parasite contamination in tropical recreational marine waters using an exponential dose response model.

	Giardia (MF)[a]		*Giardia* (FM)[b]		*Cryptosporidium* (MF)		*Cryptosporidium* (FM)	
	Risk of infection	Risk of illness	Risk of infection	Risk of illness	Risk of infection	Risk of illness	Risk of infection	Risk of illness
Beach I	1.27×10^{-1}	6.36×10^{-2}	1.76×10^{-1}	8.78×10^{-2}	4.08×10^{-3}	1.59×10^{-3}	1.51×10^{-2}	5.90×10^{-3}
	5.47×10^{-4}	2.74×10^{-4}	$<1.60 \times 10^{-3}$	$<7.99 \times 10^{-4}$	3.94×10^{-4}	1.54×10^{-4}	3.69×10^{-4}	1.44×10^{-4}
Beach II	1.38×10^{-2}	6.89×10^{-3}	2.35×10^{-2}	1.17×10^{-2}	5.32×10^{-4}	2.07×10^{-4}	$<2.54 \times 10^{-3}$	$<9.90 \times 10^{-4}$
Beach III	$<8.66 \times 10^{-4}$	$<4.33 \times 10^{-4}$	$<5.19 \times 10^{-3}$	$<2.95 \times 10^{-3}$	$<5.45 \times 10^{-4}$	$<2.12 \times 10^{-4}$	$<2.08 \times 10^{-3}$	$<8.10 \times 10^{-4}$
Beach IV	$<1.39 \times 10^{-3}$	$<6.93 \times 10^{-4}$	3.03×10^{-3}	1.51×10^{-3}	$<6.54 \times 10^{-4}$	$<2.55 \times 10^{-4}$	4.89×10^{-3}	1.91×10^{-3}

[a] Membrane filtration.
[b] Filta-Max system.

Members of the *C. coccoides* group and *Bacteroidales* belong respectively to the phylums *Firmicutes* and *Bacteroidetes*, which include dominant groups of bacterial molecular species found within the fecal microbiota of healthy humans (Tap et al., 2009) and in sewage (McLellan et al., 2013). Taken together, data from FIB, genetic markers, and human parasites indicate that the application of holistic approaches in microbial water quality assessment offers an adequate baseline from which to determine the deleterious effects of sewage pollution on coastal environments, including those that pose threats to living resources and marine life, human health, and other legitimate uses of the sea. This study also highlights the usefulness of pathogen-specific detection approaches for accurate estimation of bathing-associated disease risks in specific geographical areas, given the multiple biological and chemical interactions that control both the survival and the pathways over which microbial pathogens are transported from sources to regions of potential human exposure (Dyble et al., 2008).

Table 4 summarizes the results of the point estimate of the risk of *Giardia* and *Cryptosporidium* in tropical marine waters used for recreation. The estimated risk of illness from *Giardia* exposure at Beach I was the greatest risk and the only risk that exceeded the upper bound of the U.S. EPA criterion for recreational illness rate (0.036, U.S. EPA, 2012), used as a reference point in this study for interpreting the risk outcomes. The recreational illness rate was exceeded for both the membrane filtration and the Filta-Max methods. The lower count observed for the Filta-Max is offset by the lower recovery estimate leading to reasonably comparable estimates of risk. In addition, the higher concentration and viable fractions for *Giardia* tend to outweigh the somewhat higher dose response parameter for *Cryptosporidium*, resulting in higher risk estimates for *Giardia* in most cases.

Results of the Monte Carlo simulation are shown in Table 5. Risks for Filta-Max and membrane filtration method compared well for *Giardia*. However, for cryptosporidiosis estimated risks were roughly 5 times higher when the Filta-Max method was used than when membrane filtration was used to quantify concentration, as this method produced higher concentrations. Further work identifying which method is more accurate is warranted as this would substantially reduce uncertainty in the risk estimates. Risks for children were roughly twice risks for adults, reflecting the greater ingestion of water by children during swimming. As with the point estimate, risks for *Giardia* exceeded the risks for *Cryptosporidium* (as noted above, the higher concentrations of *Giardia* outweigh the fact that *Cryptosporidium* has a higher dose response parameter than *Giardia*).

