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Anthropogenic Fecal Pollution Affects Microbial Water Quality and Multidrug Resistance in Tropical Aquatic Environments

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Anthropogenic Fecal Pollution Affects Microbial Water Quality and Multidrug Resistance in Tropical Aquatic Environments

by

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DEDICATION

To my family, it was your unconditional love and support that made it possible for me to pursue my dreams.

To all my friends and loved ones who always encouraged me to keep pushing forward during the most difficult times. Carlos, you helped me get here. B you gave me shelter, endless support and you made me fall in love with Saint Pete. Greta and Greg, you both rescued me and offered your shoulder in the hardest moments. Ernest, you were my biggest supporter when I needed it the most.

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To all international students that decide to leave their home country to pursue a postgraduate degree, you have my admiration.

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ABSTRACT

Although an often-overlooked issue, fecal pollution at recreational beaches should concern everyone. Worldwide more than 80% of wastewater is discharged into rivers or the ocean without any treatment. Untreated and inefficiently treated wastewater can introduce disease-causing microorganisms into the aquatic environment. Fecal-borne pathogens can also originate in animal waste, and humans can be exposed to these pathogens from poorly managed animal feces that make their way into the aquatic environment. Exposure to waterborne pathogens in recreational waters is a public health hazard, as it facilitates the transmission of waterborne illness, and surface waters serve as natural reservoirs of antibiotic resistant bacteria and their genes. To ensure safe swimming conditions the World Health Organization (WHO) recommends the use of sanitary inspections, routine monitoring, and quantitative microbial risk assessment (QMRA).

The use of fecal indicator bacteria (FIB) is essential for beach management. It is expensive and logistically challenging to try to measure all disease-causing microorganisms present in environmental waters. Therefore, we rely on compliance monitoring of FIB (e.g., enterococci) to help protect people from the health risks associated with swimming in polluted waters. FIB monitoring is based on standardized methodology, and the connection of FIB levels to gastrointestinal illness in swimmers is derived from epidemiology studies. Nevertheless, one of the major drawbacks of using FIB is that we are unable to differentiate sources of fecal contamination. FIB are present in the gut of numerous warm, and even cold-blooded animals, and can persist in extra-intestinal sources like sediments and vegetation. Due to the specific nature of the association

of many pathogens with their host (for example, viruses that specifically infect humans), there is an increased risk when fecal contamination comes from humans and certain animals such as cattle (for example, *Salmonella*, *Yersinia enterocolitica*, *Listeria monocytogenesis*, and *Crytosporidium* are often found in manure:). For this reason, methods to detect host-associated microorganisms, collectively termed microbial source tracking (MST) markers are used to identify microbial contamination from feces of key animal groups that may contaminate recreational waters. For example, quantitative real-time PCR assays that target the human-specific HF183 *Bacteriodes* 16S rRNA genetic marker is often used to detect human faecal pollution in surface waters. Epidemiological studies are the gold standard when it comes to determining the human health risk associated with exposure to polluted waters. However, these studies are expensive and logistically difficult to perform. Quantitative microbial risk assessment (QMRA) is a framework that uses mathematical models to estimate the risk of infection and disease when a population is exposed to microorganisms in the environment. In this sense, QMRA is a powerful tool that can be used to improve beach management, as it helps us understand the impact that certain microorganisms in the environment will have on the health of swimmers. Together, effective monitoring to characterize water quality, accurate identification of sources of pollution, and estimation of risk posed to swimmers are paramount to support management strategies to protect human health and the environment.

In chapter two of this dissertation, the relationships among microbial indicators of fecal pollution, MST marker genes, and pathogens were analyzed in Costa Rican coastal waters. We found that regardless of season, Jacó rivers were implicated as sources of human fecal contamination based on percent exceedance of recreational water quality guidelines (RWQC), high MST marker concentrations, and occurrence of diverse waterborne pathogens. We compared and

evaluated exceedance of RWQC and performance of indicator microorganisms and MST markers with respect to pathogen detection and determined that the US EPA enterococci statistical threshold value (STV) criterion had the highest positive (78%) and negative (96%) predictive value of all indicators in ocean samples. We concluded that no one fecal indicator or MST sewage marker best correlated with pathogens; rather, the use of multiple fecal indicators and MST markers maximized pathogen correlations. We recommend a fecal pollution toolbox approach, containing at least one viral indicator for pathogen prediction at tropical beaches, as the use of viral indicators maximized pathogen correlations in both river and ocean data.

Chapter three describes a second study at the polluted beach in Costa Rica, wherein indicators, MST markers and pathogens were measured in river and ocean samples. QMRA was performed to estimate the risk of gastroenteritis from swimming in rivers in three subwatersheds at the beach. We determined that median risk from pathogens in river samples was above the USEPA benchmark of 36 illnesses per 1000 recreators (it ranged from 0.345 to 0.577). Risk of gastrointestinal illness varied at a localized scale within three different subwatersheds at the beach. Norovirus genogroup I (NoVGI) followed by adenoviruses contributed the most to risk of gastrointestinal illness in all subwatersheds. We found that FIB exceedances were higher during rainy season, but risk was greater in the dry compared to rainy season, due largely to the increased frequency of detection of NoVGI (the main driver of risk) in dry season (100% vs 41%). We concluded that substantial viral $\log_{10}$ reduction (3.8 – 4.1 dry; 2.7 -3.2 rainy) are needed to ensure safe swimming conditions in Jacó rivers.

Lastly, chapter four is a field study in which we explored the extent of ampicillin resistance and multidrug resistance of the FIB *E. coli* and *Enterococcus* spp. in Costa Rican wastewater and surface waters. *E. coli* were more frequently resistant to ampicillin (18%) than

were *Enterococcus* spp (4%). Forty two percent of *E. coli* isolates and 45% of *Enterococcus* isolates in this study were multidrug-resistant (resistant to more than 3 antibiotic classes). *E. coli* isolates that were resistant to a combination of 6 different classes of antibiotics were found frequently and exclusively in the hospital wastewater. *E. coli* isolated from the hospital wastewater were more likely to be resistant (~ 40% of isolates) to gentamicin, cefotaxime, and ciprofloxacin versus those isolated from residential wastewater, the treated (but not disinfected) effluent and the estuary where the wastewater treatment plant discharges (<25% of isolates). *Enterococcus* isolates were frequently resistant to tetracycline (>50% of isolates), erythromycin (~ 25% of isolates) and ciprofloxacin (~10% to 25% of isolates). Our results indicate that although wastewater from hospital and residential water can be important sources of antibiotic resistant bacteria, those bacteria are also present in high frequency in the estuary and highlight the importance of disinfection of the treated effluent prior to its release into the environment.

Clean water is considered an essential human right by the United Nations. Not surprisingly, clean water is a prominent need described on the worldwide sustainable development agenda. The sustainable development goal to "ensure availability and sustainable management of water and sanitation for all", reminds us of the importance of water and sanitation in the global development agenda. Clean water and sanitation for all is an ambitious goal as millions of people worldwide collect their drinking water directly from surface waters and billons lack basic or managed sanitation services. A lack of progress on eliminating wastewater pollution impedes achieving many of the sustainable development goals. Furthermore, to achieve the water and sanitation-related sustainable development goals and address antibiotic resistance, sustainable management of water resources is necessary and more likely to be attainable by focusing efforts

in areas where sanitation is lacking. Results of this research serve as a baseline for future reference that can help improve coastal management of tropical polluted beaches.

CHAPTER ONE: BACKGROUND ON POINT SOURCE WASTEWATER POLLUTION EFFECTS ON MICROBIAL WATER QUALITY AND ANTIBIOTIC RESISTANCE IN TROPICAL BEACHES

Importance of the coastal environment

Coastal ecosystems play an important role in the global economy since they provide substantial goods and services, including the rapidly growing coastal tourism industry (Burke *et al.*, 2001; Hall, 2001). According to the World Tourism Organization, tourism is one of the most important sectors in global economy, i.e., in 2019 tourism contributed to 10.3 % of global gross domestic product (GDP) (WTTC, 2021). Coastal tourism is increasing worldwide, adding pressure to the marine environment, which is one of the environments most affected by human pollution (Sánchez-Quiles and Tovar-Sánchez, 2015). For example, domestic sewage is a pollutant that has a major deleterious effect on many marine and freshwater ecosystems. Worldwide more than 80% of wastewater resulting from human activities is discharged into rivers or the ocean without any treatment (United Nations World Water Assessment Programme, 2017). Fecal pollution in the form of untreated or inefficiently treated wastewater may lead to the introduction of enteric human pathogens and antibiotic resistant bacteria into the aquatic ecosystem, which pose a serious threat to human health worldwide (Cui *et al.*, 2019; Gottlieb & Nimmo, 2011).

Environmental variables and their implications for recreational water quality

Climate change factors and population growth are expected to increasingly compromise recreational waters through increased introduction of pathogens, resulting in increased risk of human exposure to waterborne pathogens (Crimmins *et al.*, 2016). Pollution input on fresh water and marine resources is expected to be intensified by climate change factors, such as, increased precipitation, hurricanes and storm surges (Crimmins *et al.*, 2016; IPCC, 2007). The amount of runoff can affect bacterial input to aquatic environments in tropical regions (Coffey *et al.*, 2014; Milly *et al.*, 2005; Muller *et al.*, 2011; Strauch *et al.*, 2014). Climate change also poses a significant threat to sanitation infrastructure when failure due to extreme weather events causes either damage or exceedance of sanitation system capacity, which can lead to increased sewage spills and water quality deterioration (Crimmins *et al.*, 2016; Howard *et al.*, 2010).

Waterborne pathogens and antibiotic resistance are a major public health concern

Infectious disease caused by pathogenic microorganisms (i.e., bacteria, viruses, protozoa and helminths), as well as antibiotic resistant bacteria are a global health concern (Frieden, 2013; World Health Organization, 2014). The economic burden of recreational waterborne illness in the US has been estimated at $2.2- $3.7 billion annually (DeFlorio-Barker *et al.*, 2018). Between 333–1696 hospitalizations and 16–67 deaths have been estimated to occur in the US due to waterborne illness annually (DeFlorio-Barker *et al.*, 2018). Antibiotic resistance has emerged as a growing concern due to the cost of treatment of infections by antibiotic resistant bacteria, and the cost to society due to loss of productivity (Frieden, 2013; WHO, 2014). According to the CDC, antibiotic resistant bacteria in the US cause more than 2.8 million infections and 35,000 deaths per year (CDC, 2019). In addition, multidrug-resistant infections, which are more difficult to

treat, are increasing worldwide (Levy and Bonnie, 2004; Nikaido, 2009). These and other reasons make antibiotic resistance among the biggest threats to human health. (WHO, 2015).

Microbial water quality monitoring of recreational waters

Fecal indicator bacteria (FIB) are commonly used to predict the risk of pathogen presence in recreational waters and to assess microbial water quality (Griffith *et al.*, 2009; McQuaig *et al.*, 2012; US EPA, 2012). Waterborne enteric pathogens may be shed by the host through feces into the aquatic environment and may then infect other hosts (Percival *et al.*, 2004). Detection of pathogens in water can be challenging because (1) pathogens can be very diluted in water, (2) methods of detection often lack analytical sensitivity, (3) currently there is no single method to detect all pathogenic microorganisms because of physical differences between pathogen groups (Straub y Chandler, 2003). FIB are therefore used instead to indicate the presence of fecal contamination and probable presence of pathogens. Measuring FIB (i.e., *Escherichia coli*, fecal coliforms, and enterococci) concentrations in surface waters is preferred to quantifying pathogens since FIB are present at higher concentrations in wastewater, culturing techniques are straightforward and relatively inexpensive, and the testing poses little risk to laboratory personnel (Harwood *et al.*, 2005).

The predictive relationship of FIB with pathogens has not been well established in tropical settings, since recreational water quality criteria have been extrapolated from studies performed in temperate regions (Boehm *et al.*, 2009; Fujioka *et al.*, 2015). US EPA criteria used to determine whether it is safe to swim were developed based on the relationships between FIB concentrations and the rates of recreational waterborne illness taken from epidemiology studies performed at temperate beaches impacted by point sources of pollution, i.e., a single identifiable

source of pollution (e.g., a discharge pipe) (Cabelli, 1983; Cabelli *et al.*, 1982; US EPA, 2012). Furthermore, studies did not find statistically significant correlations between levels of FIB and adverse human health outcomes when beaches were impacted by non-point sources of pollution (e.g., runoff and stormwater), or showed no correlations between indicators and pathogens (Boehm *et al.*, 2009; Fujioka *et al.*, 2015; Saingam *et al.*, 2020; US EPA, 2012).

Source of pollution matters for microbial water quality purposes

Conventional FIB cannot identify the source of pollution (e.g., point vs non-point source; human vs. animal), which limits their use in monitoring water quality. FIB are not exclusively found in human feces; rather, high levels are present in the feces of a variety of animals (Ashbolt *et al.*, 2001; Nguyen *et al.*, 2018) and are able to persist and grow in the sediments and vegetation of tropical environmental waters (Anderson *et al.*, 2005; Desmarais *et al.*, 2002; Fujioka *et al.*, 1998; Nguyen *et al.*, 2018; Symonds y Breitbart, 2015). Accurate identification of the source(s) of fecal pollution in environmental waters can help to improve water management and risk prediction of being exposed to polluted waters (Ahmed and Kinzelman, 2015; Nguyen *et al.*, 2018). Risk of illness for swimming in waters polluted with sewage varies depending on the source of fecal pollution (Fujioka *et al.*, 2015). Microbial hazards include pathogens of human and animal origin (Soller *et al.*, 2014). High risk is expected when the source of pollution is human sewage due to host-specificity of many waterborne pathogens that infect only humans, i.e., viruses such as NoVGI (Fujioka *et al.*, 2015). Different risk is expected when FIB source comes from different animals since different species contain different pathogens (Brown *et al.*, 2017; Fujioka *et al.*, 2015; Soller *et al.*, 2014). Minimal risk is expected when FIB multiply in the environment, because it implicates that FIB are indigenous to microbial communities in the

environment and therefore not indicative of a recent fecal contamination in water (Byappanahalli *et al.*, 2012; Fujioka *et al.*, 2015).

Alternative indicators to conventional FIB in tropical waters, microbial source tracking of fecal pollution and reference pathogens

To account for the limitations of the conventional FIB identified in the U.S. Recreational Water Quality Criteria (RWQC) (enterococci or *E. coli*), alternative indicator microorganisms have been increasingly explored in microbial water quality testing and research (Fujioka *et al.*, 2015; US EPA, 2012). The US State of Hawaii adopted *Clostridium perfringens* as secondary tracer bacteria (to be used in addition to enterococci), but no beach action is taken using *C. perfringens* results because of the lack of associations of *C. perfringens* concentrations and health risks. (Clean Water Branch, 2020). Coliphages have been proposed as viral indicators (Fujioka *et al.*, 2015; US EPA, 2015). Reference human pathogens, like *Giardia*, *Cryptosporidium*, and norovirus, are often measured in microbial water quality studies since they provide direct evidence of human health risk (Ashbolt *et al.*, 2010; Boehm *et al.*, 2009). Microbial source tracking (MST) methods have been developed to determine sources of fecal pollution (Chase *et al.*, 2012; Harwood *et al.*, 2009). MST markers are derived from genes of host-specific microbes. Microorganisms associated with human feces and wastewater are frequent targets of MST assays, including the 16S rRNA gene of Bacteroides *dorei* (HF183) (Seurinck *et al.*, 2005) and pepper mild mottle virus (PMMoV) (Symonds *et al.*, 2018, 2016). MST markers for animal sources have also been developed; some animal-specific markers target the 16S rRNA gene of either porcine/dog/cow-associated *Bacteroidales* and shorebird markers target the 16S rRNA gene of *Catellicoccous marimammalium* (Harwood *et al.*, 2014).

Quantitative microbial risk assessment (QMRA)

The World Health Organization recommends the use of sanitary inspections, routine monitoring, and QMRA to ensure safe swimming conditions (WHO, 2021). QMRA is a framework that uses mathematical models to estimate the risk of infection and disease when a population is exposed to microorganisms (Ashbolt *et al.*, 2010), and is increasingly used to predict the risk of illness from human exposure to natural recreational waters (Federigi *et al.*, 2019). Typically, QMRA in recreational surface waters includes several steps that are well described in the QMRA wiki community portal (http://www.qmrawiki.org/). The first step is the hazard characterization, where the microorganism of interest and related health outcome, i.e., risk of gastrointestinal illness, is identified. Following is the dose-response assessment. Dose response models are mathematical equations that describe the dose response relationship for a pathogen, transmission routes, and hosts, and is based on literature of medical studies of controlled or uncontrolled experiments (outbreaks) where the dose of the pathogen that the patient receives and then their response are measured. After the best dose-response of a given pathogen is identified, the exposure assessment is used to calculate the dose of pathogen ingested, considering the pathway from the source of the pathogen (i.e., concentrations in sewage) to the actual exposure (swimming at the beach). Then in the risk characterization step, the calculated dose of ingestion (from exposure assessment) feeds into the dose-response models (dose response assessment) to predict the probability of risk per dose ingested (e.g., risk of infection, illness, or death given a known dose of a pathogen). Finally, risk management (actions needed to reduce or eliminate risks) is most effective when informed by scientific assessment of risk through risk characterization. Risk management can also be improved by the combination of QMRA with MST which provides valuable information on the extent and specific source contribution to fecal contamination of superficial

waters. This way, beach managers can prioritize and address health risks, which can't be achieved by relying on FIB compliance monitoring only (Ashbolt *et al.*, 2010; Zhang *et al.*, 2019).

Monitoring of antibiotic resistant bacteria and multidrug resistance

There is an increasing need for monitoring antibiotic resistance in environmental waters. Fecal indicator bacteria are the most widely used indicators of fecal pollution in water. Monitoring FIB that can be isolated from both the environment and the human and animal gut since they are of fecal origin but can persist in the environment, is a good strategy for understanding antibiotic resistance prevalence and multidrug resistance (Hernando-Amado and Baquero, 2019; Oliveira *et al.*, 2020). FIB are known to acquire resistance (Berendonk *et al.*, 2015). In addition, FIB such as *E. coli* and *Enterococcus* spp., have clinical relevance since they are leading causes of nosocomial infections (Collignon, 2013; Emori and Gaynes, 1993; Ortega *et al.*, 2007). The use of FIB such as *E. coli* for the surveillance of antibiotic resistance and multidrug resistance in the environment is ideal since verified methods for isolation and characterization of FIB are available, and reference laboratories can easily implement FIB in monitoring (Anjum *et al.*, 2021).

Research chapters: Objectives of the dissertation

Chapter 2: The relationships among microbial indicators of fecal pollution, microbial source tracking markers, and pathogens in Costa Rican coastal waters

Rationale: The suitability of fecal indicator bacteria (FIB) used to regulate recreational water quality, to indicate the presence of pathogens in tropical aquatic environments is still being challenged. Information on indicators of fecal pollution, microbial source tracking markers, and

pathogens in tropical environments is lacking, therefore fecal coliforms, *Enterococcus*, *C. perfringens*, somatic coliphages, F+ coliphages, *Cryptosporidium*, *Giardia*, NoVGI, AdV and *Salmonella* were measured at a tropical polluted beach to determine which microorganisms best predict the presence of pathogens.

Methodology: Microorganism concentrations were measured at a tropical beach influenced by sewage-contaminated rivers and a multivariate statistical approach was used to identify correlations between indicator microorganisms and MST markers, and pathogens in the rivers and ocean. Measure and compare FIB performance (specificity, sensitivity) with respect to pathogen presence/absence.

Hypothesis: There will be significant correlations between FIB and pathogens, but stronger correlations are expected between sewage specific MST markers and enteric pathogens, given that two of the reference pathogens measured are human-specific.

Chapter 3: Risk of gastroenteritis from swimming at a wastewater-impacted tropical beach varies across localized scales

Rationale: Disease-causing microorganisms are present in our environment, and the levels at which they will cause illness in the population is location specific. Information on microbial source tracking markers, and pathogens in tropical environments is lacking. MST markers HF183 and PMMoV were measured to determine the source of pollution and pathogens *Cryptosporidium*, *Giardia*, NoVGI, AdV and *Salmonella* were measured to determine the risk from swimming in three different subwatersheds at tropical polluted beach to estimate the risk of GI illness and pathogen reductions necessary to ensure safe swimming conditions.

Methodology: Pathogens, fecal microorganisms and MST markers concentrations were measured at three different subwatersheds and QMRA was used to estimate the risk of gastrointestinal illness of swimming in three polluted rivers, and the potential pathogen reductions necessary to ensure safe swimming conditions.

Hypothesis: Microorganisms and health risks associated with swimming in polluted rivers is expected to be vary on a localized scale due to the difference in land use surrounding each subwatershed, flow rate per river, and mixing occurring near the coast and currents that are likely to affect differently microorganisms' fate in the ocean.

Chapter 4: Multidrug-resistant E. coli and Enterococcus sp. in Costa Rican wastewater and surface waters

Rationale: Antibiotic resistant bacteria pose a threat to human health but relatively little is known about the frequency of antibiotic resistant FIB and multidrug resistance in sewage and recreational waters in tropical developing countries. Fecal indicator bacteria *Escherichia coli* and *Enterococcus* spp were cultured from hospital and residential wastewater, the treated effluent, and the receiving estuary in Costa Rica, to determine the susceptibility of FIB to several antibiotics and multidrug resistance prevalence across sites.

Methodology: Susceptibility of ampicillin resistant *E. coli* and *Enterococcus* spp. to several classes of antibiotics were measured and compared to determine if there are differences in resistance among FIB from different sources (hospital and residential wastewater), treated effluent, and the WWTP discharged into the Puntarenas estuary that is adjacent to a popular beach.

Hypothesis: Greater concentrations of ampicillin resistant FIB and higher number of isolates resistant to multiple antibiotics are expected in Hospital wastewater, which contains human

enteric pathogens that may include antibiotic-resistant bacteria originating from hospital patients, followed by the residential wastewater, and significantly lower levels of resistance are expected after secondary treatment in the treated affluent and in the environment, where discharge is further diluted into the estuary.

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CHAPTER TWO: RELATIONSHIPS AMONG MICROBIAL INDICATORS OF FECAL POLLUTION, MICROBIAL SOURCE TRACKING MARKERS, AND PATHOGENS IN COSTA RICAN COASTAL WATERS

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Relationships among microbial indicators of fecal pollution, microbial source tracking markers, and pathogens in Costa Rican coastal waters

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ABSTRACT

Tropical coastal waters are understudied, despite their ecological and economic importance. They also reflect projected climate change scenarios for other climate zones, e.g., increased rainfall and water temperatures. We conducted an exploratory microbial water quality study at a tropical beach influenced by sewage-contaminated rivers, and tested the hypothesis that fecal microorganisms (fecal coliforms, enterococci, *Clostridium perfringens,* somatic and male-specific coliphages, pepper mild mottle virus (PMMoV), *Bacteroides* HF183, norovirus genogroup I (NoVGI), *Salmonella, Cryptosporidium* and *Giardia*) would vary by season and tidal stage. Most microorganisms' concentrations were greater in the rainy season; however, NoVGI was only detected in the dry season and *Cryptosporidium* was the only pathogen most frequently detected in rainy season. Fecal indicator bacteria (FIB) levels exceeded recreational water quality criteria standards in >85% of river samples and in <50% of ocean samples, regardless of the FIB or regulatory criterion. Chronic sewage contamination was demonstrated by detection of HF183 and PMMoV in 100% of river samples, and in >89% of ocean samples. *Giardia, Cryptosporidium, Salmonella*, and NoVGI were frequently detected in rivers (39%, 39%, 26%, and 39% of samples, respectively), but infrequently in ocean water, particularly during the dry season. Multivariate analysis showed that *C. perfringens*, somatic coliphage, male-specific coliphage, and PMMoV were the subset of indicators that maximized the correlation with pathogens in the rivers. In the ocean, the best subset of indicators was enterococci, male-specific coliphage, and PMMoV. We also executed redudancy analyses on environmental parameters and microorganim concentrations, and found that rainfall best predicted microbial concentrations. The seasonal interplay of rainfall and pathogen prevalence undoubtedly influences beach users' health risks. Relationships are likely to be complex, with some risk factors increasing and others decreasing each season. Future use of multivariate approaches to better understand linkages among environmental conditions, microbial predictors (fecal indicators and MST markers), and pathogens will improve prediction of high-risk scenarios at recreational beaches.

1. Introduction

Wastewater management practices affect the health of coastal ecosystems and the well-being of people who use these water resources for their livelihood and recreational purposes (Shuval, 2003). Globally, more than 80% of domestic wastewater is discharged into rivers or the ocean without any treatment (United Nations World Water Assessment Programme, 2017). Gastrointestinal and respiratory illnesses have been frequently associated with exposure to surface water contaminated with fecal waste (World Health Organization, 2003). The annual economic burden of recreation-associated waterborne illness in countries with high sanitation coverage and treatment, like the United States (US), is estimated at $USD 2.2 – $3.7 billion (DeFlorio-Barker et al.,

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2018). This burden is likely much greater in parts of the world where safe sanitation is lacking (Prüss-Ustün et al., 2014). Adequate wastewater treatment is essential for human health and well-being, yet sanitation infrastructure is vulnerable to climate change (Howard et al., 2010) and more research is needed to understand and mitigate this impact on sanitation infrastructure (Howard et al., 2016).

Routine monitoring of fecal indicator bacteria (FIB), such as *E. coli,* fecal coliforms, and enterococci, in recreational waters has been used for decades to protect public health (Griffith et al., 2009). FIB are intended to predict pathogen presence; however, poor correlations between FIB and pathogens have been reported in many studies (e.g. Boehm et al., 2003; Harwood et al., 2005). Exclusive reliance on FIB to indicate recent fecal pollution is problematic, since there is evidence of their persistence and growth in the sediments and vegetation in environmental waters (Anderson et al., 2005; Desmarais et al., 2002; Nguyen et al., 2018; Symonds and Breitbart, 2015). Extra-intestinal sources of FIB, such as soil, sediments, and aquatic vegetation, can confound the relationship between FIB and pathogens (Badgley et al., 2011; Lamb et al., 2017; Soupir et al., 2010; Whitman et al., 2003). FIB are also present in the feces of a variety of animal species (Byappanahalli et al., 2012), which limits their predictive relationship with pathogens, as well as their efficacy in risk assessment and remediation of fecal pollution in recreational waters (Harwood et al., 2014).

Alternative indicators to conventional FIB have been increasingly explored as replacements and/or additions to water quality monitoring. The US state of Hawaii has used *Clostridium perfringens* as a tracer of fecal contamination (Fujioka et al., 2015) and coliphages have been proposed as viral indicators (US EPA, 2015; Wanjugi et al., 2018). Human pathogens, like *Giardia, Cryptosporidium*, and norovirus genogroup I (NoVGI), are increasingly included in microbial water quality studies because conventional FIB do not always correlate with their presence (Ashbolt et al., 2010; Boehm et al., 2009). Microbial source tracking (MST) methods have been developed to determine fecal pollution sources (Chase et al., 2012; Harwood et al., 2009). Microorganisms associated with human feces and wastewater are frequent targets of MST assays, including the 16S rRNA gene of a *Bacteroides* species (HF183) (Seurinck et al., 2005) and pepper mild mottle virus (PMMoV) (Symonds et al., 2018, 2016). These MST markers, which are strongly associated with domestic wastewater, were proposed as a proxy for pathogens in quantitative microbial risk assessment (QMRA) (Ahmed et al., 2018a; Zhang et al., 2019).

Most microbial water quality studies regarding FIB, alternative indicators, management criteria, and human health risks have been developed in temperate zones and are employed globally; thus, significant knowledge gaps exist with respect to tropical waters (Boehm et al., 2009; Fujioka et al., 2015; World Health Organization, 2003). Furthermore, tropical coastal beaches are useful study systems for the effects of climate change on water quality in temperate zones, as some temperate regions are likely to experience weather that is similar to tropical conditions (e.g., increased precipitation and flooding) (Trenberth, 2011). Such conditions will present challenges for wastewater treatment and collection systems and can result in widespread sewage spills (Dale et al., 2017; Howard et al., 2016).

Jacó beach, located on the Central Pacific Costa Rican coast, is characterized by a long, intense rainy season, and a short dry season (Solano and Villalobos, 2012). Known as a famous surfing destination, Jacó experiences heavy national and international tourism due to its close proximity to the greater San José metropolitan area. Tourism is central to the local economy. The tourism industry has led to intensive development of high-rise condominiums, hotels, and other infrastructure that serves tourists, which impacts local water and wastewater systems (Krause, 2012). According to the Costa Rican Institute of Tourism, tourism generated $3.8 million USD during 2018 (Instituto Costaricense de Turismo, 2018), and 68% of the tourists visited the country's coasts in 2016–2018 (Instituto Costaricense de Turismo, 2018).

An estimated 5400 reside in Jacó (personal communication, Costa Rica Social Security, Garabito Health Area; Instituto Nacional de estadística y censos, 2014). Like the majority of tropical beach towns, Jacó lacks a central sewer and sanitation system, and generally relies on on-site sanitation infrastructure (Mora, 2009; Ramírez-Sánchez et al., 2015; World Health Organization and UNICEF, 2014). These septic systems frequently fail due to intense rainfall, lack of maintenance, and limited capacity that cannot keep pace with tourism, which quintuples the population in the dry season (December to March) (Borowy, 2004). Limited sources of animal fecal pollution are present in the watershed, i.e., there is no intensive production of livestock or dense bird populations (Orozco Montoya, 2015).

Deterioration in the microbial water quality of Jacó beach was reported by the Costa Rican government institution responsible for water quality monitoring, the Costa Rican National Water Quality Laboratory (Mora, 2011, 2009), and singled out by the community and local newspapers on several occasions (Angulo, 2013; Anonymous, 2008; Lopez, 2017). Due to its popularity, Jacó is among the prioritized areas for sanitation infrastructure improvements by the Costa Rican government (Angulo, 2015). Jacó beach was chosen for this study, in part, because it is heavily used for recreation year-round. Some of the larger commercial establishments, such as hotels, have small-scale wastewater treatment plants, while much of the population has septic-leach field systems; however, there is a population that discharges directly into the surface waters or the stormwater collection system, which discharges to the rivers or the beach (Mora, 2009).

We tested two major hypotheses in this study: (1) was there seasonal variation in environmental variables that influenced fecal indicator microorganism, MST marker, and pathogen levels and relationships among them, and (2) could MST sewage markers predict the presence of pathogens better than established FIB criteria used to regulate recreational water quality. We used multivariate statistical methods that are appropriate for left-censored data (i.e., observations below the limit of detection or quantification of a given analytical method). Left-censored data are a major issue in public health-related water microbiology, because relevant pathogen concentrations tend to be below our ability to measure them in environmental waters (Ortega et al., 2009; Soller et al., 2016; Symonds and Breitbart, 2015).

2. Methods

2.1. Study site and sample collection

Jacó beach is a 4.2 km long beach located in a westward-facing bay on the Costa Rican Pacific Coast (Fig. 1). Tides at this beach are semidiurnal with a meso-tidal range (i.e., difference between high and low tide is 2 to 4 m); thus, samples were purposefully collected during outgoing (i.e., transition from high to low tide) and incoming (i.e., transition from low to high tide) tides each season. Three rivers and their ocean discharge sites were sampled on three occasions for each combination of season (rainy (October 2017) and dry (March 2018)) and tidal cycle (incoming and outgoing): Copey River, Naranjal River and Madrigal River, resulting in a total of 72 samples collected from 12 sampling events. Of the total number of samples collected, 36 were river samples and 36 ocean samples, such that 9 river and ocean samples were collected per season and tide combination.

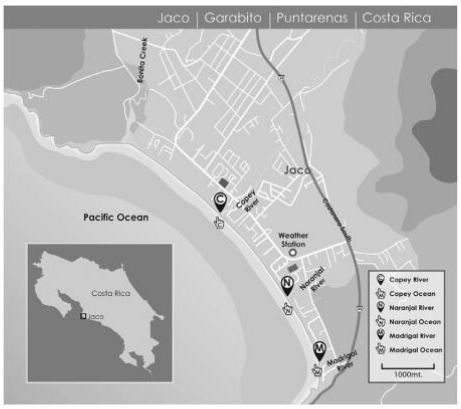

Fig. 1. Map of Jacó beach, located in the canton of Garabito and the province of Puntarenas, showing the three rivers sampled (Copey River (C), Naranjal River (N), Madrigal River (M)) and their corresponding ocean sites.

During each sampling event, water was collected from the three rivers along the beach, within three meters of the riverbank, ideally where salinity was less than 1 ppt. Additionally, water was collected in the ocean, between the swash zone and the first set of waves, just outside each river plume where the salinity was ideally 30 ppt. The direction of the river plume (northward or southward) was identified by adding a handful of Bright Dyes Standard Yellow/Green Powder (#25; Kingscote Chemicals; Miamisburg, US) to the river and observing the movement of the dyed-river plume. All samples were collected within 10 cm of the water surface. For each sampling site, two types of samples were collected: 500 mL and large-volume samples. The 500 mL-grab samples were collected in sterile containers and transported at 4 °C to the Costa Rican National Water Quality Laboratory for fecal coliform and enterococci analyses that were performed within 12 h. The large-volume water samples were concentrated on-site, as described in Section 2.2.

2.2. Sample concentration and handling

Ultrafiltration was used on-site to concentrate alternative fecal indicators, pathogens, and MST markers from ~50 L-water samples (<50 L only when filter clogged), as previously described (Smith and Hill, 2009). Clogged filters infrequently (<3% of samples) resulted in lower sample volumes. Briefly, a peristaltic pump powered by a portable generator pushed ~50 L of water through a 2-mm nylon mesh, and subsequently through a REXEED 25SX dialysis filter (Asahi Kasei, Japan). The REXEED 25SX dialysis filters were stored at 4 °C and shipped to BCS Laboratories in Gainesville, Florida for processing within 72 h of collection.

2.3. Microbiological quantification methods

Fecal coliforms were enumerated by the five tube most probable number (MPN) technique using EC media according to the American Public Health Association standard methods, section 9221 E (American Public Health Association, 2017), and following Costa Rican regulations (33903-MINAE-S, 2007) and water quality monitoring recommendations ((Mora, 2007). Enterococci enumeration was performed using membrane filtration and mEI agar according to the US Environmental Protection Agency (US EPA) Method 1600 (US EPA, 2005a).

All REXEED 25SX dialysis filters were eluted as previously described (Mull and Hill, 2012). Briefly, the filters were back-flushed with a 500-mL solution containing 0.5% Tween 80, 0.01% sodium polyphosphate, and 0.001% Antifoam Y-30 Emulsion. The final concentrate volume (460 mL) was used for different analyses as follows: F+ coliphages and somatic coliphages were cultured by US EPA Method 1601 (US EPA, 2001), *Salmonella* spp. were cultured using US EPA Method 1682 (US EPA, 2006), and *C. perfringens* were cultured using Standard Method ASTM D5916-96(2002) (American Public Health Association, 1996). *Giardia* spp. and *Cryptosporidium* spp. were also enumerated by immunomagnetic separation and microscopy by US EPA Method 1623 (US EPA, 2005b).

Finally, viruses and bacteria in the remaining 210-mL of final concentrate were further concentrated by polyethylene glycol (PEG) precipitation (Hill et al., 2010; Mull and Hill, 2012). The final concentrate was amended with 12% PEG 8000, 0.9 M NaCl, and 1% bovine serum albumin, held at 4°C for 2 h, and centrifuged at 10,000 x g for 20 min. The pellet was resuspended in 0.15 M $Na_2HPO_4 \times 7H_2O$, stored at −20°C, and shipped to the University of South Florida for molecular analysis of MST markers (HF183 and PMMoV), and NoVGI. HF183 and PMMoV were previously validated as appropriate sewage-associated markers in Costa Rica (greater than 90% sensitive and specific to domestic sewage) (Symonds et al., 2018). Limits of detection for analyses are provided in Table S1.

2.4. Molecular analyses

2.4.1. Nucleic acid extraction and qPCR analysis

DNA and RNA were extracted from all sample PEG concentrates, negative controls (extraction blanks), and DNA (salmon testes DNA (Sketa)) and RNA (feline calicivirus (FCV)) positive extraction controls using the AllPrep PowerViral DNA/RNA Kit (Qiagen; Germantown, USA) (see Section 2.4.2). All DNA samples were tested for HF183 and Sketa (Table 1). cDNA synthesis was performed by reverse transcription (RT) with the Superscript IV First-strand Synthesis System (Invitrogen; Carlsbad, USA) and all cDNA samples were analyzed for PMMoV, NoVGI, and FCV (Table 1). All samples and extraction(-RT) controls were analyzed in duplicate.

The presence of (RT-)qPCR inhibition was assessed by analyzing a 1:10 dilution of sample DNA and cDNA for the HF183 and PMMoV assays, respectively. PCR inhibition was determined by comparing the Cq (quantification cycle) of an undiluted sample to a 1:10 diluted sample. PCR inhibition was deemed to be absent if the Cq of the diluted sample was at least 2 Cq lower than that of the undiluted sample (Cao et al., 2012). No inhibition was observed during this study. All assays were run using an ABI 7500 Real-Time PCR System (Thermo Fisher Scientific; Waltham, US). All extraction blanks were negative for all (RT-)qPCR assays. Negative controls, containing no template, were included in each qPCR instrument run and all were negative.

In order to quantify the number of copies detected for each assay, standard curves were constructed from gene fragments in gBlocks (NoVGI, HF183; IDT; Coralville, US) or plasmids (PMMoV; IDT; Coralville, USA) containing the target sequences. For each assay, a ten-fold dilution series of the assay gBlock or plasmid, ranging from 10^7 to 10^1 gene copies per reaction, was analyzed in duplicate alongside samples. Linear regression analysis of the Cq and $\log_{10}$-transformed gene copy (GC) number was executed. All standard curves had >90% efficiency and >97% R^2 values. For all (RT-)qPCR assays, the calculated GC number was divided by two to account for differences between the standard material (double-stranded) and the single-stranded viruses analyzed. Targets were considered detectable but not quantifiable (DNQ) when the sample's Cq was between the analytical (assay) limit of quantification (LOQ) and limit of detection (LOD; (e.g., $Cq_{LOQ} < Cq_{sample} < Cq_{LOD}$). The original concentration in the sample, as well as the procsess LOD and LOQ, were back-calculated to take into account every step

Table 1
Reverse transcriptase-qPCR and qPCR assay primers, probes and reaction conditions.

Assay	Primers and probes (5′ – 3′)	Reaction	Reference
Human norovirus genogroup I (NoVGI)	F: CARGCCATGTTYCGYTGGATG R: CCTTAGAGCGCATCATCATTTAC [6-FAM]TGGACAGGAGAYCGCRATCT[BHQ1a~Q]	Primers: 500 nM each Probe: 250 nM Conditions: 95 °C for10 min then, 40 X (95 °C for 15 s and 60 °C for 1 min)	(Svraka et al., 2009)
HF183 *Bacteroides 16s rRNA*	F: ATCATGAGTTCACATGTCCG R: CTTCCTCTCAGAACCCCTATCC FAM-CTAATGGAACGCATCCC-MGB	Primers: 1000 nM each[a] Probe: 80 nM Conditions: 2 min at 50 °C, 10 min at 95 °C, then 40 X 15 s at 95 °C for 15 s and, 60 s at 60 °C for 1 min)	(Green et al., 2014)
Pepper mild mottle virus (PMMoV)	F: GAGTGGTTTGACCTTAACGTTTGA R: TTGTCGGTTGCAATGCAAGT [6-FAM]CCTACCGAAGCAAATG[MGBNFQ]	Primers: 400 nM each Probe: 200 nM Conditions: 95 °C for10 min then, 40 X (95 °C for 15 s and 60 °C for 1 min)	(Zhang et al., 2006; Haramoto et al., 2013)
Feline calicivirus (FCV)	F: CCGGGTGGGACTGAGTGG R: GCATAACTCGTCCGAGCGTCTC [6-FAM]CGCCTTACCGATATGAGCAGCCACATTAAC[IBRQ]	Primers: 300 nM each Probe: 200 nM Conditions: 95 °C for10 min then, 40 X (95 °C for 15 s and 60 °C for 1 min)	(Mattison et al., 2009)
Salmon testes DNA (Sketa22)	F: GGTTTCCGCAGCTGGG R: CCGAGCCGTCCTGGTC [6~FAM]AGTCGCAGGCGGCCACCGT[TAMRA]	Primers: 1000 nM each Probe: 80 nM Conditions: 95 °C for10 min then, 40 X (95 °C for 15 s and 60 °C for 1 min)	(US EPA, 2012a)

[a] Modified from citation.

in the analytical process and assumed 100% efficiency. HF183 and PMMoV were reported per 100 mL, while NoVGI was reported per L.

2.4.2. Process controls and extraction-RT efficiency

Just prior to initiating the lysis step for each round of D/RNA extraction, all PEG concentrates, as well as a sterile water calibrator, were spiked with 14 uL (140 ng) of salmon testes DNA (Sigma D7656-1 mL; 10 mg/mL in H_2O) and 5 µL (2.5×10^6 FCV PFU) FCV particles (RNA control; ATCC VR-782), according to previously published methods (Mattison et al., 2009; US EPA, 2012a). A sterile water blank (e.g., no spiking) was also used in each round of extraction. The D/RNA extraction(-RT) process efficiency for each sample was calculated as previously described (Symonds et al., 2014). Overall, the D/RNA extraction(-RT) efficiencies were good (greater than >10% recovery of Sketa and FCV); however, 7% of samples had poor RNA extraction-RT efficiency (<10% FCV recovery).

2.4.3. Environmental data

Water temperature (°C), pH, salinity (ppt), and turbidity (nephelometric turbidity unit; NTU) were measured with a YSI 556 Multiprobe (Rye Brook, USA) *in situ* at the time of sample collection. Rainfall (mm) in the last 12 h was measured using a weather station (AcuRite Pro 5-in-1 Weather Station; Lake Geneva, US) located in Jacó within 1.2 km of the sample collection sites.

2.5. Data analyses

R version 4.0.2 was used to execute all statistical analyses: descriptive statistics, hypothesis testing, and redundancy analyses, as well as correlation analyses (R Core Team, 2013). All values less than the LOD and LOQ were censored (to 1 – LOD or LOQ, respectively), and all subsequent statistical analyses for censored data were executed using the "NADA" R package and a package obtained from the Nondetects and Data Analysis training course (downloaded at https://practicalstats.teachable.com/ on June 20, 2020) (Helsel, 2019, 2011; Lee, 2017). Data censoring took into account both the non-detects (concentrations below the LOD) and positive, but not quantifiable detects (concentrations below the LOQ).

Multivariate analyses were executed to avoid problems with the increased type I error associated with multiple pair-wise comparisons and multiple hypothesis tests. Prior to all multivariate analyses, all environmental parameters were standardized by calculating their z-Score and an Euclidean distance matrix was calculated. All microbial data were natural log (ln)-normally distributed; thus, they were ln-transformed. The u-Score ranks were calculated for all the ln-transformed microbial data to account for censored observations (Helsel, 2019, 2011). To accommodate left-censored data in all multivariate analyses, Euclidean distance matrices were calculated from the microbial u-Score ranks (Helsel, 2011). Statistical significance for all analyses was assessed at $\alpha = 0.05$.

River and ocean water sampling were based upon measurements of salinity, and not a specific location, given the influence of tides on the river and ocean sampling locations. Differences by water type were confirmed by the clear clustering observed in the non-metric multidimensional scaling plot using all environmental and microbial variables (Fig. S1). Consequently, river and ocean data were analyzed separately. Non-metric multidimensional scaling was also used to identify possible differences between sub-watersheds for all of the river, as well as the ocean, environmental and microbial variables, separately. The data for each sub-watershed were grouped together for the river and ocean, respectively, given the lack of distinctive clustering by sub-watershed and the close proximity of the sub-watersheds (Figures S2 and S3).

2.5.1. Descriptive statistics

In order to describe and understand the water quality at Jacó beach, the data from river and ocean discharge sites were grouped by season (rainy vs. dry). There were 18 samples for any given water type and season. For the environmental data only, boxplots were created to describe each environmental parameter by season and by tide ($n = 9$ for each grouping). For the microbial data, boxplots were created to describe each non-censored microorganism by season. Based upon these groupings, descriptive statistics were calculated for all environmental and non-censored microbial data.

For all microbial variables that included left-censored data values, but that were <80% left-censored, descriptive statistics were estimated using Robust Regression on Order Statistics (rROS), where a lognormal distribution was assumed for modeling the left-censored portion of the distribution (observations below the process LOD or LOQ) using "NADA" R package (Helsel, 2011; Lee, 2017). Pathogen data included >80% left-censored values; thus, the frequency of detection for each pathogen was determined.

2.5.2. Testing differences in environmental and microbial data by season and tide

Given the multi-factor sampling design (by season and by tide) and the multivariate data, distance-based Redundancy Analysis (dbRDA), followed by an analysis of variance-like permutation test to assess the significance of constraints, was used to test for differences in the environmental data by season, tide, and season and tide combined (rainy/incoming vs. rainy/outgoing vs. dry/incoming vs. dry/outgoing) (Legendre et al., 2011; Legendre and Anderson, 1999). Differences in microorganism concentrations by season, tide, and season and tide combined were also tested by dbRDA, as previously described. River and ocean data were considered separately. Analyses was executed using the Vegan Package (Oksanen et al., 2016). A Manhattan distance matrix was calculated from the binomial season and tide categories. For all dbRDA analyses, each season contained 18 samples, in which nine were collected during the outgoing tide and nine were collected during the incoming tide.

2.5.3. Relationships between environmental variables and microorganisms

Redundancy analysis (RDA) is a multivariate analysis commonly used in microbial ecology to understand multivariate linear relationships (Legendre and Legendre, 2012; Ramette, 2007). Here it was executed using the Vegan package (Oksanen et al., 2016) to test the following null hypotheses for river and ocean data, separately: there is no significant multivariate linear relationship between environmental parameters and microbial data. The variance inflation factor was calculated for each set of model explanatory variables to assess the severity of multicollinearity. All variance inflation factors were less than or equal to 2; thus, minimal correlations were identified between the explanatory environmental variables.

2.5.4. Exceedance of recreational water quality criteria, pathogen presence, and indicator criteria and MST marker performance with respect to pathogen detection

All water samples were classified by their fecal coliform and enterococci concentrations according to Costa Rican and US EPA recreational water quality criteria (RWQC). Costa Rican RWQC include fecal coliform and enterococci criteria (fecal coliform criteria developed in Costa Rica; Mora, 2007), and the CR enterococci criteria are based upon the US EPA RWQC (US EPA, 2012b). Coastal waters were considered appropriate for recreation when enterococci geometric mean concentrations were below 35 CFU/100 mL and/or fecal coliform geometric mean concentrations were below 240 or 1000 MPN/100 mL for marine or freshwater, respectively. The US EPA RWQC statistical threshold value (STV; 130 CFU/100 mL) was used to define when water samples were not appropriate for swimming per US EPA RWQC. Since Costa Rica's recommendations do not include a threshold value for a single sample, the geometric mean criterion was used to classify each water sample.

To explore the predictive relationship between FIB RWQC and pathogen presence, water samples were binned as follows: (1) FIB above or below RWQC and (2) any pathogen detected, or no pathogen detected. The relationship between RWQC exceedance and pathogen detection was subsequently calculated as follows: (1) % true-positive (TP; RWQC exceedance + pathogen detection); (2) % true-negative (TN; RWQC not exceeded and pathogen not detected); (3) % false-positive (FP; RWQC exceedance but pathogen not detected); and (4) % false-negative (FN; RWQC not exceeded but pathogens detected). For each criterion, the following were calculated: the sensitivity (indicating how frequently the criterion was exceeded when at last one pathogen was detected) was determined as:

$$\frac{TP}{(TP+FN)} \tag{1}$$

the specificity (indicating how frequently FIB levels were below the criterion when no pathogen was detected) was calculated as

$$\frac{TN}{(TN+FP)} \tag{2}$$

the positive predictive value (indicating how frequently a RWQC exceedance co-occurred with detection of at least one pathogen) was calculated as

$$\frac{TP}{(TP+FP)} \tag{3}$$

and the negative predictive value (indicating how frequently FIB levels below the criterion co-occurred with detection of 0 pathogens) was calculated as

$$\frac{TN}{(TN+FN)} \tag{4}$$

A similar exercise was carried out to compare the predictive ability of MST sewage markers, HF183 and PMMoV, to indicate pathogen presence given the lack of livestock activities and point sources of treated and undertreated domestic wastewater (Mora, 2009; Orozco Montoya, 2015). In this case, the MST markers were binned according to whether they were above or below the process LOD, or according to MST marker criteria, which were based on QMRA estimates of human health risks associated with recreational water exposure (Ahmed et al., 2018a; Crank et al., 2019). Ahmed et al. (2018a) calculated that 3220 HF183 GC/100 mL and 544 PMMoV GC/100 mL are associated with the US EPA benchmark for recreational waters (36 illnesses/1000 swimmers). For PMMoV only, Crank et al. (2019) estimated that 5054 PMMoV GC/100 mL was associated with the US EPA benchmark for recreational waters (30 illnesses/1000 swimmers).