The mean risks from the Monte Carlo analysis are generally below the point risk estimates due largely to differences in the ingestion assumptions. For the point estimates a conservative (health-protective) estimate of 100 mL ingestion was used while for the Monte Carlo analysis a distribution with a median of 16 mL and a 97th percentile of 53 mL was used for adults, and a distribution with a median of 37 mL and a 97th percentile of 90 mL was used for children. It is generally appropriate to make health-protective assumptions for point estimates and to relax these assumptions in a Monte Carlo analysis (given that the upper bound of the Monte Carlo output will reflect health protective assumptions). The upper bound (95th percentile) of the giardiasis risks to children that are estimated by Monte Carlo analysis exceed the EPA criterion for recreational risk (0.036). It is also notable that at the lower bound all of the risks are well below the U.S. EPA risk criterion, suggesting that at some times and locations risk is minimal. Nevertheless, these results are an indication that risks due to recreational contact with marine waters in these areas can approach or exceed levels of concern, particularly for children. As such, these risks merit attention from researchers and public health officials.

In conclusion, the risks of parasitic diseases acquired while undertaking water-based recreation in tropical marine waters contaminated with domestic sewage can be estimated using quantitative microbial risk assessment. Through this framework, the expected dose and the distribution of doses of *Giardia* and *Cryptosporidium* were accurately determined based on a combination of experimental and computation methods which allowed making better estimates of the risks of recreational exposure to these waterborne protozoan pathogens.

The data reveal that the risks of infection for *Giardia* are significantly higher than those for *Cryptosporidium* based on the high frequency of detection and concentration of potentially infectious (DIC+, DAPI + PI−) *Giardia duodenalis* cysts found in marine waters, which may be epidemiologically associated with the pool of common microbial pathogens and diseases that occur in the human population that lives along the coastline. Thus, the application of molecular-based methods (i.e., genetic methods) to investigate the occurrence of infectious disease agents in tropical marine waters allows better understanding of the distribution and ecology of specific waterborne pathogens, including awareness of the

Table 5
Monte Carlo simulation results for risk.

	Risk of illness for adults mean (5th–95th percentiles)	Risk of illness for children mean (5th–95th percentiles)
Giardiasis (MF)[a]	4.9×10^{-3} (5.6×10^{-7}–1.4×10^{-2})	9.2×10^{-3} (1.4×10^{-6}–3.7×10^{-2})
Giardiasis (FM)[b]	5.5×10^{-3} (3.5×10^{-6}–1.9×10^{-2})	1.1×10^{-2} (9.7×10^{-6}–4.9×10^{-2})
Cryptosporidiosis (MF)	1.9×10^{-4} (4.7×10^{-7}–6.5×10^{-4})	4.7×10^{-4} (8.8×10^{-7}–1.5×10^{-3})
Cryptosporidiosis (FM)	9.3×10^{-4} (2.1×10^{-6}–3.2×10^{-3})	2.2×10^{-3} (4.0×10^{-6}–7.2×10^{-3})

[a] Membrane filtration.
[b] Filta-Max system.

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multiple but sometimes overlooked transmission routes that occur in the tropics.

The detection of genetic markers of sewage pollution (human-specific *Bacteroidales* marker [HF183] and *Clostridium coccoides*) along with culture-based methods for enumeration of FIB (enterococci and *C. perfringens*) provided a suitable approach to ascertain the level and to pinpoint the major source of fecal pollution occurring in the study area. While clearly more research is needed to fully understand the relationship between sewage-borne pathogens and surrogate indicators in tropical marine waters, this research highlights advantages from applying microbial source tracking tools for identifying sources of marine pollution in the tropics. Sewage is a major contributor of pollution problems with pathogens in tropical coastal areas, therefore rapid identification and evaluation of the extent of sewage pollution plus environmental surveillance of multiple microbial pathogens is crucial in order to control and eradicate the effects that increasing anthropogenic impacts have on marine ecosystems and human health.

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