2.5.5. Correlations between fecal indicator microorganisms and MST markers, and pathogens

The Mantel test, a multivariate approach, was used to identify correlations between indicator microorganisms and MST markers (one matrix), and pathogens (second matrix) in the rivers and ocean, separately (Legendre and Legendre, 2012). This approach was executed using the Kendall method with the Vegan package (Oksanen et al., 2016), and included 9999 permutations. Euclidean distance matrices, one for the indicator microorganisms and MST markers, and the second one for the pathogens, were calculated and the correlation between the two matrices was analyzed. In order to identify the best subset of indicator microorganism and MST markers that maximized the correlations with the

Table 2
Frequency of pathogen detection (percentage of positive observations) in river ($n = 36$) and ocean ($n = 36$) samples in the rainy ($n = 18$) and dry seasons ($n = 18$). The theoretical process limit of detection and quantification is included in Table S1.

Pathogen	Percentage of Positive Samples (number of positive samples)					
	Rivers ($n = 36$)			Ocean ($n = 36$)		
	By season		Regardless of season	By season		Regardless of season
	Rainy ($n = 18$)	Dry ($n = 18$)		Rainy ($n = 18$)	Dry ($n = 18$)	
Giardia	33% (6)	44% (8)	39% (14)	11% (2)	0%	6% (2)
Cryptosporidium	78% (14)	0%	39% (14)	33% (6)	0%	17% (6)
[a]*Salmonella*	18% (3)	35% (6)	26% (9)	6% (1)	0%	3% (1)
NoVGI	0%	78% (14)	39% (14)	6% (1)	0%	3% (1)

[a] *Salmonella* $n = 17$ during dry season in river and ocean, therefore $n = 35$ by season and 70 by water type.

pathogen concentrations, iterative Mantel tests with the Kendall method were executed using the BIO-ENV procedure (Clarke and Ainsworth, 1993) from the Vegan package (Oksanen et al., 2016).

3. Results

The environmental data varied by season, and to a lesser extent, by tide (Figure S4). The mean rainfall was 0.88 mm and 21.42 mm in the dry and rainy season, respectively. The mean physical-chemical water parameters were as follows for the river in the dry and rainy season, respectively: water temperature (28.84 °C and 27.07 °C), salinity (0.23 ppt and 0.37 ppt), and turbidity (3.95 NTU and 24.06 ± NTU). In the ocean data, mean physical-chemical water parameters were as follows for the dry and rainy season, respectively: water temperature (29.80 °C and 27.43 °C), salinity (31.40 ppt and 27.60 ppt), and turbidity (23.19 NTU and 64.96 NTU).

With respect to tides, median turbidity was greatest during the incoming tide in the rivers (16.5 NTU) and the outgoing tide in the ocean (21.5 NTU; Figure S4). Median river and ocean water temperatures were greatest during the outgoing tide (30.2 °C and 30.55 °C, respectively). The median salinities were similar in the river (~0.2 ppt) and ocean (~32 ppt), respectively, regardless of season or tidal stage. However, increased variability was observed in the rainy season in which higher salinities (>0.4 ppt) were identified in the river, and lower salinities in the ocean (<30 ppt), particularly on the outgoing tide.

Similarly, the microbial data varied by season. In the river, measurements of enterococci, male-specific coliphage, and HF183 were at least one order of magnitude greater during the rainy season (Fig. 2 and Table S2). Furthermore, the frequency of detection of most pathogens (*Giardia, Salmonella* and *Cryptosporidium*) was greatest during the rainy season, except for NoVGI, which was only detected during dry season (Table 2). In the ocean, enterococci, fecal coliforms, male-specific coliphage, and HF183 were at least one order of magnitude greater during the rainy season, and somatic coliphage had greater concentrations during the dry season (Fig. 2, and Tables S2). The overall pathogen detection frequency was greatest in the ocean during the rainy season (Table 2).

3.1. Hypothesis testing for differences associated with seasons and tides

River and ocean samples were collected on six occasions in the rainy and six occasions in the dry season as well as during outgoing (i.e., transition from high to low tide) and incoming (i.e., transition from low to high tide) tides. Thus, analyses testing the differences in environmental parameters and microorganisms by season and tide were executed for the river and ocean data, separately (for each water type, $n = 9$ for each season and tide combination).

3.1.1. Differences in environmental parameters by season and tide

River: Significant differences in river environmental parameters between seasons ($F = 7.3483$, $p = 0.001$) and tides ($F = 4.519$, $p = 0.003$) were identified by dbRDA separately. Significant differences in river environmental parameters were also identified by the multi-factor hypothesis test for season and tide using dbRDA ($F = 5.9689$, $p = 0.001$), in which water temperature ($F = 9.7402$, $p = 0.001$) and rainfall ($F = 6.2202$, $p = 0.001$) significantly contributed to the differences observed. Turbidity ($F = 0.4917$, $p = 0.751$) and salinity ($F = 1.9470$, $p = 0.061$) were not significant contributors.

Ocean: Significant differences in environmental parameters between seasons were also identified by season ($F = 14.845$, $p = 0.001$) and tide ($F = 3.3044$, $p = 0.015$) by dbRDA separately. Significant differences in ocean environmental parameters were also identified by the dbRDA multi-factor hypothesis test for season and tide ($F = 6.0915$, $p = 0.001$), in which water temperature ($F = 8.7430$, $p = 0.001$) and rainfall ($F = 8.0718$, $p = 0.001$) most significantly contributed to the differences observed. Turbidity ($F = 0.4717$, $p = 0.852$) and salinity ($F = 1.1654$, $p = 0.354$) were not a significant contributors.

3.1.2. Differences in microbial parameters by season and tide

River: Significant differences in river microorganism concentrations were identified between seasons ($F = 12.667$, $p = 0.001$), and not tides ($F = 1.8465$, $p = 0.116$), using dbRDA. Significant differences in river microorganisms concentrations were identified by the dbRDA multi-factor hypothesis test for season and tide combined ($F = 3.1006$, $p = 0.001$), in which *Cryptosporidium* concentrations significantly contributed the most to the differences observed ($F = 5.5225$, $p = 0.003$), followed by enterococci ($F = 4.4098$, $p = 0.007$) and NoVGI ($F = 3.8663$, $p = 0.014$).

Ocean: Significant differences in ocean microorganism concentrations between seasons ($F = 6.5674$, $p = 0.001$), but not between tides ($F = 1.5659$, $p = 0.179$) were identified by dbRDA. Somatic coliphage contributed the most to the differences observed between seasons ($F = 5.9577$, $p = 0.028$), followed by PMMoV ($F = 3.9262$, $p = 0.064$). Significant differences in ocean microorganism concentrations were identified by the dbRDA multi-factor hypothesis test for season and tide ($F = 2.1354$, $p = 0.003$); however, no specific microorganism(s) significantly contributed to the differences identified.

3.2. Relationships between environmental variables and microorganisms

River: There was a significant relationship between environmental variables and microorganisms in the river, in which the RDA model explained 12.34% of the overall microorganism variability ($F = 2.1969$, $p = 0.001$, adjusted $R^2 = 0.1234$) (Fig. 3). All of the variability was explained by the first canonical axis (RDA1; $p = 0.001$).

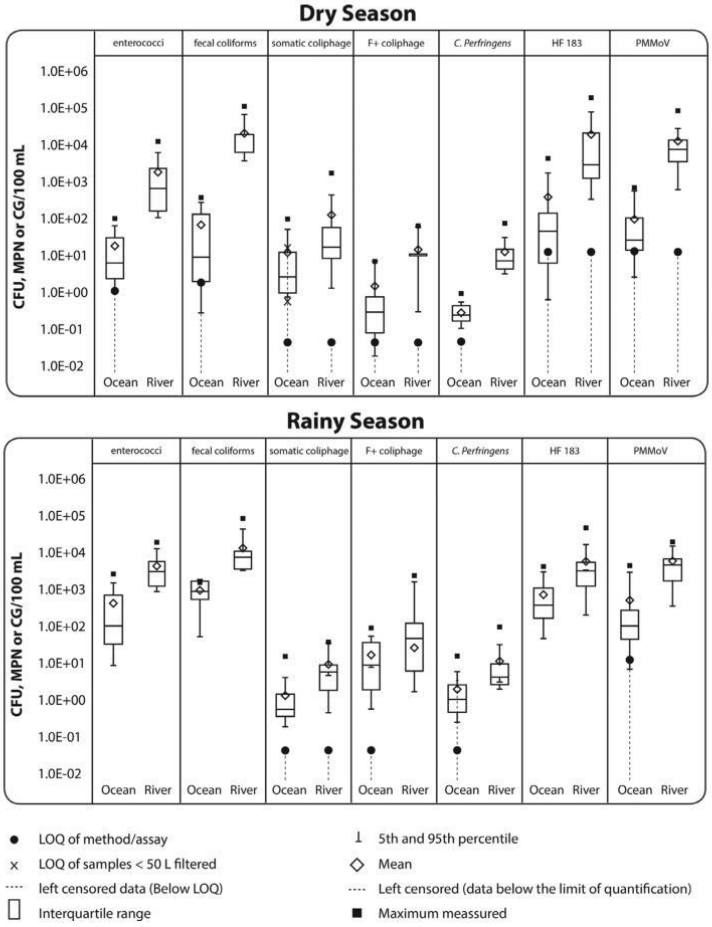

Fig. 2. Fecal indicator microorganism and microbial source tracking marker concentrations in dry (top) and rainy (bottom) seasons in oceans and rivers. For left-censored data, the method limit of quantification (LOQ) is identified (dark circle) along with the the left-censored data (dashed lines).

Rainfall and salinity significantly explained the variability in microbial concentrations ($F = 2.8307$, $p = 0.006$ and $F = 2.619$, $p = 0.003$, respectively). Water temperature and turbidity did not significantly explain microbial variability ($F = 0.7604$, $p = 0.617$ and $F = 0.9993$, $p = 0.459$, respectively) in the model. In the RDA ordination plot, all microorganisms clustered where salinity was lowest. Enterococci, male-specific coliphage and *Cryptosporidium* grouped together and were clustered with higher rainfall and turbidity, and decreasing temperature. Contrastingly, fecal coliforms, *C. perfringens*, somatic coliphage, *Salmonella*, and NoVGI grouped together with increasing temperature and decreasing rainfall and turbidity.

Ocean: There was also a significant relationship between environmental parameters and microorganisms in the ocean, in which the RDA model explained 26.44% of microorganism variability ($F = 4.0561$, $p = 0.001$, adjusted $R^2 = 0.2644$) (Fig. 4). All of the variability was explained by the first canonical axis (RDA1; $p = 0.001$). Rainfall ($F = 3.1391$, $p = 0.025$), water temperature ($F = 3.0286$, $p = 0.035$), and salinity ($F = 2.8416$, $p = 0.024$) were the best predictors of microorganism concentrations in the ocean. Turbidity was not predictive of microorganism concentrations ($F = 0.8766$, $p = 0.508$) in the model. In the RDA ordination plot, microorganism concentrations were typically clustered where salinity and water temperature were lowest. Enterococci, fecal coliforms, *C. perfringens*, male-specific coliphage, HF183, PMMoV, *Cryptosporidium*, and *Giardia* grouped together according to increasing rainfall and decreasing water temperature, while somatic coliphage clustered with decreasing rainfall and increasing temperatures.

3.3. Exceedance of RWQC and performance of indicator microorganisms and MST markers with respect to pathogen detection

FIB concentrations in 89 – 100% of the river samples exceeded RWQC regardless of the standard to which they were compared (Table 3). Fecal coliform and enterococci geometric mean concentrations were 9.04×10^3 CFU/100 mL and 1.26×10^3 MPN/100 mL, respectively. FIB levels in the ocean samples exceeded RWQC much less frequently (25 – 44%), with fecal coliform and enterococci geometric mean concentrations of 9.68×10^1 CFU/100 mL and 2.98×10^1 MPN/100 mL, respectively. With respect to MST markers,

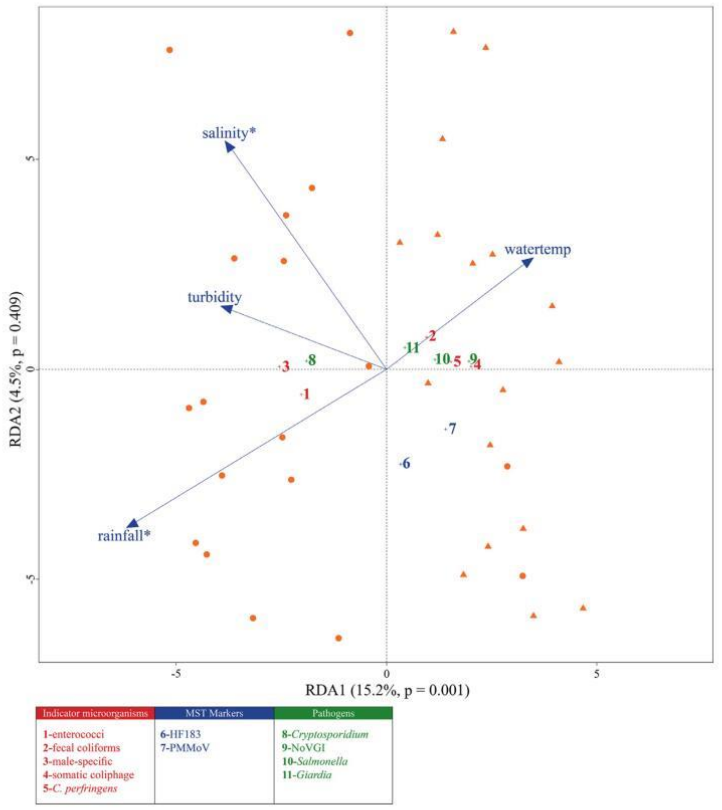

Fig. 3. Redundancy analysis ordination plot of river data, showing the relationships between environmental parameters (arrows) and microorganisms (numbers; fecal indicators = red, MST markers = blue, and pathogens = green). Data points are identified by season (circle = rainy; triangle = dry). The first axis significantly described all the variability ($p = 0.001$). Significant predictor environmental variables are identified with a * ($\alpha = 0.05$).

Table 3
Percent of samples exceeding recreational water quality criteria (RWQC) by water type according to Costa Rican and US Environmental Protection Agency recommendations. Performance of the FIB criteria with respect to pathogen detection are evaluated by sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The theoretical process limit of detection and quantification are in Table S1.

FIB	FIB% exceedance (# above RWQC)		Prediction	Pathogen detection (%)		Criteria	Reference
	[b]River	[b]Ocean		[b]River	[b]Ocean		
Fecal coliforms	100% (36)	44% (16)	Sensitivity	100	88	CR seawater - 240 MPN/ 100 mL geometric mean	(Mora 2017)
			Specificity	0	68		
			PPV	89	44	CR freshwater - 1000 MPN/ 100 mL geometric mean	(La Gaceta, 2017)
			NPV	11	95		
Enterococci	100% (36)	31% (11)	Sensitivity	[a]ND	88	CR seawater - 35 CFU/ 100 mL geometric mean	(Mora 2017)
			Specificity	[a]ND	75		
			PPV	[a]ND	50		
			NPV	[a]ND	50		
Enterococci	89% (32)	25% (9)	Sensitivity	91	88	US EPA sea- and freshwater - 130 CFU/ 100 mL STV[c]	(US EPA 2012)
			Specificity	25	93		
			PPV	91	78		
			NPV	25	96		

[a] ND – not determined because the indicator is not used to regulate freshwater in Costa Rica.
[b] $n = 36$ samples per water type.
[c] STV = Statistical threshold value.

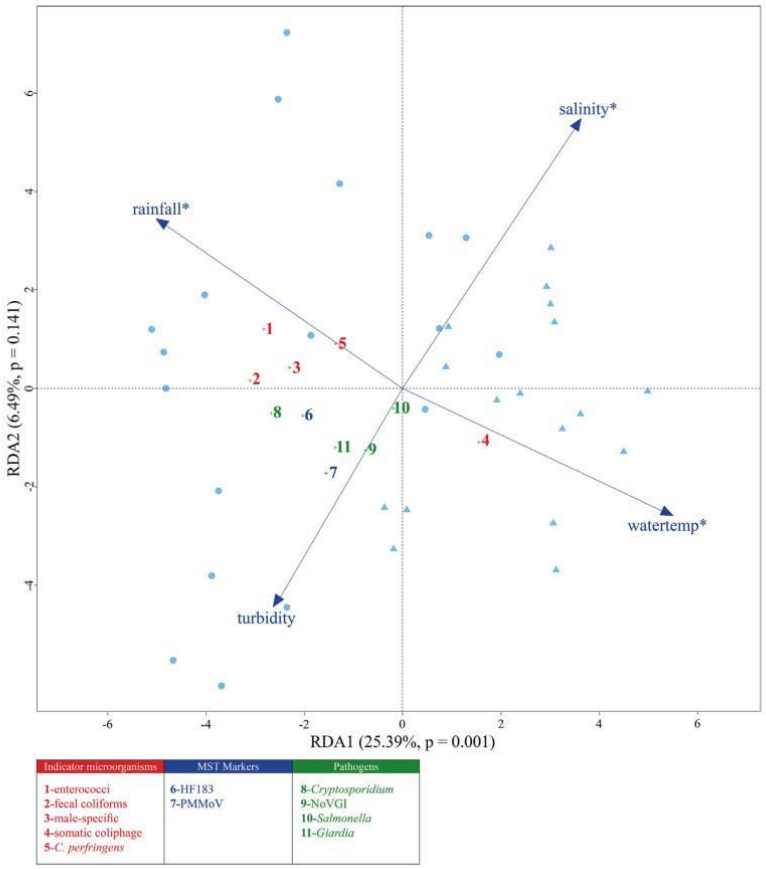

Fig. 4. Redundancy analysis ordination plot of ocean data, showing the relationships between environmental parameters (arrows) and microorganisms (numbers; fecal indicators = red, MST markers = blue, and pathogens = green). Data points were identified by season (circle = rainy; triangle = dry). The first axis significantly described all the variability ($p = 0.001$). Significant predictor environmental variables are identified with a * ($\alpha = 0.05$).

HF183 and PMMoV were detected in 100% of river samples, as well as 88.9% and 91.7% of ocean samples, respectively. The mean HF183 and PMMoV concentrations in the rainy season were 5.7×10^3 and 5.3×10^3 GC/100 mL in the rivers, respectively, compared to 7.9×10^2 and 5.1×10^2 GC/100 mL in the ocean, respectively (Table S2). During the dry season, mean HF183 and PMMoV concentrations in the rivers were 3.8×10^2 and 1.0×10^2 GC/100 mL, respectively, compared to 7.9×10^2 and 5.1×10^2 GC/100 mL in the ocean, respectively (Table S2).

The ability of indicator microorganisms to predict pathogen presence was assessed by binning indicator observations into above or below RWQC (i.e., criteria for enterococci or fecal coliforms), and by binning pathogen detection results (e.g., at least one pathogen detected or no pathogens detected in a sample). Performance metrics (sensitivity, specificity, positive predictive value, and negative predictive value) were then calculated (see Methods for details) (Table 3; Table S3). Regardless of the criterion used, enterococci and fecal coliforms were sensitive to pathogen presence in both water types (88 – 100%; i.e., indicator criteria were generally exceeded when pathogens were present). Specificity was low in river samples (0 – 25%), but higher in ocean samples (68–93%), due largely to infrequent observations of FIB below RWQC in river samples (0% for CR fecal coliforms and enterococci criteria; 11% for the US EPA STV). Positive predictive values were higher in river (89 –91%) than in ocean (44–78%) samples, indicating greater confidence that indicator microorganisms above RWQC in river samples would correctly indicate pathogen presence compared to ocean samples (Table 3). Negative predictive values were low in the river (11- 25%), again driven by very few indicator microorganism observations below the RWQC. In the ocean, the negative predictive values were highest for enterococci (STV standard) and fecal coliforms (95- 96%) and were much lower for the Costa Rican enterococci criteria (50%%.

The ability of MST markers to predict pathogen presence in water samples was explored in a similar manner (Table 4). When MST markers were binned as above (positive) or below (negative) the LOD, sensitivity was 100% in river and ocean samples, indicating that the MST marker was always observed when at least one pathogen was detected. When binning criteria for MST markers was changed to reflect the QMRA-estimated criteria byAhmed et al. (2018a), sensitivity dropped to 88% for PMMoV and 53% for HF183 in river samples, and dropped to 0% for both

Table 4
Performance of the sewage-associated microbial source tracking (MST) markers with respect to pathogen detection evaluated by sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). MST markers were binned positive or negative by comparison to the process limit of detection (LOD) or to QMRA-derived benchmark values from Ahmed et al. (2018) or Crank et al. (2019). The theoretical process limit of detection and quantification are included in Table S1.

MST markers	Performance Criterion	Process LOD[a], this study		(Ahmed et al., 2018a)[b]		(Crank et al., 2019)[c]	
		River	Ocean	River	Ocean	River	Ocean
PMMoV	Sensitivity	100	100	88	0	40	0
	Specificity	0	11	0	86	100	100
	PPV	89	24	88	0	100	NP[d]
	NPV	11	100	0	75	0	78%
HF183	Sensitivity	100	100	53	0	NA[e]	NA[e]
	Specificity	0	14	25	93	NA[e]	NA[e]
	PPV	89	25	85	0	NA[e]	NA[e]
	NPV	11	100	6	76	NA[e]	NA[e]

[a] HF183 and PMMoV process LOD 6 GC/ 100 mL.
[b] HF183 3220 GC/100 mL and PMMoV 544 GC/100 mL (Ahmed et al., 2018a).
[c] PMMoV 5054 GC/100 mL (Crank et al., 2019).
[d] NP – not possible because no samples were above the benchmark for QMRA risk.
[e] NA – not applicable because no benchmark criteria exist.

markers in ocean samples. Thus, this drop in sensitivity by using QMRA-estimated criteria indicated an increased frequency of false-negative relationships (MST marker below criteria but pathogen present). For PMMoV, the more stringent criteria estimated by Cranks et al. (2019) produced a much lower sensitivity in river samples (40% compared to 88%) compared to Ahmed's criteria.

Specificity was low for both MST markers when they were binned according to the LOD (0 −14%), but was improved by binning according to QMRA criteria, particularly in ocean samples. For example, specificity of HF183 in ocean samples increased from 14% (LOD binning) to 93% (QMRA binning). PMMoV followed a similar pattern, indicating that the more stringent criteria for defining a MST marker positive decreased the rate of false-positives (MST marker positive, no pathogens detected). Positive predictive values for MST markers under all criteria were markedly better in river compared to ocean samples, indicating greater confidence that a positive MST result would be co-observed with a pathogen in river samples compared to ocean, while negative predictive values showed the opposite trend.

3.4. Correlations between indicator microorganisms and MST markers and pathogens

A significant correlation between indicator microorganisms and MST markers, and pathogens was identified by the multivariate Mantel test for river ($\tau = 0.131$, $p = 0.0004$) and ocean datasets ($\tau = 0.1699$, $p = 0.0008$). *C. perfringens*, somatic coliphage, male-specific coliphage, and PMMoV were the subset of indicator microorganisms and MST markers that maximized the correlation with pathogen concentrations in the rivers ($\tau = 0.1529$, $p = 0.0003$). In the ocean, enterococci, male-specific coliphage, and PMMoV were the subset of indicator microorganisms and MST markers that maximized correlations with the pathogen concentrations ($\tau = 0.2710$, $p = 0.0001$). Fig. 5 is a conceptual diagram that illustrates these results.

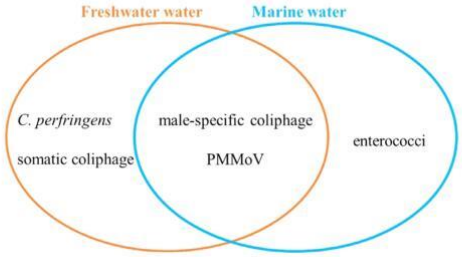

Fig. 5. Summary of the subset of indicator microorganisms and MST markers identified by the BIO-ENV procedure that best maximized correlations with pathogens in the river ($\tau = 0.1529$, $p = 0.0003$) and ocean waters ($\tau = 0.2710$, $p = 0.0001$).

4. Discussion

Jacó, like the majority of tropical beaches, is a popular beach whose recreational waters receive treated and undertreated domestic wastewater (Mora, 2009; World Health Organization and UNICEF, 2014). Our analyses consisted of multivariate approaches that were appropriate for the left-censored data sets typical of environmental pathogens, which allowed for their analysis in spite of frequent undetectable concentrations (Helsel, 2011). Since the power of statistical analyses are compromised when a large percentage of the data are censored, future studies are needed to corroborate the findings presented here. Using multivariate analyses, we identified that the environmental parameters were significantly different by season and tide, and explained 12% and 26% of the microbial variability in the river and the ocean, respectively. The percentage of variability explained by environmental parameters is similar to other studies (Laureano-Rosario et al., 2017). It also likely reflects the constant discharge of domestic wastewater to the rivers, which was identified by frequently high levels of MST markers *Bacteroides* HF183 and PMMoV in river samples, and to a lesser extent, in the ocean waters. A previous study identified illegal sewage connections to the stormwater system that drained into the river study areas (Mora, 2009).

4.1. Environmental variables explain some variability in pathogens

While correlations among variables are not necessarily indicative of cause-and-effect relationships, they nonetheless provide important information about relationships among variables in complex natural systems. These relationships can be used to develop hypotheses that may be tested in manipulative experiments; however, this type of scientific inquiry is extremely difficult to carry out in a representative manner in scenarios as complex and variable as anthropogenically-impacted beaches. Multivariate statistical analyses yield more insight about complex systems than univariate analyses because they consider multiple independent and dependent variables simultaneously. As such, they produce a more holistic analysis that is also less subject to the type I statistical er-

rors that easily arise from conducting multiple univariate analyses (Grace, 2002). We therefore used multivariate statistics to analyze relationships of microorganisms with environmental variables, and with each other, throughout this study. An additional complexity in analyses of relationships among human pathogens in anthropogenically-impacted environments is that pathogens may be contributed to water bodies by sewage, or by the feces of a variety of animals (Harwood et al., 2014; Korajkic et al., 2018; Nguyen et al., 2018). Fecal material derived from various sources will doubtlessly be affected differently by environmental variables, contributing to discrepant relationships that would not be expected in simpler systems.

Rainfall, which was significantly different between seasons, was a significant predictive variable of microbial concentrations in river and ocean models. Previous studies also found a positive relationship between rainfall and fecal-associated microorganisms (Ahmed et al., 2018b; Curriero et al., 2001; Laureano-Rosario et al., 2017; Shehane et al., 2005). Interestingly, contrasting associations between rainfall and the fecal microorganisms occurred in the rivers and ocean. In the rivers, *Cryptosporidium* increased with increasing rainfall, while NoVGI was highest when rainfall was lowest. While pathogen detection was infrequent in the ocean, *Cryptosporidium* tended to increase when rainfall was higher. Heavy rainfall often results ito increased runoff and saturation of soils, which can cause leakage from septic-leach field systems. Such leakage may increase pathogen concentrations in surface waters, particularly those pathogens that are usually removed (e.g., unicellular parasites) (Hofstra and Vermeulen, 2016; Neumann et al., 2015).

Of the microorganisms, *Cryptosporidium* made the greatest contribution to the differences observed between seasons, as it was only detected during rainy season in the river and ocean. Although this pathogen is not exclusive to humans, limited agricultural and livestock activities occur in the Jacó beach watershed (Orozco Montoya, 2015). It is likely that the high percentage of septic-leach field systems, combined with intense rainfall, led to increased *Cryptosporidium* concentrations in the rainy season. The other protozoan parasite analyzed, *Giardia,* was detected equally across seasons and as frequently as *Cryptosporidium* overall. *Cryptosporidium* prevalence in human populations is strongly correlated with precipitation, particularly in tropical locations (Jagai et al., 2009). Our results suggest that *Giardia* prevalence in fecal-contaminated surface waters is not as affected by season as *Cryptosporidium,* perhaps because of the presence of a source other than sewage. *Giardia* infection in canines is not uncommon, even in well-cared for dogs (Hascall et al., 2016). Many dogs roam Jacó beach and canine-associated parasites have been frequently detected in Jacó beach sand (Castro et al., 2009).

Salmonella and NoVGI were detected most frequently during the dry season in the rivers, which likely reflects the increased volume of sewage generated when tourism is greatest and possibly the seasonality of the pathogen in the population. Seasonal variability in NoVGI concentrations in domestic wastewater is understudied in the tropics; nevertheless, there is evidence of high concentrations year-round on the Costa Rican Pacific coast (Symonds et al., 2017), and increased concentrations have been identified in the dry season in the San José Greater Metropolitan Area (Chacon et al., 2020). It is likely that NoVGI was detected most frequently in the rivers during the dry season due to increased tourism in the area (Borowy, 2004), which leads to increased wastewater generated, and also increases the potential for the introduction of NoVGI cases from other countries or areas within Costa Rica. Furthermore, an outbreak of gastrointestinal illness occurred in Jacó during the dry season sampling period, in which NoV was among the suspected causative agents (Ministerio de Salud, 2018). It is also likely that lower river flow in the dry season (indicating less dilution by rainwater), as well as lower turbidities, improved the likelihood of NoVGI detection in the rivers during the dry season (Mull and Hill, 2012).

Salinity was a dominant predictive variable in the river and ocean models, generally correlating negatively with pathogens. This relationship was likely due to the greater inflow of untreated and partially-treated sewage directly to the rivers; thus, lower salinity waters almost always contained more pathogens given the higher percentage of riverwater. Tidal stage also influenced salinity by changing the relative proportion of river and ocean water in samples, which was visually apparent in the rainy season when the river discharge created a marked brown mass of low-salinity water along the coast. This relationship was also identified by previous studies, where higher FIB and *Salmonella* levels were negatively correlated with salinity (Ortega et al., 2009; Walters et al., 2011).

Water temperature was a significant predictor of fecal microorganisms in the ocean, in which they clustered according to decreasing temperature. Several studies have also determined an inverse relationship between fecal microorganisms and water temperature, and have found associations between bacterial die-off and increasing water temperature (Walters et al., 2011) (Faust et al., 1975; Vasconcelos and Swarmz, 1976). Although water temperature was significantly different between seasons in both river and the ocean, it was not a significant predictor of microorganism variability in the rivers.

4.2. Performance of indicator criteria and MST markers with respect to pathogen detection

Recreational water quality at Jacó and many other tropical beaches is regulated by FIB criteria originally developed in temperate climates. Some have recommended regionally specific guidelines for tropical beaches due to the influence of climate on microbial ecology and ineffective wastewater treatment in low-income communities (e.g. Verhougstraete et al., 2020). While epidemiological studies are needed to identify and validate RWQC in the tropics, we were able to relate FIB RWQC from the United States and Costa Rica, as well as sewage-associated MST markers, with pathogen detection in this study.

The predictive capabilities of the CR fecal coliform and enterococci geometric mean RWQC, and the US EPA enterococci STV RWQC with respect to pathogen presence were similar, and most had high sensitivity (RWQC exceedance when at least one pathogen was detected). This result was unexpected because fecal coliform concentrations are less likely to be correlated with human health outcomes from exposure to recreational water in comparison to enterococci in marine and freshwaters (US EPA, 2012b, 2000; Wade et al., 2003). Enterococci were more specific with respect to pathogen presence in river and ocean samples compared to fecal coliforms, indicating fewer false-positive results when using enterococci criteria (FIB above RWQC when pathogens were not detected).

The predictive capability of MST markers HF183 and PMMoV to identify pathogen presence was also assessed. MST marker detection was used as a permissive criterion for calling a marker positive, while criterion based on QMRA estimates of exceeeding the US EPA benchmark were used as a more rigorous criterion . The permissive LOD criterion (detection) produced 100% sensitivity (marker always present when at least one pathogen was detected), but very low specificity (marker frequently positive when no pathogen was detected); thus, it appears that low levels of the markers are unlikely to be associated with sewage-borne pathogens, particularly in the ocean where positive predictive values were low.

Ahmed et al. (2018a) calculated that 3220 HF183 GC/100 mL and 544 PMMoV GC/100 mL are associated with the US EPA bench-

mark for recreational waters (36 gastroenteritis illnesses/1000 swimmers). For PMMoV only, Crank et al. (2019) estimated that 5054 PMMoV GC/100 mL is associated with the approximate US EPA benchmark for recreational waters (30 illnesses/1000 swimmers). Both studies based risk in part on estimated NoV sewage concentrations from the literature, which may not accurately describe the NoV prevalence in Jacó sewage. Binning MST marker levels according to these QMRA estimates (greater than or less than the benchmark) markedly increased specificity with respect to prediction of pathogen presence in river and ocean samples compared to binning MST markers by LOD (detected or not detected). The sensitivity of PMMoV towards pathogens using the Ahmed et al. (2018a) benchmark was markedly greater than that for the Crank et al. (2019) estimate, suggesting that, at Jacó beach, the higher PMMoV criterion elevated the false-negative results (MST marker below criterion, pathogens detected). This comparison illustrates the variability of QMRA estimates developed in different geographic regions. The data collected in this study will provide the opportunity to develop a regionally-specific QMRA, and will also be augmented by data from subsequent sampling efforts.

4.3. Fecal pollution toolbox approach in tropical surface waters

Relationships between fecal microorganisms are complex, making the predictive capabilities of one fecal indicator or MST marker alone inadequate to accurately indicate pathogen presence (Fujioka et al., 2015; Symonds and Breitbart, 2015; Viau et al., 2011a). In this study, for example, fecal microorganisms formed two distinct clusters based on the influence of environmental parameters in RDA ordination plots in both rivers and the ocean. Furthermore, no one fecal indicator or MST marker best correlated with pathogens in the river or ocean waters. Rather, the use of multiple fecal microorganisms best correlated with pathogens, which was also previously observed for tropical streams (Viau et al., 2011a).

In this study, different subsets of the fecal microorganisms best correlated with pathogens: *C. perfringens*, somatic and male-specific coliphage, and PMMoV in the rivers and enterococci, male-specific coliphage, and PMMoV in ocean waters (Fig. 5). The best subset of indicator microorganisms and MST markers always included at least two viruses and one FIB regardless of the water type. The presence of PMMoV and male-specific coliphage in both the river and ocean subsets of fecal microorganisms that best correlated with pathogens further highlights the importance of these microorganisms as water quality indicators (Fujioka et al., 2015; Nappier et al., 2019; Symonds et al., 2018; Vergara et al., 2015; Viau et al., 2011a, 2011b). It also further emphasizes the importance of including viral indicators to increase the likelihood of identifying the presence of pathogens (Symonds and Breitbart, 2015).

For the river and ocean samples, different FIB were identified in the best subset of fecal microorganisms that maximized correlations to pathogens. *C. perfringens* was best for freshwaters when accompanied by three viral indicators, which may reflect previous observations that *C. perfringens* is a useful fecal indicator in tropical environments (Viau et al., 2011; Fujioka et al., 2015). Enterococci and two viral indicators best correcated with pathogens in the ocean. Previous studies have identified a significant relationship with enterococci concentrations and swimmer gastrointestinal illnesses in marine waters with point source pollution in temperate latitudes, as well as in the tropics (Lamparelli et al., 2015; Verhougstraete et al., 2020). Our study further supports enterococci as an indicator of fecal contamination and human health risks in marine tropical waters. Future studies are needed to confirm the most appropriate combination of fecal indicators, as well as their specific RWQC, for Jacó and other tropical beaches.

5. Conclusions

- Regardless of season, Jacó rivers were implicated as sources of human fecal contamination based on percent exceedance RWQC, high MST sewage marker concentrations, and occurrence of diverse waterborne pathogens.
- Environmental variables explained <26% of the microbial variability; rainfall was the most important predictor at Jacó beach, while salinity and water temperature were also significant in river and ocean models.
- FIB and MST sewage markers had high positive predictive values for pathogens in rivers when RWQC or QMRA-estimated criteria were used as benchmarks. The US EPA enterococci STV criterion had the highest positive (78%) and negative (96%) predictive value of all indicators in ocean samples.
- *C. perfringens*, somatic and male-specific coliphages, and PMMoV together maximized correlations with pathogens in the rivers, while male-specific coliphages, PMMoV and enterococci together maximized correlations with pathogens at ocean sites.
- No one fecal indicator or MST sewage marker best correlated with pathogens; rather, the use of multiple fecal indicators and MST markers maximized pathogen correlations. A fecal pollution toolbox approach, containing at least one viral indicator, is recommended for pathogen prediction at tropical beaches. Further research is needed to confirm the constituents of this toolbox.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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CHAPTER THREE: RISK OF GASTROENTERITIS FROM SWIMMING AT A WASTEWATER-IMPACTED TROPICAL BEACH VARIES ACROSS LOCALIZED SCALES

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Key Highlights

1. Pathogen levels differed by subwatershed across 2.4 km of beach.

2. Norovirus and adenovirus drove health risk for swimmers.

3. QMRA found that swimmers' health risk was higher in dry (tourist) season.

4. Viral log reduction up to 4.1 is needed for safe swimming in Jacó rivers.

Abstract

Population growth and changing climate are expected to increase human exposure to pathogens in tropical coastal waters. We examined microbiological water quality in three rivers within 2.3 km of each other that impact a Costa Rican beach and in the ocean outside their plumes during the rainy and dry season. We performed quantitative microbial risk assessment to predict the risk of gastroenteritis associated with swimming and the amount of pathogen reduction needed to ensure safe conditions. Recreational water quality criteria based on enterococci were exceeded in >90% of river samples, but in only 13% of ocean samples. Multivariate analysis grouped microbial observations by subwatershed and season in river samples, but only by subwatershed in the ocean. The modeled median risk from all pathogens in river samples was between 0.345 and 0.577, tenfold above the USEPA benchmark of 0.036 (36 illnesses/1000 swimmers). Norovirus genogroup I (NoVGI) contributed most to risk, but adenoviruses raised risk above the threshold in the two most urban subwatersheds. Risk was greater in the dry compared to rainy season, due largely to the greater frequency of NoVGI detection (100% vs 41%). Viral log10 reduction needed to ensure safe swimming conditions varied by subwatershed and season, and was greatest in dry season (3.8 – 4.1 dry; 2.7 -3.2 rainy). QMRA that accounts for seasonal and local variability of water quality contributes to understanding the complex influences of hydrology, land use, and environment on human health risk in tropical coastal areas and can contribute to improved beach management.

Introduction

The release of untreated and inadequately treated domestic wastewater into rivers and the ocean is a global threat to the health of both humans and the environment (DeFlorio-Barker *et al.*, 2018; Fleming *et al.*, 2006). An estimated 90 million illnesses per year are caused by exposure to pathogens in recreational water in the United States (US), where sewage is routinely treated and disinfected (DeFlorio-Barker *et al.*, 2018). This situation is likely worse in many other countries that have less wastewater treatment infrastructure (Prüss-Ustün *et al.*, 2014). Less than 65% of domestic wastewater is collected in Latin American countries and the Caribbean, of which only 41% is disinfected (UN Habitat & WHO, 2021).

Recreational water quality criteria (RWQC) used to assess the safety of recreational waters are based on fecal indicator bacteria (FIB), including enterococci, fecal coliforms and *E. coli*, which are used as surrogates for waterborne pathogens (US EPA, 2012a). Although FIB can originate from human feces, they can also be found in the feces of other animals and in extra-intestinal sources, such as vegetation and sediments, disrupting their relationships with pathogens (Anderson *et al.*, 2005; Byappanahalli *et al.*, 2012; Desmarais *et al.*, 2002; Nguyen *et al.*, 2018). Alternative indicators such as *Clostridium perfringens* and somatic and F+ coliphages suffer from many of the same drawbacks of conventional indicators (Boehm *et al.*, 2009; Cabelli, 1978; Fujioka *et al.*, 2015; US EPA, 2015, 2012a). The information provided by general fecal indicators is increasingly supported by data on host- or source-associated microbial source tracking (MST) microorganisms or genes in research studies and actions (Harwood *et al.*, 2014; Nguyen *et al.*, 2018). Many MST methods for human/sewage fecal pollution have been developed, among them the extensively-validated *Bacteroides* gene marker HF183 (Ahmed *et al.*, 2016, 2008; Bernhard and Field, 2000; Boehm *et al.*, 2013; Gawler *et al.*, 2007; Odagiri *et al.*, 2015;

Seurinck *et al.*, 2005). Pepper mild mottle virus (PMMoV) is a plant virus that is ubiquitous and present at high levels in sewage (Rosario *et al.*, 2009; Symonds *et al.*, 2019, 2018). HF183 and PMMoV have been used as complementary bacterial and viral markers, respectively, for sewage in several studies (González-Fernández *et al.*, 2021; Harwood *et al.*, 2014; Symonds *et al.*, 2017, 2016).

Epidemiology studies are the "gold-standard" for estimating disease burden associated with water exposure, and they provide the definitive information on human health risk required to develop evidence-based guidance for public health protection (Hedberg & Maher, 2019). It is, however, logistically challenging and very expensive to perform robust epidemiology studies that can detect a given health effect. Quantitative microbial risk assessment (QMRA) is a more feasible mathematical modeling approach used to predict the impact that pathogens in polluted waters may have on the health of exposed individuals (Ashbolt *et al.*, 2010). QMRA provides a quantitative estimate of the probability of illness resulting from a specific exposure, such as submerging one's head underwater, and can help guide management strategies that maximize human health protection at beaches.

The QMRA framework starts by identifying the microbial hazard(s) and defining exposure, which for beach recreators involves defining distributions for pathogen concentration(s) in the water and the volume of water ingested while recreating. While pathogens can be measured directly, they are typically present at low concentrations in environmental waters. Thus, a variety of left-censored data techniques (e.g., Robust Regression on Order Statistics (rROS), 95th percentile estimation based on surrogate) are often required to define the distribution of pathogens in the model (Helsel, 2011; Orner *et al.*, 2021; Verbyla *et al.*, 2016). The volume of water ingested is typically assumed from the distribution defined by swimming pool studies from the US

(Dufour *et al.*, 2006). Additionally, uncertainties and assumptions are inherent to all dose-response models (Haas *et al.*, 2014), particularly for norovirus genogroup I (NoVGI) where a ~1000-fold difference in risk of infection is determined depending upon the use of the disaggregate or aggregate model (Orner *et al.*, 2021; Schmidt, 2015; Sunger *et al.*, 2019; Van Abel *et al.*, 2017). Despite the assumptions of each model parameter and the introduction of uncertainty, QMRA and its interpretation can be improved through effective model construction from source to exposure, the use of distributions and not point estimates, identification of sensitive system components to ensure that parameters are representative, and measurement of model parameter sensitivity (Haas *et al.*, 2014; Petterson & Ashbolt, 2016).

Few studies executed in the tropics have measured indicators, MST-markers, and pathogens or repeatedly measured the same site over time (Kongprajug *et al.*, 2021; Vadde *et al.*, 2019). This study builds upon a prior microbial water quality study and a QMRA performed at Jacó beach (González-Fernández *et al.*, 2021; Orner *et al.*, 2021). Jacó is a heavily-visited beach that lacks a central sewer and sanitation system, and relies on on-site sanitation infrastructure (Mora, 2009; Ramírez-Sánchez *et al.*, 2015; WHO & UNICEF, 2014). The field work of our previous study occurred during the rainy season of 2017 and the dry season of 2018 and will be referred to as the 2018 study, while our current study took place in the same location during the rainy season of 2018 and the dry season of 2019 and will be referred to as the 2019 study. In this 2019 study, we measured FIB, sewage-associated MST markers, and pathogen levels in three of the five rivers that convey wastewater contamination to Jacó Beach and estimated the risk of gastrointestinal (GI) illness from swimming in the rivers. We also determined differences among microbes at a more localized scale; per subwatershed, and in both rivers and in the ocean outside their plumes for a subset of microbes. Using QMRA, we modeled the viral log10 reduction value

(LRV) required to lower viral concentrations to achieve safe recreational water quality in the rivers (Orner *et al.*, 2021).

Material and Methods

Study site and study design

Jacó is a highly visited beach that is known to be polluted by domestic wastewater (González-Fernández *et al.*, 2021). It is located in the province of Puntarenas in the Central Pacific coast of Costa Rica. The beach is 4.2 km long, and most water recreation activities (surfing and swimming) occur near three of the rivers that discharge on the southern end of the beach. Copey, Naranjal and Madrigal rivers, including their respective ocean discharges, each comprise a separate subwatershed, which we sampled on a localized scale. River and ocean water of each subwatershed were sampled 13 times each during the rainy (Aug – Oct, 2018) and dry seasons (Jan – Mar, 2019), which resulted in 39 river samples and 39 ocean samples per season, 78 per water type (river and ocean) and a total of 156 water samples (Table 1).

Sample concentration and handling

Microorganisms in the river samples (salinity ~ 0) were concentrated on site from 50 L of water by ultrafiltration using REXEED 25SX dialysis filters as previously described (González-Fernández *et al.*, 2021; Mull & Hill, 2012). The filters were stored at 4 °C and shipped on Techni Ice™ to BCS Laboratories in Gainesville, Florida for processing within 72 h of collection. Dialysis filters were eluted and back-flushed with a 500-mL solution containing 0.5% Tween 80, 0.01% sodium polyphosphate, and 0.001% Antifoam Y-30 Emulsion as previously described, yielding 460 ml eluate (~1,000-fold concentration factor). A proportion of eluate was further concentrated by PEG precipitation as previously described (González-Fernández *et al.*, 2021).

Ocean samples were not analyzed for pathogens due to the infrequent detection of pathogens in ocean samples during our previous study (González-Fernández *et al.*, 2021), and were therefore processed differently. Ocean samples were collected just outside of the river plume (salinity generally >30 ppt) where ocean depth was approximately 1 m.

Microorganisms in the ocean samples were filtered from a 500-mL grab sample onto a 0.45-µm, mixed cellulose ester, 47-mm diameter filter (Type HAWP; Millipore, Billerica, MA) using a modified can crusher, sterile syringe, and syringe filter holder as previously described (Symonds *et al.*, 2014). PEG concentrates from river samples and membrane filters from ocean samples were stored at -80 °C and were thawed only once for nucleic acid extraction. Simultaneously, 500-mL grab samples were collected from all river and ocean sites and transported on ice to the Costa Rican National Water Quality Laboratory for fecal coliform and enterococci analyses within 12 hours of collection (Table 1).

Microbial analysis

Pathogens (NoVGI, human adenovirus (AdV), *Giardia* and *Cryptosporidium*), human wastewater-associated MST markers (HF183 and PMMoV), and bacterial (enterococci, fecal coliforms, *C. perfringens*) and viral (somatic and F+ coliphage) fecal indicators were quantified in river samples, while only HF183 and FIB (enterococci and fecal coliforms) were quantified in ocean samples (Table 1). From the concentrated eluate of river samples, somatic coliphages and F+ coliphages were cultured by the US Environmental Protection Agency (US EPA) Method 1601 (US EPA, 2001), *C. perfringens* were cultured using Standard Method ASTM D5916-96(2002) (American Society for Testing & Materials, 1996). *Salmonella* spp. were cultured from the concentrated eluate of river samples by inoculating 5 replicates of 20-mL, 10-mL and 1-mL

volumes for the most probable number (MPN) analysis. *Salmonella* spp. was quantified and confirmed following US EPA Method 1682 (US EPA, 2006). *Giardia* spp. and *Cryptosporidium* spp. were enumerated by immunomagnetic separation and microscopy by US EPA Method 1623.1 (US EPA, 2005a) (Table 1). Fecal indicator bacteria in river and ocean samples were cultured from 500-mL grab samples. Fecal coliforms were enumerated by multiple tube fermentation according to the American Public Health Association standard methods, section 9221 E (American Public Health Association, 2017) and enterococci enumeration was performed using membrane filtration according to US EPA Method 1600 (US EPA, 2005b). NoVGI, AdV, HF183, PMMoV and extraction efficiency controls (See section 1.5) were all quantified from nucleic acid using quantitative PCR (qPCR) (Table 2).

Nucleic acid extraction (-reverse transcription)

Nucleic acid was extracted from PEG concentrates and membrane filters from river and ocean water samples using the AllPrep PowerViral DNA/RNA Kit (Qiagen; Germantown, USA) and the PowerWater DNA Kit (Qiagen), respectively, following the manufacturer's instructions. An extraction control consisting of nuclease-free water was processed for each batch of samples. All river DNA samples were tested for AdV, HF183 and salmon testes DNA (Sketa; positive process control; Table 2). Reverse transcription (RT) was performed using random hexamers and the Superscript IV First-strand Synthesis System (Invitrogen; Carlsbad, USA) immediately following nucleic acid extraction, and all river cDNA samples were analyzed for PMMoV, NoVGI, and feline calicivirus (FCV; positive process control) (Table 2). Ocean DNA samples were only tested for HF183 and salmon testes DNA (Sketa; positive process control) (Table 2). DNA and cDNA were stored at -20 °C for no longer than ten months for all pathogens and MST markers prior to qPCR.

Extraction (-reverse transcription) controls and efficiency calculation

An extraction negative control (sterile water added to an extraction tube and carried through the entire process) was included during each extraction round to test for contamination. The positive and negative controls represent a control for the following processes: D/RNA purification in all samples, and reverse transcription only in river samples. To determine the efficiency of nucleic acid extraction, each sample as well as a sterile water calibrator was spiked with salmon testes DNA and FCV (ATCC VR-782, lot #63847341), and efficiency was calculated according to previously published methods (Mattison *et al.*, 2009; Symonds *et al.*, 2014; US EPA, 2012b). Mean recovery of Sketa and FCV was 82% and 81% respectively.

(RT-)qPCR analyses

Quantitative reverse transcription polymerase chain reaction [(RT-)qPCR] analyses were executed to determine the concentrations of microbial targets in river water (original volume = 50 L) and ocean water (original volume = 500 mL) samples. All samples were analyzed following previously published assays in 25-µL reactions using TaqMan™ Environmental Master Mix 2.0 (Table 2). Negative controls containing no template were included in each qPCR instrument run and all were negative. All assays were run using an ABI 7500 Real-Time PCR System (Thermo Fisher Scientific; Waltham, USA).

Standard curves for qPCR analyses were constructed from gBlocks Gene Fragments (IDT; Coralville, USA). For each assay, ten-fold serial dilutions of the gBlock standard, ranging from 10,000 to 10 gene copies (GC) per reaction, were analyzed in duplicate alongside samples and no-template controls. For each instrument run, log10-linear regression analysis of the quantification cycle (Cq) value and gene copy (GC) number associated with each point in the ten-fold

dilution series was executed to produce a standard curve. To adhere to the qPCR MIQE guidelines, data were only generated from qPCR instrument runs when standard curves had 90 to 110% efficiency and R2 values were ≥0.97 (Bustin *et al.*, 2009).

Microbial targets were considered quantifiable when duplicate measurements were within the assay dynamic range and the standard deviation was less than 1 Cq. If no fluorescence was observed in duplicate reactions during 40 cycles, the quantity measured was classified as 'less than the assay limit of detection' (<LOD). Samples in which only one technical replicate amplified were rerun. The sample was considered positive but below the limit of quantification (LOQ) when the target was detected in at least two out of the four reactions, and when the mean Cq was greater than the assay LOQ.

(RT-)qPCR inhibition testing

qPCR inhibition was assessed by analyzing a 1:10 dilution of target nucleic acids for a DNA (HF183) and a cDNA (PMMoV) assay. PCR inhibition was determined by comparing the Cq of an undiluted sample to that of a 1:10 dilution. PCR inhibition was deemed to be absent if the dilution Cq was at least 2 Cq lower than that of the undiluted sample (Cao *et al.*, 2012). No inhibition was observed during this study.

Estimation of microbial concentrations from (RT-)qPCR Cq values

To account for inter-plate variability across all qPCR runs for a specific assay, the 'pooled approach' was used to estimate the mean GC in each sample using WinBUGS software V1.4.3 (Imperial College and Medical Research 107 Council, UK) (Sivaganesan *et al.*, 2010). This approach uses Markov chain Monte Carlo simulations to calculate a single, standard curve equation (also known as a calibration equation) from all of the standard curves generated during

the experiment (4 to 9 instrument runs, depending on the assay). For PMMoV, NoVGI, and FCV, all mean copy numbers were divided by two to take into account the differences between the double-stranded gBlock standard curve material and the viruses' single-stranded genomes.

Based upon all of the standard curves generated during this study, each assay's limit of detection (LOD) and limit of quantification (LOQ) were calculated (Table 1). The assay LOD was defined as the lowest quantity at which amplification occurs 95% of the time within 40 cycles, calculated as previously described (Verbyla *et al.*, 2016). The assay LOQ was defined as the lowest quantity that was reliably measured 100% of the time within the dynamic range of the assay. The process LOD, process LOQ and sample concentration were back-calculated to include all of the steps in the molecular and microbial concentration processes (GC/L).

Environmental data

Water temperature (°C), pH, salinity (ppt), and turbidity (nephelometric turbidity unit; NTU) were measured with a YSI 556 Multiprobe (Rye Brook, USA) *in situ* at the time of sample collection. Rainfall (mm) in the12 h, 24 h, 48 h, 72 h and 96 h periods prior to sampling were calculated with hourly rainfall data provided by AyA Piedra Bruja weather station located in Jacó.

Data analyses

Left-censored data were considered to be observations below the limit of detection (<LOD) or between the LOD and the limit of quantification (LOQ), which are considered detectable but not quantifiable (DNQ). Between 0% and 100% of observations for each pathogen (NoVGI, AdV, *Cryptosporidium* and *Giardia*) were left-censored per subwatershed (Table S1 and S2). MST marker observations (HF183 and PMMoV) in contrast, were 0% to 50% left-

censored, and FIB observations (enterococci and fecal coliforms) were not left-censored per sub-watershed (Table S1, S2 and S3). Alternative fecal indicator microorganisms (*C. perfringens*, somatic and F+ coliphages were 0 to 23% left-censored (Table S1 and S2). Fecal coliform observations were the only ones that included right-censored data (>LOQ; 6%). Percentage of observations that were not quantifiable, arranged in order of non-detects (below the assay LOD), detected but not quantifiable (above assay LOD and below the assay LOQ) and quantifiable data (above assay LOQ) are also provided by season in Figure 1 and 2. Data were entered in a two-tier censoring scheme that considered both the non-detects (<LOD) and DNQ observations. All values less than the LOD or LOQ were censored to LOD -1 or LOQ - 1, respectively (Helsel, 2011). The u-scores, which are the numerator in computation of Kendall's tau correlation and the basis for the Mann-Whitney test, were calculated for all the ln-transformed interval microbial data to account for censored observations (Helsel, 2011). All environmental parameters were standardized by calculating their z-score and creating a Euclidean distance matrix. R version 4.0.2 was used to execute all statistical analyses: descriptive statistics, hypothesis testing, redundancy analyses, QMRA and sensitivity (correlation) analyses (R Core Team, 2013).

Descriptive statistics (mean and standard deviation) were calculated by season and sub-watershed for all environmental and non-censored microbial data. If data were <80% left censored (observations below the process LOD or LOQ), descriptive statistics were estimated with Robust Regression on Order Statistics (rROS) using the "NADA" R package (Helsel, 2011; Lee, 2017). When observations were > 80% censored only the 90th and 95th percentiles were reported. The frequency of detection (>LOD) for each pathogen was determined.

Differences by river and season

Since data were equally collected in three subwatersheds during the rainy and dry seasons, a two-way permutational multivariate analysis of variance (PERMANOVA) was performed to determine if there is a significant subwatershed-by-season interaction effect in the concentration of all measured microorganisms in the river (enterococci, fecal coliforms, *C. perfringens*, somatic and F+ coliphages, *Giardia*, *Cryptosporidium*, NoVGI and AdV) and ocean (enterococci, fecal coliforms and HF183) data separately using the vegan R package (Oksanen *et al.*, 2016). PERMANOVA's assumption of homogeneity of multivariate dispersion was examined and determined to be met prior to testing for differences among groups, and a pairwise PERMANOVA with corrections for multiple testing (holm p-value adjustment method) was used to determine which groups varied from one another. Discriminant analysis using the canonical analysis of principal components (CAP) was performed with the Biodiversity R package to produce an ordination diagram to visualize differences in observations among subwatersheds and seasons (Kindt, 2019).

Redundancy analysis- Effect of environmental variables on fecal microorganisms

Relationships among environmental parameters and fecal microorganisms were explored by employing an Akaike information criterion (AIC) based stepwise variable selection procedure with a redundancy analysis (RDA) approach to build an optimal model (most parsimonious) and test the following null hypothesis: There is no significant effect of the following selected variables: antecedent rainfall (12, 24, 48, 72 and 96 h prior to sampling), turbidity, and/or salinity, on fecal microbial concentrations in Jacó's subwatersheds. RDAs to test for multivariate linear relationships between environmental parameters and all measured fecal microorganisms were built

for river and ocean data separately, with both rainy and dry season data combined. To reduce the number of species (microorganisms) plotted on the river RDA and help with interpretation, a multiple regression of species with linear scores of ordination axes was calculated and species were plotted based on goodness of fit. PMMoV, followed by *Salmonella*, NoVGI, F+ coliphage, and *C. perfringens*, were most highly associated with the first two RDA axes ($r2 > 0.4$) and are labelled in the river ordination plot (Table S4A, Figure S1). These analyses were performed using the vegan R package (Oksanen *et al.*, 2016).

Risk of gastroenteritis and viral log10 reduction values required for safe swimming

QMRA was used to determine the risk of illness for swimmers in the rivers at Jacó beach in terms of risk from individual pathogens and cumulative risk from all measured pathogens (Sunger *et al.*, 2019). The exposure assessment was defined for an individual who was submerged in the river (wetting head or face) and was calculated by river and season as previously described (Orner *et al.*, 2021). Swimmer exposure in the river (dose ingested, d) was calculated by multiplying the assumed volume of water ingested (US EPA, 2010) (defined as a distribution, for mixed adult and child populations in swimming pools; Table S5) by the pathogen concentration derived from measurements in the river water (Table S5 and S6). Briefly, log10-normal distributions were used to describe pathogen concentrations in the river and the volume ingested (see SI for details; Table S5 & S6). When data were left-censored, the pathogens' log10-mean and log10-standard deviation concentrations were estimated using either robust rROS statistics (<80% left-censored; NADA package; Helsel, 2021) or 95th percentile estimation methods (>80% left-censored) (Orner *et al.*, 2021; Verbyla *et al.*, 2016). For each model parameter described as a distribution, a random set of 10,000 Monte Carlo values was used to account for model variability and uncertainty. To convert AdV gene copies to median tissue culture

infectious dose (TCD50) for the AdV dose ingested, the dose was multiplied by a harmonization factor described by a uniform distribution (Flint *et al.*, 2009; Heider and Metzner, 2014; Kundu *et al.*, 2013) (Table S5).

Risk of infection for each pathogen (Pinf) was calculated using the estimated dose ingested and the following dose-response models and model parameters: fractional Poisson for *Cryptosporidium* (Messner & Berger, 2016) and NoVGI (Messner *et al.*, 2014), exponential for *Giardia* (Rose *et al.*, 1991), beta function for *Salmonella* (Teunis *et al.*, 2010), and the exact beta Poisson for AdV (P. Teunis *et al.*, 2016) (Table S5). Norovirus GI was measured in this study because most data used to develop the NoV dose-response curve were from NoVGI feeding studies (Messner *et al.*, 2014) and this genogroup was previously identified as prevalent in the region (Symonds *et al.*, 2017). The risk of infection from NoVGI was calculated using the aggregate and disaggregate values for the model parameter (μ) separately because there is currently no consensus in the literature on the most appropriate value to use (Schmidt, 2015; Schoen *et al.*, 2020; Sunger *et al.*, 2019; Van Abel *et al.*, 2017). Susceptibility to NoVGI (P) has a human genetic component (Nordgren *et al.*, 2016) and was modified to reflect Costa Rica's population (Morera *et al.*, 2003) as recommended (Van Abel *et al.*, 2017). Subsequently, risk of GI illness (Pill) was calculated by multiplying the risk of infection by the morbidity ratio (DuPont *et al.*, 1995; Haas *et al.*, 2014; Rose *et al.*, 1991; Soller *et al.*, 2017) (Table S5). The cumulative risk of GI illness was then calculated by summing the risk of illness for each reference pathogen (NoVGI, AdV, *Salmonella*, *Giardia*, and *Cryptosporidium*) (Orner *et al.*, 2021; Sunger *et al.*, 2019).

Descriptive statistics describing the risk of illness were calculated for each pathogen, as well as cumulatively, for all measured pathogens for each river and season. Risk of illness was compared against the US EPA health benchmark (36 illnesses/1000 swimmers) (US EPA,

2012a). The sensitivity of the QMRA was evaluated by using Spearman rank order correlation analyses between simulated input parameters derived from the literature (Boehm *et al.*, 2018; Maier *et al.*, 2009; Messner *et al.*, 2014; Messner and Berger, 2016; Rose *et al.*, 1991; Stone *et al.*, 2008; Symonds *et al.*, 2017; P. Teunis *et al.*, 2016; Van Abel *et al.*, 2017) (Table S7) and the cumulative probability of illness from all measured pathogens ($\alpha = 0.05$). Finally, since NoVGI and AdV contributed the most to cumulative risk of illness, the viral surface water log10 reduction value (LRV) needed to ensure safe swimming conditions in the rivers along Jacó beach were calculated for each river and season.

For this analysis, swimming conditions were considered safe when the 95th percentile of viral cumulative risk of GI illness was less than 0.036 (US EPA health benchmark recommendation 1) (US EPA, 2012a) as previously described (Orner *et al.*, 2021). The LRV was calculated separately using the NoVGI disaggregate or aggregate model for each river and season. Briefly, if the 95th percentile of the cumulative risk of illness of NoVGI and AdV exceeded the health benchmark, then viral concentrations in the exposure assessment were reduced by 0.1-log10. Subsequently, the risk of infection and illness were modeled with the same QMRA previously described until the 95th percentile of the cumulative viral risk of illness fell below the health benchmark (Orner *et al.*, 2021; Symonds *et al.*, 2014).

Results

Jacó rivers are a source of wastewater pollution for Jacó beach. Exceedance of recreational water quality criteria (RWQC) for FIB occurred in the vast majority of river samples (90% to 97%), regardless of the regulatory criteria used, however, frequency of exceedance was lower and more variable in ocean samples (0 to 54%) (Table 3). RWQC exceedances in ocean samples

occurred much more frequently in the rainy (26% to 54%) than in the dry season (0% to 38%; Table 3). Most pathogens were frequently detected in the rivers (39% to 100% of observations) (Table S8) at high concentrations (i.e., 2.85 x 103 GC/L of NoVGI and 5.87 x 102 GC/L of AdV in the dry season) (Table S9); however, *Cryptosporidium* was detected in only 5 out of 78 river samples (Table S8). Pathogens were not assessed in the ocean, but mean HF183 concentrations were 3.33 x 104 GC/L in the rainy season and 2.78 x 104 GC/L in the dry season (Table S10), demonstrating year-around contamination by wastewater at Jacó beach.

Differences in microbial concentrations by subwatershed and season

Copey, Naranjal and Madrigal rivers coupled with their associated ocean waters were each considered a separate subwatershed. Analysis was conducted separately in the river and ocean samples of each subwatershed. Microbial concentrations measured in Jacó rivers varied significantly by season (rainy vs. dry) and by river (Copey, Naranjal and Madrigal) according to the multivariate PERMANOVA analysis, which identified a significant interaction effect between river and season (Table S4B). Pairwise comparisons with Holm p-value correction for multiple testing were performed for all combined river and season interaction factors (e.g., Copey*dry, Copey*rainy) to identify which factors were significantly different (Table S4C). Canonical axes that best discriminate among microbial variables by site and season are shown in the ordination plot generated from CAP analysis (Figure 3). Microorganisms that contribute the most to differences in season/river groupings have arrows of greater magnitude and their direction is more horizontal along canonical axis I, which explained the greatest portion of the variability in the model (49.32%). Descriptive statistics and frequency of detection determined for the raw data are also shown in Figure 4 (Table S1 and S2) and Table 4:

Samples clustered distinctly by season and to a lesser extent by river. Increased concentrations of enterococci and AdV were the main drivers of clustering among samples from Naranjal River in the rainy season (Figure 3). Microbial levels in Naranjal River samples collected in the rainy season were significantly different from those in all other river*season combinations (Table S4C). For example, the highest mean concentrations of AdV (1.45 x 103 GC /L) and enterococci (4.35 x 104 CFU/L) were measured in Naranjal River during the rainy season (Figure 3 and 4, Table S1), coinciding with the highest frequency of AdV detection (69%) (Table 4). AdV was consistently present year-round in at least one sample from each river, but it was infrequently detected in Madrigal River regardless of season (only 8% frequency), while detection frequency ranged from 38% to 62% in all other river/season combinations (Table 4). Enterococci mean concentrations were elevated one to two orders of magnitude above regulatory criteria in all rivers regardless of season but were typically at least one order of magnitude greater in the rainy versus dry season (Figure 4, Table S1 and S2).

High concentrations of HF183 and *C. perfringens* were the main drivers of the clustering observed among Copey River samples in the dry season (Figure 3). Copey River samples in the dry season were significantly different than samples from all pairs of river/season factors except for Naranjal River in the dry season (Table S4C). The Copey River dry season samples were characterized by the highest mean levels of HF183 (6.30 x 104 GC/L) and *C. perfringens* (8.87 x 102 CFU/L) (Figure 4, Table S2). The concentrations of these microorganisms were always greater in the dry season samples compared to the rainy season samples in all rivers (Figure 4, Table S1 and S2).

NoVGI and *Salmonella* were detected in 100% of the samples in all rivers during the dry season (Table 4). Increasing levels of *Salmonella* and NoVGI were the main drivers of the

clustering among Madrigal River samples in the dry season (Figure 3). Madrigal River samples in the dry season were significantly different from observations from all rivers sampled during the rainy and dry seasons, with the exception of samples from Naranjal River in the dry season (Table S4C). The highest mean concentrations of *Salmonella* (1.74 x 102 CFU /L) and NoVGI (4.52 x 103 GC /L) were found in Madrigal River in the dry season (Table S1 and S2). In the rainy season, NoVGI was infrequently detected in Copey (23% detection) and Madrigal (15% detection) rivers in comparison with Naranjal River (85% detection), and when detected, observations were often below the assay limit of quantification (Table 4). Mean concentrations of *Salmonella* in the rainy season samples were at least one order of magnitude greater at Madrigal River versus Copey and Naranjal rivers; Figure 3 (Table S1).

Fecal coliforms, enterococci and HF183 measured in the ocean samples were significantly different among ocean sites (Table S4B) (i.e., just outside the river plumes of Copey, Naranjal and Madrigal rivers), but not by season (Table S4D). Microbial concentrations at the Copey ocean site were significantly higher than those at Naranjal and Madrigal (Table S3 and S4D). Most (94.37%) of the variation among the ocean sites sampled was explained by canonical axis 1, where higher concentrations of HF183 and FIB were identified as the drivers of the observed clustering among samples from the ocean in the Copey subwatershed (Figure 5). Although HF183 diverges from FIB in Figure 4, canonical axis II only accounts for 5% of explained variability. The highest mean levels of enterococci (1.38 x 103 CFU/L), fecal coliforms (8.70 x 103 MPN/L) and HF183 (5.06 x 104 GC/L) were detected in the Copey ocean sites (Figure 5 and 6, Table S3).

Relationship of environmental variables to microbial concentrations

RDA analysis found that a small, but significant, fraction of the variability in microbial concentrations was explained by environmental parameters (Table S4E and S4F). RDAs were performed separately for river and ocean data, and the selected models were those with the lowest AIC, composed of a subset of environmental parameters that best represented the relationship between microbial concentrations and environmental parameters. Variables that significantly optimized the relationship between microorganisms in the river RDA were rainfall 48 h prior to sampling and water temperature, and both had a significant effect on microbial concentrations (Table S4E, Figure S1). Greater concentrations of *Salmonella*, followed by NoVGI, F+ coliphage and PMMoV, were typically found in samples with low or no antecedent rainfall (Figure S1). In the ocean RDA, optimal selected variables were water temperature and rainfall 48 h and 96 h prior to sampling (Figure S2, Table S4F), yet only water temperature and rainfall 48 h prior sampling were significant predictors (Figure S2, Table S4F). Enterococci, fecal coliforms and HF183 clustered with a gradient of increasing rainfall and decreasing water temperature (Figure S2).

Quantitative microbial risk assessment for GI illness due to swimming per season and river

Although the risk of infection from NoVGI was calculated using both the aggregate and disaggregate model parameter (μ), we focus our results section on the conservative disaggregate norovirus model which predicts higher risk to swimmers. Results for the aggregate model are shown in the supplementary material section (Figure S3 and S4, Table S11) and both models are compared in the discussion section. The cumulative risk of GI illness from exposure to all measured pathogens varied by season and river. Median cumulative risk exceeded the US EPA benchmark (36/1000, or 0.036) for safe recreation regardless of river (Figures 7 and S4). NoVGI

contributed the most to risk, followed by AdV, regardless of season and river (Figure 7 and S4). Median risk of GI illness from NoVGI always exceeded 0.036, while median risk due to AdV alone exceeded the benchmark only in Copey and Naranjal rivers in the dry season and in the Naranjal River during the rainy season (Figure 7). The contributions of *Giardia, Cryptosporidium,* and *Salmonella* to the cumulative risk of GI illness varied by river and season but were always much lower than the risk posed by viral pathogens (Figure 7 and S4). The QMRA was most sensitive to the exposure parameters, particularly the concentrations of *Cryptosporidium*, *Giardia*, and NoVGI, and volume ingested, followed by the risk characterization (dose-response) parameters (Figure S5). Since pathogen recovery efficiency was not among the most significantly correlated parameters with total pathogen risk of illness, this model parameter was not considered in the exposure assessment (Orner *et al.*, 2021).

Viral log10 reduction values needed for safe swimming conditions per subwatershed

Jacó rivers require reductions in the concentration of viruses for safe swimming conditions to be met (i.e., for the 95th percentile of the cumulative risk of illness to be lower than U.S. EPA health benchmark of 36 illness per 1000 recreators). The viral LRV was calculated by season and river based on cumulative NoVGI and AdV risk of illness calculated with the QMRA (Table 5). The greatest viral LRV was calculated in the dry season for Madrigal River (4.1-log10), followed by Naranjal River (3.9-log10) and Copey River (3.8-log10) (Table 5). LRVs during the rainy season showed a different pattern were Naranjal River was highest (3.2-log10), followed by Copey River (3.0-log10) and Madrigal River (2.7-log10).

Discussion

This study analyzed pathogen levels to estimate the risk of gastroenteritis from waterborne pathogens among swimmers at a tropical beach that receives domestic wastewater pollution from septic systems and direct discharge from sewer pipes into rivers within the watershed. It builds upon QMRA studies using pathogen measurements from other latitudes, and improves upon others by measuring specific pathogens in the rivers, rather than relying on surrogates (indicator organisms or MST markers) or estimating pathogen concentrations from the literature (Ashbolt *et al.*, 2010; Boehm *et al.*, 2015; McBride *et al.*, 2013; Sunger *et al.*, 2019). By analyzing microbial levels and estimating risk of GI illness in each river, we were able to discern differences in pathogen levels and human health risk even though all rivers were highly polluted by wastewater and were located less than 2.5 km from one another. Despite using actual pathogen measurements to define distributions in the exposure assessment, it is important to note that risk of GI illness was significantly influenced by the volume of ambient water ingested. The ingestion parameter was derived from swimming pool studies that may or may not reflect human behavior and exposure to pathogens in a river. Future studies are needed to better understand and define this parameter in different contexts to improve the estimates of risk of GI illness to swimmers.

In our previous (2018) study, microbial concentrations in Jacó rivers varied seasonally (rainy versus dry) (González-Fernández *et al.*, 2021); however, differences by river were not explored. In the current study, high levels of most microorganisms were found in Jacó rivers during the dry season, but in the rainy season Naranjal River was differentiated from Copey and Madrigal rivers due to high concentrations of enterococci and AdV and more frequent detection of NoVGI. Median cumulative risk of GI illness was also greater during the dry season, in

agreement with a study that utilized the 2018 data for QMRA (Orner *et al.*, 2021). Increased tourism in the dry season months increases the volume of wastewater discharged to surface waters, while decreased rainfall weakens natural river flow, resulting in less dilution of microbial pollutants. Furthermore, some seasonal workers who support the tourism industry are known to stay in informal settlements located in Copey and Naranjal during peak tourism season in dry months (Borowy, 2004; González-Fernández *et al.*, 2021). These factors undoubtedly contribute to elevated levels of NoVGI, *Salmonella*, HF183, PMMoV, somatic coliphages and *C. perfringens* in all rivers during the dry season and generally elevated risk for swimmers in the rivers. Higher levels of most microorganisms, including NoVGI, MST markers, *C. perfringens* and somatic coliphages in Copey River and Naranjal River compared to Madrigal River are also likely to be influenced by land use, as these rivers have more urban development and a small percentage of the population lives in informal settlements without access to sanitary infrastructure. The Madrigal River subwatershed, in contrast, is characterized by more cattle grazing lands and lower human population density, as well as the presence of horses used for recreation on the beach (Orner *et al.*, 2021; Orozco Montoya, 2015; Sánchez-Gutiérrez *et al.*, 2020).

In the ocean, where pathogens were not measured due to documented low concentrations (González-Fernández *et al.*, 2021), HF183 and FIB levels varied in samples from each subwatershed but not by season. HF183 and FIB levels were higher in Copey and Naranjal ocean sites compared to Madrigal, reflecting the trend of greater pathogen concentrations in the rivers with more urban land use. A risk-based threshold for HF183 of 525 GC/100 ml was defined in a California QMRA study as the concentration of HF183 for which the median estimated risk of gastroenteritis from swimming is 32/1000 (Boehm & Soller, 2020) due to cumulative risk from exposure to AdV, NoV, *Giardia*, *Cryptosporidium*, *E. coli* O157:H7, *Campylobacter* and

Salmonella. We applied this benchmark to HF183 values measured in our study and determined that HF183 concentrations in 73% of the river samples exceeded the 525 GC/100 ml threshold, compared to only 26% exceedance in the ocean samples. The lower HF183 levels in the ocean reflect dilution and dispersion of microorganisms from the source (rivers) to the ocean and these physical processes are known to vary greatly both spatially and temporally (Horner-Devine *et al.*, 2015). From river samples that exceeded the 525 GC/100 ml threshold, 47% were from dry season data versus 26% from rainy season data. Interestingly, exceedance in the ocean was higher in the rainy season (9% were from dry season data versus 17% from rainy season data). Previously, Orner *et al.* (Orner *et al.*, 2021) identified significant differences in dilution and dispersion from these same rivers into the ocean that significantly varied by subwatershed and season and noted instances of limited dilution/dispersion in the rainy season, which may explain why exceedance was higher in the rainy season.

The variability in microbial concentrations explained by environmental variables decreased between study years, from 12.3% in the 2018 study (González-Fernández *et al.*, 2021) to 7.5% in the 2019 study, but was consistently a minor factor. Rainfall and water temperature were the only significant predictors of microbial levels in both years. In both studies, higher levels of *Salmonella* and NoVGI were associated with lower rainfall and decreased water temperature in rivers, while enterococci levels were positively associated with rainfall (González-Fernández *et al.*, 2021). Correlations with 12 h antecedent rainfall were observed in our 2018 study at Jacó, but different cumulative antecedent rainfall variables were not explored (González-Fernández *et al.*, 2021). Forty-eight-hour antecedent rainfall was the rainfall variable selected in the most parsimonious models that best captured the relationship between microorganism and the set of environmental variables measured [i.e., cumulative rainfall (12, 24, 48, 72 and 96 h prior to

sampling), water temperature and turbidity] in river and ocean samples from the 2019 study. Previous studies have identified positive correlations between FIB and environmental factors that include similar rainfall time-lags (Chase *et al.*, 2012; Harwood *et al.*, 2014; Laureano-Rosario *et al.*, 2021).

While enterococci concentrations were positively correlated with rainfall in the 2018 and 2019 sampling campaigns, this relationship was true for Cryptosporidium only during the first year of sampling (González-Fernández *et al.*, 2021). *Cryptosporidium* was detected in 78% of rainy season samples and was not detected during the dry season in our 2018 study, while in our current 2019 study it was detected in 8% of the rainy season samples in the Copey and Madrigal rivers and 5% of the dry season samples in Copey River. In other tropical locations, *Cryptosporidium* prevalence in human populations has been positively correlated with wetter months and precipitation (Abeywardena *et al.*, 2015; Jagai *et al.*, 2009). However, *Cryptosporidium* has many non-human sources that include mammals, birds, reptiles, amphibians and fish (Abeywardena *et al.*, 2015). A strong influence of livestock and agricultural activity with the cultivation of rice, beans and corn has been established upstream of Copey River but is yet to be determined for all other river areas (Mattey Trigueros *et al.*, 2017). However, we consistently observed cattle and horses in the Madrigal River, where land use was dominated by pasture lands. The difference in the frequency of detection of *Cryptosporidium* in the 2018 vs 2019 study could likely to be related to Hurricane Nate, which occurred a week prior to our earlier rainy season sampling campaign and caused severe flooding in Jacó (Chaves *et al.*, 2017). Hence, flushing of *Cryptosporidium* into surface waters from animal and human sources could explain the increased frequency of detection noted during the 2018 study.

Pathogen levels measured in Jacó rivers led to a high estimated risk of GI illness to swimmers, particularly when the disaggregate parameter in the NoVGI dose-response model was used. In the absence of one best norovirus dose–response model, calculating risk of illness caused by NoVGI separately using the aggregate and disaggregate values, which represent a broad estimate of NoVGI infectivity, is considered a good practice (Orner *et al.*, 2021; Sunger *et al.*, 2019; Van Abel *et al.*, 2017). The median cumulative risk of illness from all measured pathogens was at least one order of magnitude less when the NoVGI aggregate value was used compared to the disaggregate value. This is due to the dose-response model where the probability of infection is inversely proportional to μ (mean aggregate size) and is over a thousand times greater when viral particles are assumed to be aggregated as compared to when they are assumed to be disaggregated (Messner *et al.*, 2014; Teunis *et al.*, 2008). Viral aggregation in the environment is governed by complex processes that are not well understood, but are dependent on the type of virus, concentration and chemistry of salts in solution, and pH (Gerba & Betancourt, 2017). However, it is likely that NoVGI is disaggregated in surface waters due to the relationship between the suspension pH and the viral isoelectric point (Grant, 1994; Hamadieh *et al.*, 2021) and this approach is usually preferred for recreational water quality (Crank *et al.*, 2019; Soller *et al.*, 2016).

QMRA estimated that NoVGI and AdV contributed more than the bacterial and protozoan pathogens to the cumulative risk of GI illness for swimmers in the rivers. The relative contribution of NoVGI to cumulative risk depended on the mean aggregate size parameter, i.e., NoVGI contributes more to cumulative risk than AdV using the disaggregate value. AdV is the greatest contributor to risk when the aggregate value is used. A study conducted in O'ahu, Hawaii (US) found that risk of viral GI illness (enterovirus, AdV, NoVGI, and NoVGII) was

significantly greater than from bacterial exposure (*Salmonella* and *Campylobacter*); it assumed a disaggregate NoVGI model and argued that viral aggregation is insignificant in natural waters (Viau *et al.*, 2011). Furthermore, other studies have identified viruses, specifically NoVGI, as contributing the most to risk of GI illness to swimmers (Boehm *et al.*, 2018; McBride *et al.*, 2013).

QMRA has been applied in a variety of studies to understand the impact of swimming in contaminated waters and has recently been reviewed by Federigi *et al.* (2019). However, most research has focused on temperate regions (mainly US and Canada), with fewer studies performed in recreational tropical waters (Federigi *et al.*, 2019). Much higher cumulative risk (up to 0.577) was determined for Jacó rivers in this study compared to other QMRA studies in tropical fresh and marine waters where viral pathogens were measured. For example, Viau *et al.* (Viau *et al.*, 2011) estimated median cumulative risk of illness from exposure to a stream discharge at a beach in Hawai'i to range from 0 to 0.02 and Soller *et al.* (Soller *et al.*, 2016) estimated an average of swimming-associated illness of 0.002 in coastal Puerto Rico. The greater risk of GI illness estimated for recreation in Jacó rivers compared to these studies is likely due to Jacó's lack of sanitation infrastructure and the direct proximity of the town and rivers to the beach (González-Fernández *et al.*, 2021; Mora, 2009; Orner *et al.*, 2021). Nevertheless, it is important to mention that when the less conservative approach is adopted using the aggregate value in this study, the median cumulative risks do not exceed the US EPA benchmark in rainy season in Copey and Madrigal River.

Log reduction values for pathogens facilitate translation of QMRA estimates of risk to actionable goals for safe recreation at beaches. The LRV is an expression of the magnitude of viral reduction needed to achieve safe swimming conditions. Because NoVGI is unlikely to be

aggregated in natural waters, we focus our discussion on LRVs estimated using the disaggregate model. Microbial concentrations in the rivers needed to be reduced by at least 10,000-fold in the dry season and 1,000-fold in the rainy season. Viral LRVs calculated in all rivers for this study were greater than pathogen LRVs published from the 2018 sampling campaign, except for Copey River during dry season (Orner *et al.*, 2021). Differences in LRVs calculated between years are most certainly dependent upon how many, and which specific pathogens, are included in risk assessment. Orner *et al.* (Orner *et al.*, 2021) used cumulative risk of illness due to NoVGI, *Cryptosporidium*, *Giardia* and *Salmonella* for LRV calculations, while we calculated viral LRVs that considered cumulative risk due to NoVGI and AdV in this study. However, because both studies determined that *Cryptosporidium*, *Giardia* and *Salmonella* contributed very little to cumulative risk of illness it is likely that higher LRVs in the 2019 study (this paper) are related not only to increased detection of NoVGI, but the addition of AdV measurements.

Context-appropriate scenarios to achieve safe swimming conditions according to EPA recommendation 1 (36 illness per 1000 recreators) for Jacó have been previously identified by Orner *et al.* (Orner *et al.*, 2021). Two sanitation options were recommended for Jacó which remove nutrients and pathogens, and would therefore contribute to conditions suitable for recreation: (1) A wastewater treatment plant, which has a sanitary sewer, and primary, secondary, tertiary treatment and disinfection prior to the effluent being discharged to surface water or (2) a combination of centralized and decentralized treatment, where part of the population uses a septic tank with infiltration of the effluent into the ground, and another part is served by a wastewater treatment plant. Costa Rica is also taking important steps toward improved sanitation practices and has approved international financing for a wastewater sanitation project in Jacó (Diario oficial La Gaceta, 2018).

It is possible that the risk of GI illness is actually greater than that modeled in this paper because the QMRA model is most sensitive to pathogen concentration (Figure S5) and losses inevitably occurred during each step of the methods for each pathogen, from concentration to measurement (Mull & Hill, 2012). This caveat is applicable to all QMRA studies. Previous studies using similar methods have reported process recovery efficiencies as follows: MS2 (66 and 73%), *C. parvum* (49 and 83%), enterococci (85%), *E. coli* (81%), for *E. faecalis*, 78%; and *C. perfringens* (63 and 57%) (Mull and Hill, 2012; Smith & Hill, 2009). The average D/RNA extraction (-reverse transcription) efficiency observed in this study was 82% for Sketa (DNA) and 81% for FCV (RNA). Many other factors can also contribute to under- or over-estimation of the risk of infectious disease given that the QMRA is also sensitive to volume ingested, as well as the dose-response model parameters (Figure S5). Finally, it is likely that the risk of GI illness caused by NoV was underestimated in this study since only NoVGI was measured, and NoVGII was likely also prevalent (Eftim *et al.*, 2017).

The 2018 and 2019 studies support the transdisciplinary collaboration between microbiologists and anthropologists that will link human behavior at beaches with health outcomes (Workman *et al.*, 2021). It is an integral component of the development of a holistic water management approach in collaboration with the Costa Rican Institute of Aqueducts and Sewers that also relied on field work of students from the University of Costa Rica. Ethnographic analyses will improve our understanding of beachgoer behavior, which will allow us to develop better estimates of ingestion volumes, routes of exposure to pathogens, and pathogen influence on human health risk. This collaboration emphasizes using local knowledge to inform experimental design and interpretation of data. Rapid feedback of our findings to Costa Rican agencies, which are integral partners in this project, improve local efforts to protect beachgoer health, and support current

plans of the Costa Rican government to improve wastewater treatment in cities like Jacó (PGR, 2022). This study provides a baseline for microbial water quality and health risk for beachgoers against which the cost of improvements in water quality can be weighed. This collaboration provides a template to government agencies in other countries, which may choose to follow Costa Rica's leadership by exploring the extent of wastewater impacts on beaches so that they can allocate resources to improve water quality and protect public health.

Conclusions

- This study found differences in the magnitude of fecal microorganisms, pathogens, and human health risk by season, and at a local scale (<2.5 km), highlighting the value of detailed knowledge of the sources and seasonality of pollution at a given beach.
- The median risk of gastroenteritis for swimmers in the rivers estimated by QMRA was at least 0.317 (317 illnesses/1,000 swimmers).
- NoVGI and AdV contributed much more to risk of gastroenteritis from swimming in Jacó rivers than *Cryptosporidium*, *Giardia* and *Salmonella*.
- The NoV mean aggregate size parameter (μ) in the dose−response model greatly impacts QMRA risk predictions and LRVs needed for safe swimming conditions.
- Overall, microbial concentrations were greatest in the dry season; nevertheless, microbial concentrations were great enough in the rainy season such that excess risk to swimmers was present year-round.

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Tables

Table 1: Sampling design, concentration and analytical methods and their process limit of detection (pLOD) and quantification (pLOQ) in the rivers and ocean. See methods for citations for specific assays

Water Type (n)	Season (n)	Area (n)	Concentration Method	Microbe	pLOD	pLOQ	Units	Analytical Method
River (78)	Rainy (39) Dry (39)	Copey (13) Naranjal (13) Madrigal (13)	Ultrafiltration	*C. perfringens*	4.5	NA	CFU/L	Standard Method ASTM D5916-96(2002) [a]
				somatic coliphage	4.2	NA	PFU/L	US EPA Method 1601[a]
				F+ coliphage	4.2	NA	PFU/L	US EPA Method 1602[a]
				HF183	168.22	1210.21	GC/L	HF183 Bacteroides 16S rRNA qPCR [b]
				PMMoV	128.2	1155.2	GC/L	pepper mild mottle virus RT-qPCR assay [b]
				Giardia	0.04	NA	Cysts/L	IMS[c]/ microscopy US EPA Method 1623.1 [c]
				Cryptosporidium	0.04	NA	Oocysts/L	IMS/ microscopy by US EPA Method 1623.14 [c]

				Salmonella	1.7 CFU/L	NA	CFU/L	US EPA Method 1682 [a]
				NoVGI	128.23	1210.21	GC/L	human norovirus genogroup I RT-qPCR [b]
				AdV	394.53	1155.2	GC/L	adenovirus qPCR assay [b]
			Membrane filtration	enterococci	1	NA	CFU/100 mL	US EPA Method 1600 [a]
			Most probable number (MPN)	fecal coliforms	1.8	NA	MPN/100 mL	APHA Standard Methods, Section 9221 [a]
Ocean (78)	Rainy (39) dry (39)	Copey (13) Naranjal (13) Madrigal (13)	Membrane filtration	HF183	695	5000	GC/L	HF183 Bacteroides 16S rRNA qPCR [b]
			Membrane filtration	enterococci	1	NA	CFU/100 mL	US EPA Method 1600 [a]
			Most probable number (MPN)	fecal coliforms	1.8	NA	MPN/100 mL	APHA Standard Methods, Section 9221 E [a]

[a] Culture.
[b] QPCR
[c] IMS – immunomagnetic separation.

Table 2: (RT-)qPCR assay primers probes and reaction conditions. Modified from Gonzalez-Fernandez *et al.* (2021)

Assay	Primers and Probes (5' – 3')	Reaction	Reference
Human adeno-virus (AdV), all 51 types	F: GCCCCAGTGGTCTTACATGCACATC R: GCCACGGTGGGGTTTCTAAACTT [6-FAM]TGCAC-CAGACCCGGGCTCAGGTACTCCGA[TAMRA]	Primers: 250 nM each Probe: 250 nM Conditions: 95 °C for10 min then, 40 X *(95 °C for 15 s and 60 °C for 1 min)	(Ahmed *et al.*, 2016; Heim *et al.*, 2003)
Human no-rovirus GI (NoVGI)	F: CARGCCATGTTYCGYTGGATG R: CCTTAGACGCCATCATCATTTAC [6-FAM]TGGACAGGA-GAYCGCRATCT[BHQ1a~Q]	Primers: 500 nM each Probe: 250 nM Conditions: 95 °C for10 min then, 40 X *(95 °C for 15 s and 60 °C for 1 min)	(Svraka *et al.*, 2009)
HF183 *Bacteroides*	F: ATCATGAGTTCACATGTCCG R: CTTCCTCTCAGAACCCCTATCC	Primers: 1000 nM each Probe: 80 nM	

16S rRNA	FAM-CTAATGGAACGCATCCC-MGB	Conditions: 2 min at 50°C, 10 min at 95°C, then 40 X 15 s at 95°C for 15 s and, 60 s at 60 °C for 1 min)	(Green *et al.*, 2014)
Pepper mild mottle virus (PMMoV)	F: GAGTGGTTTGACCTTAACGTTTGA R: TTGTCGGTTGCAATGCAAGT [6-FAM]CCTACCGAAGCAAATG[MGBNFQ]	Primers: 400 nM each Probe: 200 nM Conditions: 95 °C for10 min then, 40 X (95 °C for 15 s and 60 °C for 1 min)	(Haramoto *et al.*, 2013)
Feline calicivi-rus (FCV)	F: CCGGGTGGGACTGAGTGG R: GCATAACTCGTCGGAGGTGTC [6-FAM]CGCCTTACGGATATGAG-CAGCCACATTAAC[IBRQ]	Primers: 300 nM each Probe: 200 nM Conditions: 95 °C for10 min then, 40 X (95 °C for 15 s and 60 °C for 1 min)	(Mattison *et al.*, 2009)
Salmon testes DNA	F: GGTTTCCGCAGCTGGG R: CCGAGCCGTCCTGGTC	Primers: 1000 nM each Probe: 80 nM	

(SKeta22)	[6~FAM]AGTCGCAGGCGGCCACCGT[TAMRA]	Conditions: 95 °C for10 min then, 40 X (95 °C for 15 s and 60 °C for 1 min)	(US EPA, 2012b)

Table 3: Frequency of exceedance of recreational water quality criteria (RWQC) according to Costa Rican (CR) and US Environmental Protection Agency (US EPA) recommendations in the rivers and the ocean (Mora, 2007; US EPA, 2012a) in the rainy and dry seasons. STV = statistical threshold value and GM= geometric mean. CR = Costa Rica

	River (n=78)		**Ocean (n=78)**	
Regulatory Standard	**Dry (n=39)**	**Rainy (n=39)**	**Dry (n=39)**	**Rainy (n=39)**
Enterococci US EPA STV[a]	90 % (35)	97 % (38)	0 %	26 % (10)
Enterococci CR-GM[b]	NA[e]	NA	21 % (8)	46 % (18)
Fecal coliform CR-GM[c] & d	95 % (37)	97 % (38)	38 % (15)	54 % (21)

[a]>130 CFU/100 mL criterion for river and ocean samples.
[b]>35 MPN/100 mL criterion for river samples.
[c]>1000 MPN/100 mL criterion for river samples.
[d]>240 MPN/100 mL criterion for ocean samples.
NA[e]> CR Does not apply, Costa Rican enterococci GM criterion is only used in marine waters.

Table 4: Frequency of pathogen detection (percentage of positive observations) in rivers (n = 78) in the rainy (n=39) and dry seasons (n=39) per river sampled (n=13)

Pathogen	**Rainy Season**		
	Copey % (n)	**Naranjal % (n)**	**Madrigal % (n)**
Giardia (cysts/L)	77% (10)	85% (11)	85% (11)
Cryptosporidium (Oocysts/L)	15% (2)	0	8% (1)
Salmonella (CFU/L)	77% (10)	69% (9)	77% (10)
NoVGI (GC/L)	23% (3)	85% (11)	15% (2)
AdV GC/L)	38% (5)	69% (9)	8% (1)
Pathogen	**Dry Season % (n)**		
	Copey % (n)	**Naranjal % (n)**	**Madrigal % (n)**
Giardia (cysts/L)	100% (13)	92% (12)	46% (6)
Cryptosporidium (Oocysts/L)	15% (2)	0	0
Salmonella (CFU/L)	100% (13)	100% (13)	100% (13)
NoVGI (GC/L)	100% (13)	100% (13)	100% (13)
AdV GC/L)	62% (8)	46% (6)	8% (1)

Table 5: Viral log10 reduction values (LRVs) needed to ensure safe swimming conditions at Jacó beach by river, and season. The LRV range represented was based upon the cumulative viral risk of GI illness to swimmers calculated using the norovirus genogroup I (NoVGI) disaggregate model

River	Viral $\log_{10}$ Reduction Value	
	Dry Season	Rainy Season
Copey	3.8	3
Naranjal	3.9	3.2
Madrigal	4.1	2.7

Figures

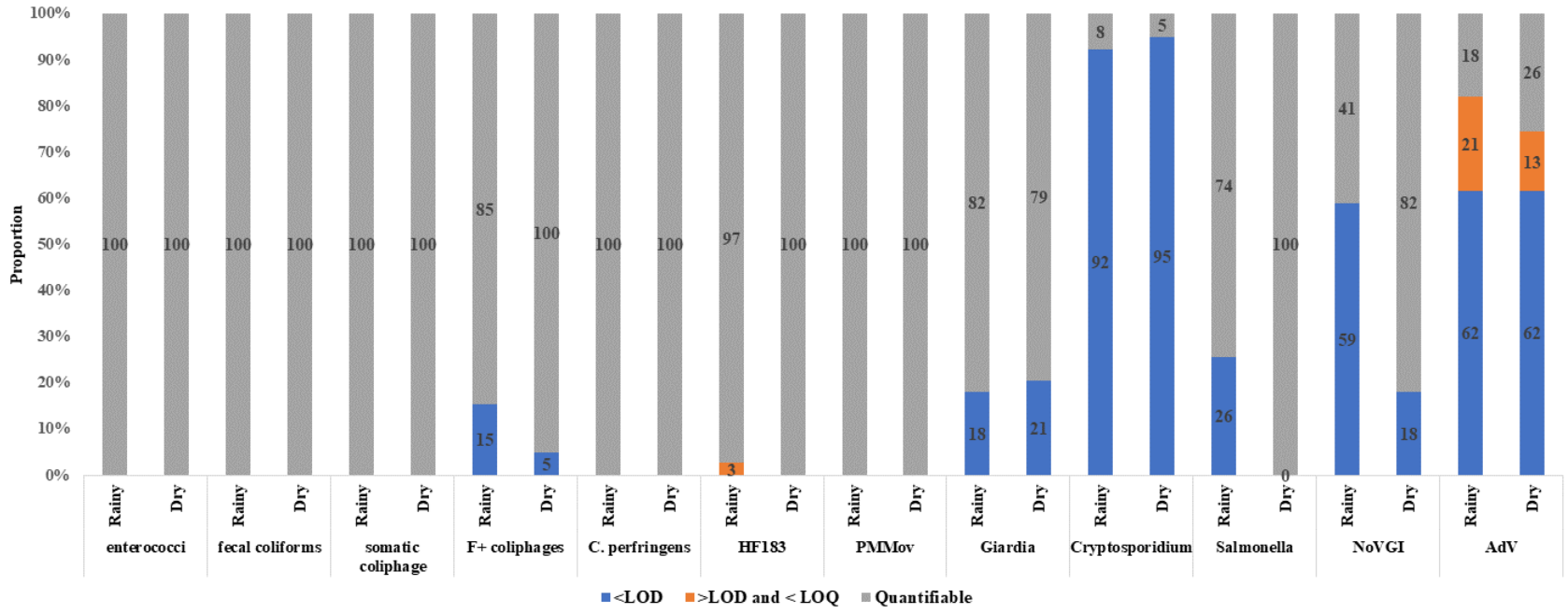

Figure 1: Comparison of left censored observations for each microbial variable in river samples during the rainy(n=39) and dry season (n=39). Percent censored refers to the percentage of observations that were not quantifiable, arranged in order of non-detects (below the assay LOD), detected but not quantifiable (above assay LOD and below the assay LOQ) and quantifiable data (above assay LOQ)

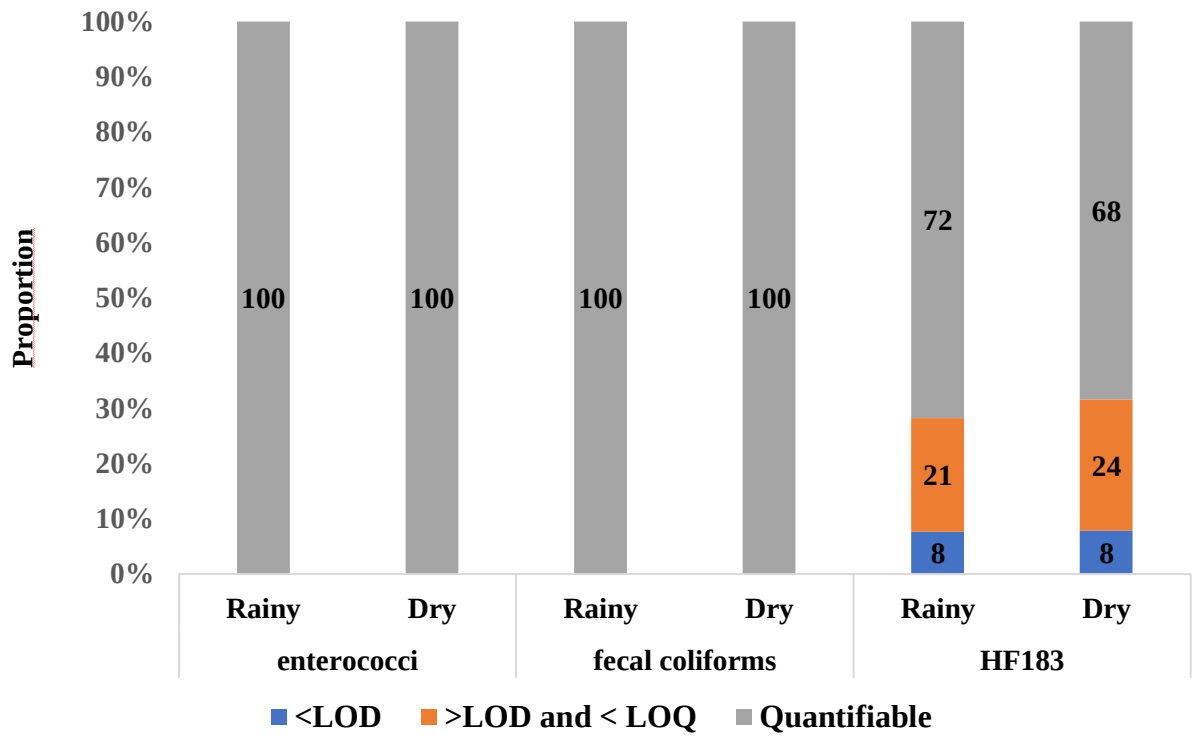

Figure 2: Comparison of left censored observations for each microbial variable in ocean samples during the rainy (n=39) and dry season (n=39). The y-axis refers to the percentage of observations that were not detected (below the assay LOD), detected but not quantifiable (above assay LOD and below the assay LOQ) and quantifiable data (above assay LOQ)

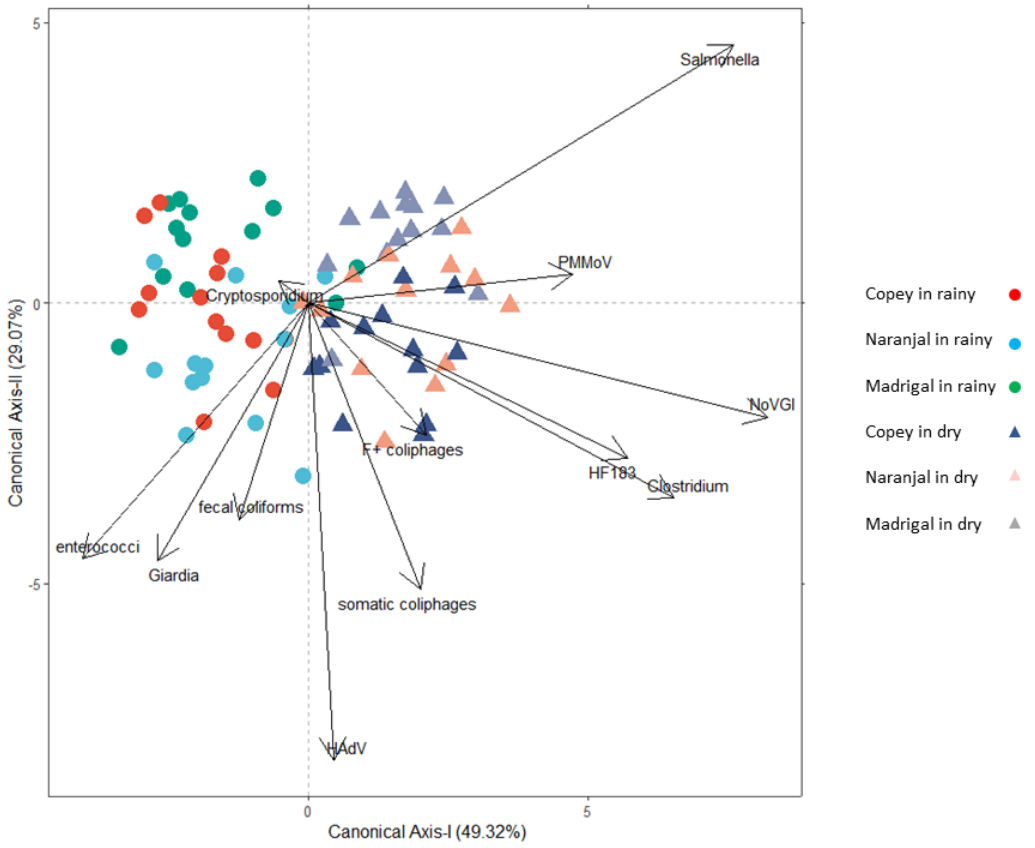

Figure 3: Canonical analysis of principal components (CAP) ordination plot showing canonical axes that best discriminate microbial variables (enterococci, fecal coliforms, somatic and F+ coliphage, *C. perfringens*, *Salmonella*, *Giardia*, *Cryptosporidium*, NovGI, AdV) across river sites: Copey (CP), Naranjal (NJ) and Madrigal (MD), and season: rainy (circle) and dry (triangle) seasons. Fecal microorganism's vectors are depicted in the correlation biplot as arrows

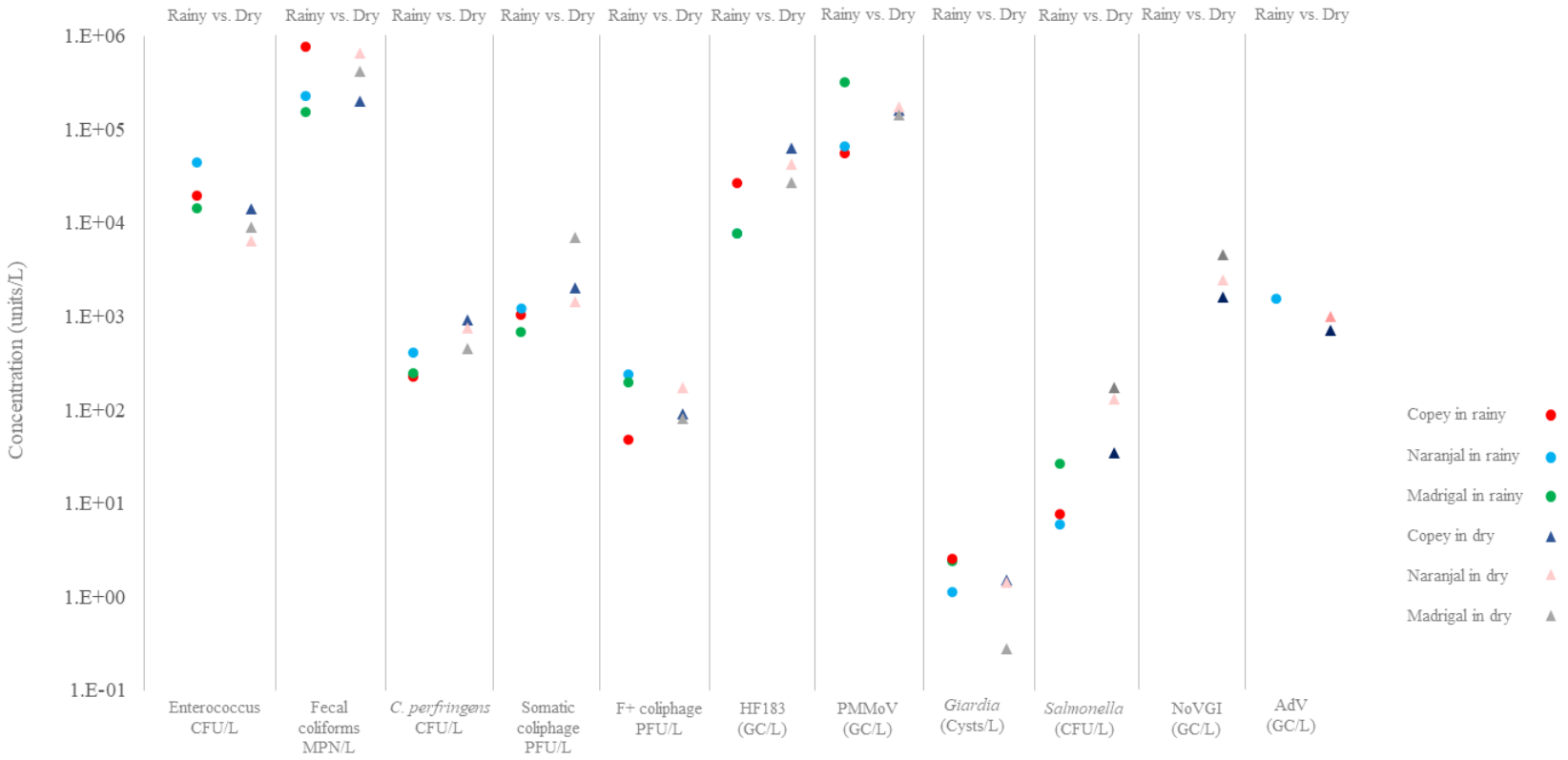

Figure 4: Fecal indicator microorganism, microbial source tracking marker and pathogen mean concentrations across river sites: Copey, Naranjal and Madrigal, in rainy (circle) and dry (triangle) seasons

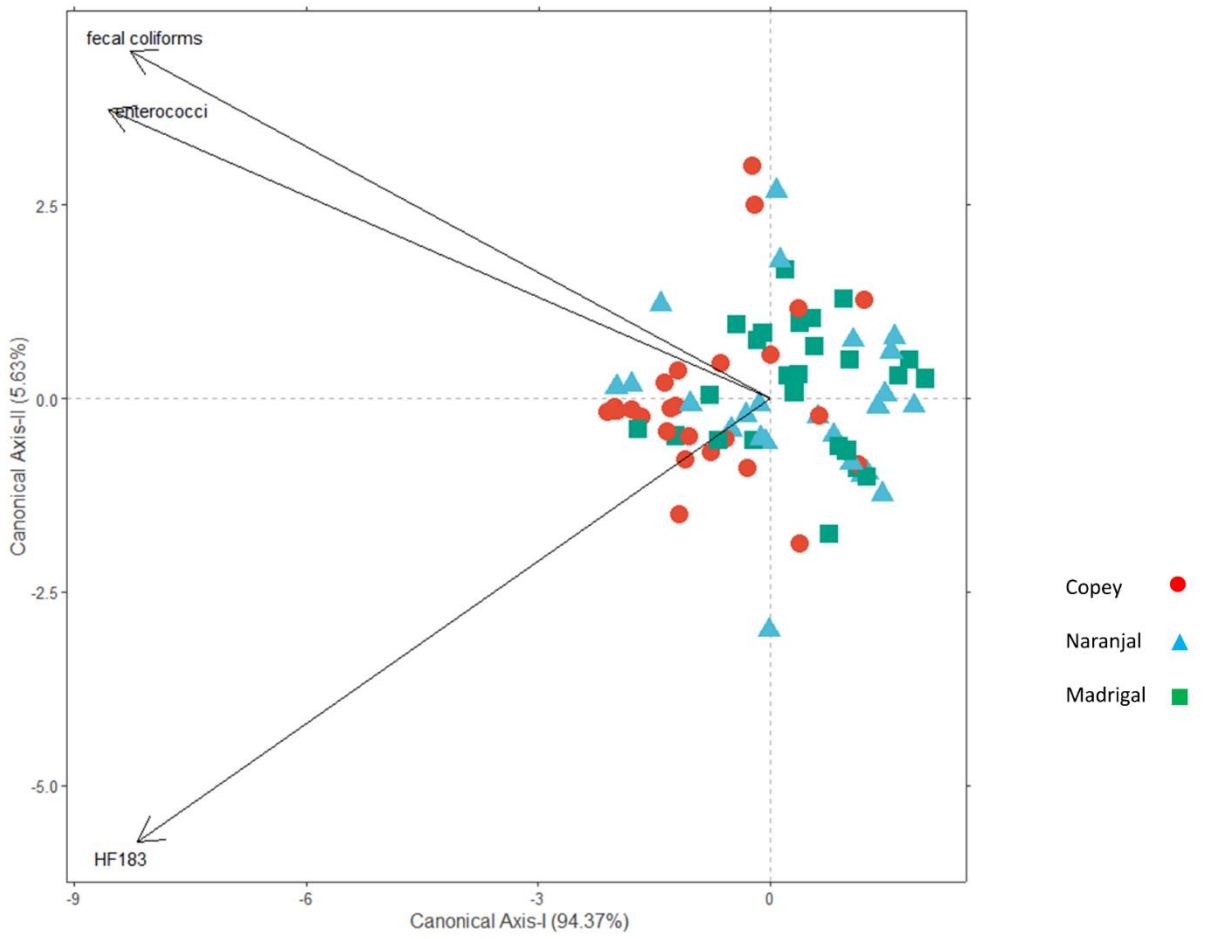

Figure 5: Canonical analysis of principal components (CAP) ordination plot showing canonical axes that best discriminate microbial variables (enterococci, fecal coliforms and HF183) across ocean areas: Copey, Naranjal and Madrigal, regardless of season. Fecal microorganism's vectors are depicted in the correlation biplot as arrows

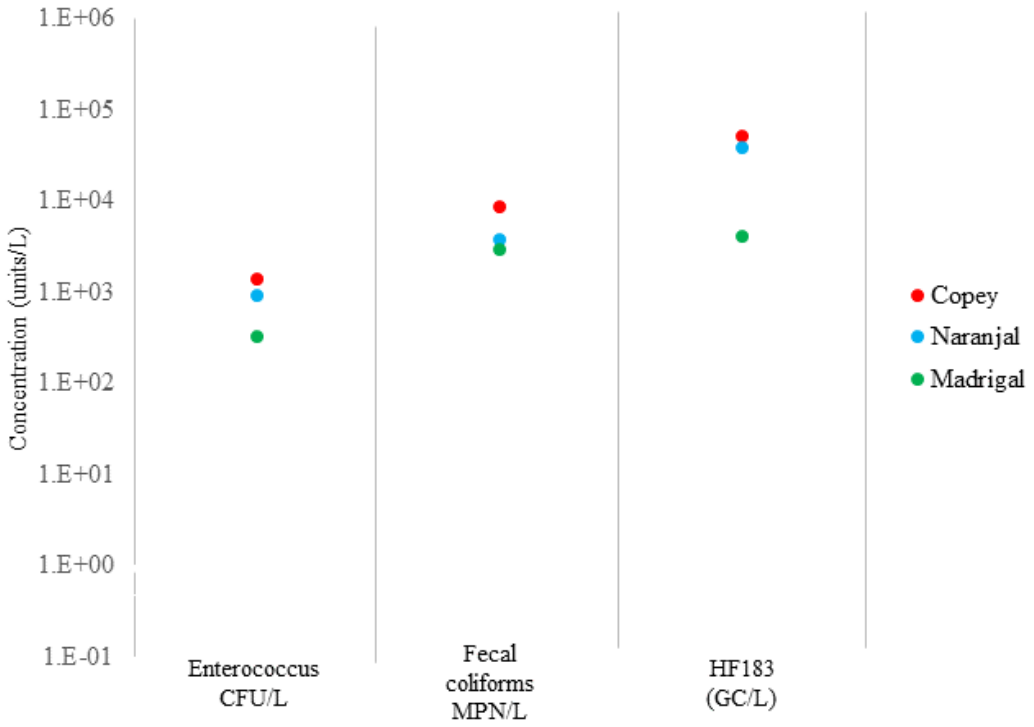

Figure 6: Fecal indicator microorganism and HF183 mean concentrations in Copey (red), Naranjal (blue) and Madrigal (green) in the ocean, regardless of season

Figure 7: Median risk of gastrointestinal (GI) illness to swimmers for each river and season by pathogen, as well as cumulatively. Estimates for NoVGI and cumulative risk were calculated with the disaggregate NoVGI model. Color scales indicate how the median value of risk compared to the US EPA Recreational Water Quality health benchmark (recommendation 1: 36 illness per 1000 recreators), with red exceeding the criteria. Microbial abbreviations are as follows: adenovirus (AdV), norovirus disaggregate model (NoVGI), all pathogens (Total)

CHAPTER FOUR: MULTIDRUG-RESISTANT E. COLI AND ENTEROCOCCUS SPP. IN COSTA RICAN WASTEWATER AND SURFACE WATERS

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A.G.F and V.J.H. conceived ideas, A.G.F and E.B oversaw experiments in Tampa, FL, A.G.F. conducted statistical analyses, and A.G.F wrote the paper with comments and edits from E.B and V.J.H

Abstract

Population Infections from antibiotic-resistant bacteria (ARB) are a global health concern. Humans can be exposed to ARB in recreational waters, particularly those that are impacted by wastewater discharges. Relatively little is known about the frequency of ARB in wastewater and surface waters in tropical countries. Here, *E. coli* and *Enterococcus spp.* were cultured with and without ampicillin from untreated wastewater originating from a hospital or neighborhood, from treated effluent and from the receiving estuary in Puntarenas, Costa Rica. Although no significant differences in the proportion of ampicillin-resistant fecal indicator bacteria (FIB) to total

FIB among sampling sites was observed, ampicillin-resistant *E. coli* concentrations were significantly greater in residential wastewater compared to hospital wastewater (mean concentrations were 6.4 $\log_{10}$ CFU/100 ml versus 4.9 $\log_{10}$ CFU/100 ml). Resistance of ampicillin-resistant FIB to additional antibiotics was also tested. Greater frequency of resistance (more than 50% of isolates) to tetracycline in both FIB, and to trimethoprim- sulfamethoxazole in *E. coli* was determined in all sites when compared to all antibiotics tested. *E. coli* isolated from the hospital wastewater were most frequently resistant (~ 40% of isolates) to gentamicin, cefotaxime, and ciprofloxacin versus all other sites (<25%), while *Enterococcus* isolates from the estuary were more frequently resistant to tetracycline (>50% of isolates), erythromycin (~ 25%) and ciprofloxacin (~10% to 25% of isolates). Forty two percent of ampicillin-resistant *E. coli* isolates and 45% of ampicillin-resistant *Enterococcus* isolates were multidrug-resistant (resistant to three or more antibiotic classes) at least one antibiotic in three or more antibiotic categories. *E. coli* isolates that were resistant to a combination of 6 different classes of antibiotics were found frequently and exclusively in the hospital influent. Nevertheless, high levels of antibiotic resistant bacteria in treated effluent and the estuary are the most problematic sources for human contact, since it could impact the health of local populations and tourists and suggests that antibiotic stewardship efforts in the country should include the fate of antibiotic-resistant bacteria from wastewater treatment plant (WWTP) into the aquatic environment.

Introduction

Antibiotic resistance is a major global health threat. A systematic analysis of global antibiotic resistance estimated 1.27 million deaths due to antibiotic resistance in 2019 (Murray *et al.*, 2022). Also concerning is the continued rise in infections caused by multidrug-resistant bacteria (i.e., resistant to at least one antibiotic in three or more antibiotic categories) (Basak *et al.*, 2016),

as these infections are more difficult to treat, and are likely to lead to poor outcomes for the patients (Nikaido, 2009). Antibiotic resistance is a global problem that has critical consequences for low- and middle income countries (Sulis *et al.*, 2022; Zheng *et al.*, 2021). Antibiotic resistance is a leading cause of death around the world, with the highest burdens in low-resource settings, which have a higher burden of infectious disease due to poverty and weak health systems (Murray *et al.*, 2022; Pokharel *et al.*, 2019). In Costa Rica, antibiotic resistance is a national public health problem. Antibiotic resistant bacteria and multidrug-resistant bacteria have been reported in hospitals and the community throughout the territory and in the livestock sector (Gobierno del Bicentenario Costa Rica, 2018; INCIENSA, 2022). Clinical data regarding antibiotic resistant bacteria of public health concern in Costa Rica are readily available, while data on antibiotic resistant bacteria isolated from wastewater and the environment is scarce (INCIENSA, 2022).

Aquatic ecosystems contain a pool of antibiotic resistant bacteria and resistance genes, making them potential reservoirs of antibiotic resistance and habitat-s where antibiotic resistance could be disseminated (Suzuki *et al.*, 2017). Untreated or inefficiently treated wastewater from wastewater treatment plants (WWTP), particularly those that receive hospital wastewater, have been described as hot spots for the accumulation and spread of antibiotic-resistant bacteria and their genes in the environment (Amarasiri *et al.*, 2020; Guo *et al.*, 2017; Paulus *et al.*, 2019; Rizzo *et al.*, 2013). The use of reclaimed water for agricultural irrigation, as well as the use of animal manure to fertilize crops leads to the spread of resistance through soil (Christou *et al.*, 2017; Ghosh & LaPara, 2007) and into the aquatic environment through coastal runoff (Hatosy & Martiny, 2015).

Antibiotic resistant bacteria in bathing waters pose a threat to human health (Leonard *et al.*, 2022). Human exposure to antibiotic resistant bacteria in water may result in hard-to-treat infections. For example, studies have identified risk to recreational bathers of exposure to antibiotic-resistant *E. coli* (Leonard *et al.*, 2015, 2018). Increased risk on the skin infections is expected due to elevated levels of antibiotic resistance genes found in skin of individuals who swam in the ocean (Nielsen *et al.*, 2021). Although studies have reported the presence of clinically relevant antibiotic resistant bacteria and their genes in recreational waters (Leonard *et al.*, 2022), potential human health risks from exposure to aquatic environments with antibiotic- resistant bacteria and antibiotic resistance genes is still scarce, because information on dose-response curves and exposure assessment data are needed to conduct a quantitative microbial risk assessment (QMRA) (Pepper *et al.*, 2018).

Relatively little is known about the frequency of antibiotic resistant bacteria in wastewater and recreational waters in tropical countries (Makkaew *et al.*, 2021). Most studies have been performed in temperate regions, where environmental conditions (i.e., rainfall) affect microbiological water quality differently (Strauch *et al.*, 2014). The influence of environmental variables on the distribution of resistance genes and a gradient of increased relative abundances of resistance genes in middle latitudes compared to high and low latitudes has been previously reported (Zheng *et al.*, 2021). Antibiotic contaminants in low- and middle-income economies characteristic of many tropical countries is higher when compared to high-income countries (Sulis *et al.*, 2022; Zheng *et al.*, 2021). Factors such as lower GDP per capita, insufficient sanitation infrastructure, limited access to healthcare, and antibiotic misuse have been invoked to explain the discrepancy (Morgan *et al.*, 2011; Pokharel *et al.*, 2019; Sulis *et al.*, 2022; Vila and Pal, 2010; WHO, 2014).

‘Fecal indicator bacteria (FIB) that are commonly found in animal feces and in the environment are opportunistic pathogens (Stec *et al.*, 2022) that can be used to study antibiotic resistance in wastewater and surface waters (Hernando-Amado *et al.*, 2019; Oliveira *et al.*, 2020; Von Baum & Marre, 2005). FIB, including *Escherichia coli* and *Enterococcus spp.*, are easy to culture, and are used to assess microbial water quality (Griffith *et al.*, 2009; McQuaig *et al.*, 2012; US EPA, 2012a). Currently, the World Health Organization (WHO) Global Antimicrobial Resistance and Use Surveillance System (GLASS) (WHO, 2015), has a module directed towards extended- spectrum *β*- lactamase producing *E. coli* as a key indicator for global surveillance of antibiotic resistance (WHO, 2021, 2020). *E. coli* can readily acquire antibiotic resistance genes by horizontal gene transfer, and it frequently acquires extended- spectrum *β*-lactamases, which are an important cause of multidrug resistance in gram negative bacteria worldwide (Moghaddam *et al.*, 2015; Von Baum & Marre, 2005).

Deterioration of water quality at Puntarenas beach and in Puntarenas estuary in Costa Rica has been reported and well documented over the last 30 years (Anonymous, 2008; Marín Alpízar, 2006; Mora Alvarado, 2002). Puntarenas, Costa Rica is an appropriate test site to explore the prevalence of antibiotic resistance through wastewater treatment stages and the extent to which these bacteria are discharged into the environment because it enables testing for differences in susceptibility to multiple classes of antibiotics of *E. coli* and *Enterococcus* spp. among potential sources (hospital and residential wastewater), and WWTP effluent discharged into Puntarenas estuary that is adjacent to a popular beach. Identifying regionally specific patterns of resistance in Costa Rica is likely to improve our understanding of antibiotic resistance and subsequently inform decision and policy makers to develop locally relevant interventions.

Methods

Study site

Wastewater samples were collected in the city of Puntarenas on the central Pacific coast of Costa Rica (Figure 8). The population of the city of Puntarenas reached 41,528 inhabitants according to the 2011 census (INEC, 2011). Wastewater from several residential areas and Monseñor Sanabria Hospital enter El Roble Wastewater Treatment Plant (WWTP). After secondary treatment, and without disinfection, the effluent is discharged into Puntarenas estuary that drains into the Gulf of Nicoya (Figure 8). Several upgrades have been made to El Roble WWTP since it was built. Last published data indicated that the plant has a flow of 5 011 200 L per day and uses activated sludge technology (Mora-Alvarado and Portuguez-Barquero, 2016). Four sampling sites were chosen: the untreated wastewater discharged from the hospital, the wastewater from one of the residential areas, the treated effluent discharged from the WWTP (9°58'54.3"N, 84°44'18.8"W), and Puntarenas Estuary (9°59'02.0"N, 84°46'54.9"W), which receives the effluent.

Sampling and culture of FIB

Samples of 500 mL were collected at each of the four sites in Puntarenas, Costa Rica. Four sampling events occurred between October and December 2019. The estuary was sampled from shore with an open sampling container attached to a rope that was thrown 5 m into the estuary. The container was pulled back to shore, and the water was immediately poured into a sterile container. The samples were transported on ice to the Laboratorio Nacional de Aguas (National Water Laboratory) in Tres Rios, Cartago, Costa Rica. There, the bacteria were concentrated by membrane filtration following US EPA Method 1603 for enumeration of *E. coli* (US EPA, 2009)

and US EPA Method 1600 for enumeration of *Enterococcus* spp. (US EPA, 2005). The volume that was filtered (1, 10 or 100 mL) varied depending on the sampling site and 1:10 dilutions were made, when necessary, to obtain countable numbers of colonies. After membrane filtration, filters were placed on mTEC agar plates without antibiotic to measure total *E. coli*, and on mTEC amended with 16 µg/mL ampicillin to measure *E. coli* with intermediate resistance to ampicillin. Filters were placed on mEI agar plates without antibiotic to measure total *Enterococcus*, and on mEI amended with 16 µg/mL ampicillin to measure ampicillin-resistant *Enterococcus* spp. Ten colonies per site for each FIB were selected from ampicillin-amended plates at each sampling event and subcultured to brain heart infusion (BHI) with ampicillin (16 µg/mL), and ampicillin-resistant FIB were stored individually in cryovials containing 50% glycerol. These cryovials were stored at 4°C for a period of ≈ 2 weeks and shipped at room temperature to the University of South Florida (USF) for further antibiotic-resistance testing.

Phylogenetic confirmation of ampicillin-resistant isolates

Ampicillin-resistant isolates were stored at -80°C upon receipt at USF. Before further testing, the putative ampicillin-resistant *E. coli* and *Enterococcus spp.* were subcultured three times on brain heart infusion agar with ampicillin to ensure isolation of a pure culture. An isolated colony was picked from the most recent subculture and resuspended in 50 µL of nuclease free water, which was then boiled to extract the DNA via the boil lysis method. Polymerase chain reaction (PCR) of the *uidA* gene, which is specific to *E. coli*, was used to confirm the species of putative *E. coli* isolates (US EPA, 2009). qPCR for the 23S rRNA gene was used to confirm the genus of putative *Enterococcus* spp. isolates (US EPA, 2012b). During shipping and a freezer malfunction some isolates were not recovered, therefore fewer than the anticipated 160 putative *E. coli* and *Enterococcus* isolates were recovered. All putative ampicillin-resistant *E.*

coli (n= 116) and *Enterococcus* spp. (n= 58) isolates were confirmed to species or genus, respectively. Confirmed isolate number per site for *E. coli* and *Enterococcus* was as follows: Hospital influent (n = 27 *E. coli* and 11 *Enterococcus*), residential influent (n= 29 and 13), effluent (n = 28 and 18) and estuary (n= 28 and 16).

Frequency of resistance of E. coli and Enterococcus to ampicillin

We estimated the frequency of ampicillin-resistant *E. coli* and *Enterococcus spp,* at each site as the ratio between the concentration of bacteria on ampicillin-amended plates (ampicillin-resistant) divided by the concentration on unamended plates (total).

Multidrug-resistance testing

The susceptibility of isolates to additional antibiotic classes was determined using the Kirby-Bauer disc diffusion assay, and resistance to ampicillin was also confirmed by this procedure. The antibiotics used for the Kirby-Bauer disc diffusion assay were chosen from the CLSI test groups (A, B, C, O, U) (CLSI, 2016) (Table S1 and S2). Group A is comprised of antibiotics that are used for primary testing, while group B are used selectively (for example when the microorganism is resistant to antibiotics in Group A). Group U is comprised of antibiotic that are primarily used to treat urinary tract infections (UTIs). Group C are antibiotics used when reporting to infection control as an epidemiological aid. Group O antibiotics are not tested routinely in the United States. Multiple antibiotic classes, that included antibiotics that have clinical relevance or that are frequently used in Costa Rica were selected. *E. coli* isolates were tested against ampicillin, ciprofloxacin, cefotaxime, amoxicillin with clavulanate, gentamycin, tetracycline and trimethoprim sulfamethoxazole, while *Enterococcus* spp. isolates were tested against ampicillin, ciprofloxacin, vancomycin, tetracycline, linezolid, gentamycin and erythromycin.

Isolates that had been stored in the -80℃ freezer after confirmation as *E. coli* or *Enterococcus* spp. were recovered onto BHI plates amended with 16 µg/ml ampicillin (CLSI, 2016). Isolated colonies were picked using an inoculating loop and placed into 1 mL of a 0.85% saline solution. The density of bacteria in the solution was measured using a Nanodrop instrument, by measuring absorbance at 625 nm (Bauer *et al.*, 1966). If necessary, saline or bacteria were added to achieve the ideal absorbance range (0.08-0.13 AU). Immediately following the absorbance measurement, a sterile swab was dipped into the prepared inoculum and streaked over a prepared Mueller-Hinton plate (Bauer *et al.*, 1966). Antibiotic discs were placed on the plates using sterile twicers, and incubated at 35℃ for *E. coli* isolates or 41℃ for *Enterococcus* spp. for 24 hours. Briefly, the size (in millimeters) of each zone of inhibition was measured and recorded. The resistance or susceptibility of the bacteria to each antibiotic was determined according to CLSI standards (CLSI, 2016). Although *E. coli* and *Enterococcus* were isolated using ampicillin concentrations representing intermediate and full resistance, respectively (see methods section *Sampling and culture of FIB*), all *E. coli* isolates tested for multidrug-resistance (n=112) were confirmed to be fully ampicillin-resistant, hence will be referred to as ampicillin resistant *E. coli* isolates in this study**.**

Statistical data analysis

Frequency of resistance of E. coli and Enterococcus to ampicillin

We explored the relationship between the frequency of resistance to ampicillin (dependent variable) and FIB and sampling sites (independent variables) by means of a beta regression analysis using the betareg package (Cribari-Neto & Zeileis, 2010). A new variable frequency was calculated as the ratio between the concentration of *E. coli* or *Enterococcus* on ampicillin-

amended plates (ampicillin-resistant) divided by the concentration on unamended plates (total) for each observation. Since the frequency of resistance of FIB to ampicillin ranges between 0 to 1, the model assumes that the data follows a beta distribution. Pseudo-R-squared produced by the *summary* function in *betareg* were used as a measure for goodness of fit, and the test for the *p*-value for the model was produced using the *lrtest* function in the *lmtest* package (Zeileis & Hothorn, 2002). Lastly, after fitting the model we performed a post hoc comparison among groups by estimating and comparing marginal means (Least-Squares Means) derived from the model.

Differences in levels of ampicillin-resistant FIB among sampling sites

Concentrations of ampicillin-resistant *E. coli* and *Enterococcus* spp. were analyzed using a one-way ANOVA to determine if there were significant differences in ampicillin-resistant FIB levels among sampling sites. FIB data were $\log_{10}$-transformed to approximately conform to a normal distribution. Groups being compared were determined to have equal variance using the Bartlett test before performing ANOVA analysis. Tukey's HSD test, which accounts for the probability of making one or more Type I errors, was used as posthoc analysis to test for significant differences in all pairwise site comparisons.

Susceptibility of ampicillin-resistant FIB to other antibiotics

A binary variable was created where fully resistant isolates were designated with a 1, and intermediate resistant and susceptible isolates were designated with a 0. We fitted a linear logistic regression model to predict frequency of resistance of FIB to antibiotics (binary dependent variable) with sampling site and antibiotic (categorical independent variables) and the interaction between both factors (formula: Resistance ~ Site * Antibiotic). An analysis of deviance was performed to compare how much the logistic regression model improved by adding the predictors

when compared to the null model (a model without predictors) and calculated a p-value to test if the independent variables provide a statistically significant improvement on the null model. The Akaike information criterion (AIC) was used to compare different possible models and determine which model explains the greatest amount of variation using the fewest possible independent variables. Tjur's coefficient of discrimination was used as a measure for goodness of fit (Tjur, 2009). Ninety-five percent confidence intervals (CIs) and p-values were computed using a Wald z-distribution approximation.

Frequency of multidrug resistance

A new binary variable was created where multidrug resistant FIB isolates were designated with a 1 (resistant to three or more antibiotic classes) and isolates resistant to less than 3 antibiotic classes were designated with a 0. We fitted a linear logistic regression model (estimated using maximum-likelihood) to predict frequency of multidrug resistance with sampling site (multidrug resistance ~ site). An analysis of deviance was performed to compare how much the regression model improved by adding the predictor when compared to the null model (a model without predictors) and calculated a p-value to test if the independent variables provide a statistically significant improvement on the null model. The Cragg-Uhler (Nagelkerke) coefficient of discrimination was used as a measure for goodness of fit. Ninety-five percent confidence intervals (CIs) and p-values were computed using a Wald z-distribution approximation.

Results

Frequency of ampicillin-resistant E. coli and Enterococcus in wastewater, effluent, and the estuary

E. coli were more frequently resistant to ampicillin than were *Enterococcus* spp. (Figure 9). Ampicillin-resistant *E. coli* across all sites represented 18% of total *E. coli*, while ampicillin-resistant *Enterococcus* spp. represented 4% of total *Enterococcus* spp. A beta regression was used to test if FIB type (*E. coli* versus *Enterococcus*) and sampling sites significantly predicted the frequency of resistance of cultured bacteria to ampicillin (Figure 9). Indicator type (*E. coli* versus *Enterococcus* spp.) significantly predicted the frequency of resistance to ampicillin (β = 1.4, p-value = 0.0001). Sampling site was not a significant predictor (β = -2.2, p =0.1788) in the regression model.

Mean $\log_{10}$ concentrations of ampicillin-resistant *E. coli* and *Enterococcus* were compared among sites. Ampicillin-resistant *E. coli* concentrations in residential wastewater were significantly greater than those at any other site (Figure 10, Table S3 and S4). Mean concentrations of ampicillin resistant *E. coli* were 6.4 $\log_{10}$ CFU/100 ml in residential wastewater, followed by 4.9 $\log_{10}$ CFU/100 ml in hospital wastewater, 4.6 $\log_{10}$ CFU/100 ml in treated effluent and 3.9 $\log_{10}$ CFU/100 ml in the estuary. No significant differences in mean $\log_{10}$ concentrations of ampicillin-resistant *Enterococcus* spp. were observed among sites (Figure 10, Table S3). Mean concentration of *Enterococcus* among all sites ranged 3.1 to 3.6 CFU/ 100 ml.

Susceptibility of ampicillin-resistant FIB to other antibiotics

Susceptibility testing against seven additional antibiotics (Table S1 and S2) was performed on confirmed ampicillin-resistant FIB isolates. The frequency of confirmed *E. coli*

(n=112) and *Enterococcus* (n=58) isolates that were susceptible, intermediate, or fully resistant to each antibiotic is shown by site on Figure 11 and Figure 12 respectively. No *Enterococcus* isolates were resistant to vancomycin or linezolid, and most *E. coli* isolates were susceptible (82%) or displayed intermediate resistance (11%) to amoxicillin with clavulanic acid, therefore these antibiotics were not included in the logistic regression model.

The frequency of additional resistance phenotypes observed in ampicillin-resistant *E. coli* isolates was significantly different among antibiotic tested and sites (Figures 4 and 6, Table 6), and a significant interaction effect between site and antibiotic was identified (Table 6). The regression model (formula: resistance ~ site * antibiotic) explained 31% of the variance, and the model significantly predicted frequency of resistance (p-value = 2.2e-16). Typically, *E. coli* had greater odds of being resistant to trimethoprim sulfamethoxazole (log odds antibiotic [SXT] = 2.71) and tetracycline (log odds antibiotic [TET] = 3.48), when compared to other antibiotics, and isolates from hospital wastewater had greater log odds of being resistant to antibiotics tested (log odds site [Hospital] = 2.50) (Table 7).

Resistance of *E. coli* isolated from hospital wastewater to specific antibiotics was different compared to all other sites (Figure 13). *E. coli* isolated from hospital wastewater were significantly less likely to be resistant to tetracycline than *E. coli* isolated from other sites (log odds site[hospital]*antibiotic [TET] -2.92) (Figure 11 and 13, Table 7). While the frequency of resistance to tetracycline and trimethoprim sulfamethazine was high regardless of site (>0.5), typically resistance to tetracycline was highest followed by SXT, except for hospital wastewater (Figure 13), where this relationship is inverse. Conversely, frequency of resistance to gentamicin, cefotaxime and ciprofloxacin was low (<0.2), except for the hospital wastewater (>0.4) (Figure 11 and 13, Table 7).

Like *E. coli*, site and antibiotic were significant predictors of resistance in *Enterococcus* isolates (Figure 12, Table 6), but no significant interaction effect was determined between sampling sites and antibiotics (p-value = 0.58573) (Table 6). The regression model (formula: resistance ~ site + antibiotic) explained 31% of the variance, and the model significantly predicted frequency of resistance (p-value = 7.775e-15). *Enterococcus* isolates from the estuary (log odds site[estuary] = 1.50), and the treated effluent (log odds site[effluent] = 1.13), had greater log odds of being resistant than isolates from the hospital or the residential wastewater (Figure 12, Table 8). Ampicillin-resistant-*Enterococcus* had greater log odds of tetracycline resistance (log odds site [TET] = 3.93) compared to the other antibiotics tested (Figure 12, Table 8).

Multidrug resistance in wastewater, effluent, and the estuary

More than 45% of *E. coli* and *Enterococcus* isolates were multidrug-resistant (resistant to three or more antibiotic classes) according to our testing scheme (Figure 14). The frequency of multidrug-resistant *E. coli* and *Enterococcus* was highest in hospital wastewater compared to other sites (Figure 14). The regression model (formula: multidrug resistance ~ site + FIB) explained only 6% of the variance, but the model significantly predicted frequency of resistance (p-value = 0.03746). Sampling site was significantly associated with the frequency of multidrug resistance in FIB (*E. coli* + *Enterococcus*) (p-value= 0.04908), while FIB was not a significant predictor in the logistic regression model (p-value=0.14887). *E. coli* and *Enterococcus i*solates from the hospital wastewater were more likely to be multidrug-resistant than those from other sites (log odd site[hospital] = 3.63) (Table 9). The greatest number of resistance phenotypes (6 out of 7) in any one isolate was found in *E. coli* isolated from hospital wastewater (n=7), while *Enterococcus* isolates from the estuary had the greatest number of resistance phenotypes in any one isolate (5 out of 7) (n=1) (Figure 15).

E. coli isolated from hospital wastewater were most frequently resistant to a combination of six antibiotics (AMP + TET + SXT + GEN+ CIP + CTX) (Figure 15 and 16), while *E. coli* from the residential wastewater (5 isolates) were typically resistant to a combination of 2 antibiotics (AMP + TET), the treated effluent (9 isolates) and the estuary (9 isolates) were typically resistant to a combination of only two (AMP +TET) or three antibiotics (AMP + TET + SXT) (Figure 15 and 16). *Enterococcus* isolates from hospital wastewater were most frequently resistant to a combination of three antibiotics (AMP + TET + CIP) (4 isolates), while *Enterococcus* from the residential wastewater were frequently resistant to ampicillin only (5 isolates) and treated effluent (7 isolates) and the estuary (6 isolates) were often resistant to a combination of two antibiotics (AMP + TET) (Figure 15 and 17).

Discussion

The presence and persistence of antibiotic resistant bacteria in wastewater treatment plants and hospital wastewaters have led to their description as hot spots for the environmental release of antibiotics and the development of resistant bacteria (Guo *et al.*, 2017; Hultman *et al.*, 2018; Pazda *et al.*, 2019; Rizzo *et al.*, 2013). Many high-income countries have implemented antimicrobial stewardship programs; however, such measures are uncommon in low- and middle-income countries. These areas face unique socioeconomical challenges, antibiotic resistance frequency is higher, and wastewater treatment efficacy varies drastically (Kaiser *et al.*, 2022; Pierce *et al.*, 2020). As surveillance of antibiotic resistance becomes more important, our results highlight the different patterns of resistance of FIB that are opportunistic pathogens and are also used for monitoring water quality in wastewater and the environment in Costa Rica.

Resistance of E. coli and Enterococcus to ampicillin in wastewater, effluent, and the Puntarenas estuary

Ampicillin-resistance frequencies of FIB isolated from wastewater and surface waters in this study are similar or lower than those reported in other regions. For example, in some European countries (Greece, Poland and Netherlands) the frequency of resistance to ampicillin in *Enterococcus* was reported at between 7.3 and 16% (Kolokotsa *et al.*, 2021; Scheurer *et al.*, 2015; Taučer-Kapteijn *et al.*, 2016) Resistance to ampicillin in *E. coli* was 34% in Poland, 19% in Japan and 5% in the United States (Łuczkiewicz *et al.*, 2010; Ma *et al.*, 2022; Mukherjee *et al.*, 2021) It is important to mention that although some dilution is observed in FIB levels in the treated effluent when compared to hospital and residential wastewater in this study, differences were not significant. El Roble wastewater treatment does not have a disinfection step prior to releasing the secondarily treated effluent into the environment. In countries like Europe or the United States, this is uncommon, for example some of these studies report at least a 2-log reduction of FIB after disinfection of treated effluent with chlorine (Kolokotsa *et al.*, 2021; Łuczkiewicz *et al.*, 2010). In this study, FIB resistance to ampicillin was ~ 18% for *E. coli* and ~4% for *Enterococcus*, and no other studies on resistance of FIB to ampicillin in wastewater were found in Costa Rica. One study in 2004 isolated *E. coli* from freshwater close to a hospital discharge in the metropolitan area and found that 45% of isolates were resistant to ampicillin, but intermediate and fully-resistant data were pooled and *E. coli* confirmation was performed using the API 20E system (Tzoc *et al.*, 2004). Similar or lower resistance of FIB to ampicillin in Costa Rica compared to European countries and the United States is unexpected, as regulation of antibiotic use in Costa Rica is less stringent than in more developed countries. For example, Costa Rican law has required a prescription for the sale of antibiotics since 1998, nevertheless illegal

acquisition of antibiotics is a concern that has drawn the attention of the local media (Cordero Parra and Parra, 2019; Diario Oficial La Gaceta, 1998; unknown, 2021).

We found higher mean levels of ampicillin-resistant *Escherichia coli* in residential wastewater compared to hospital wastewater, yet almost identical mean levels of ampicillin-resistant *Enterococcus* spp. in residential and hospital wastewater. It has been suggested that most of the ampicillin-resistant *E. coli* in wastewater probably originated within the community and not from hospitals, given that ampicillin resistant *E. coli* are common in people who have not taken antibiotics or stayed in a healthcare facility (Linton *et al.*, 1974). A systematic review showed that *E. coli* isolated from healthy individuals in community settings in low- and middle-income countries were frequently resistant to ampicillin in hospital wastewater (70%) and in community wastewater (73%) (Nji *et al.*, 2021). The same cannot be said for *Enterococcus*, for example, an increase in ampicillin resistant enterococcal infections in hospitals has been previously reported in the Netherlands (Top *et al.*, 2008), and more than 90% *of E. faecium* isolates from hospitals in the United States exhibited ampicillin resistance (Weiner *et al.*, 2016).

Susceptibility of ampicillin-resistant FIB to other antibiotics

Together, complementary data on antibiotic resistance in the FIB *E. coli* and *Enterococcus* provide a more complete landscape of antibiotic resistance in Puntarenas. Greater frequency of resistance to tetracycline (in both FIB) and trimethoprim- sulfamethoxazole (*E. coli*), and to a lesser extent resistance to gentamicin (FIB), cefotaxime (*E. coli*), ciprofloxacin (FIB) and erythromycin (*Enterococcus*) compared to amoxicillin with clavulanic acid (resistant strains were rarely detected) was determined in our study. Tetracycline, trimethoprim- sulfamethoxazole, gentamicin, cefotaxime and ciprofloxacin are commonly used in Costa Rica. For example,

estimates of national consumption of antibiotics, which were calculated from importation manufacture data, depicted ciprofloxacin, trimethoprim–sulfamethoxazole and cefotaxime to be among the antibiotics with a higher daily dose per 1000 inhabitants, while gentamicin was one of the main imports used for crops (Gobierno del Bicentenario Costa Rica, 2018). Although tetracycline was not listed in Costa Rican estimates of national consumption, oxytetracycline, a member of the tetracycline class of antibiotics, is one of the most commonly imported antibiotics for agriculture in the country. Tetracycline is known to be used in the food industry (i.e., pigs, chicken and tilapia fish) in Costa Rica (Gobierno del Bicentenario Costa Rica, 2018; Gutiérrez *et al.*, 2010). Although vancomycin resistance in *Enterococcus* is of concern in many regions (Ayobami *et al.*, 2020; Markwart *et al.*, 2019; Ping *et al.*, 2021), no *Enterococcus* isolated in this study were resistant to vancomycin.

Higher resistance of *E. coli* to trimethoprim- sulfamethoxazole in low- and middle-income countries when compared to high income countries such as the US and Sweden has been previously reported. It is a relatively inexpensive drug that has been in use for many decades and is widely used in low- and middle-income countries to treat various infections (Huovinen *et al.*, 1995). For example, it was estimated by the International WhoNET surveillance program that out of >20000 *E. coli* isolates, 41 to 62% of isolates from central America and Asia were resistant to trimethoprim- sulfamethoxazole, versus 9 to 23% of isolates that were resistant in the US and Sweden (Huovinen *et al.*, 1995) Resistance to trimethoprim- sulfamethoxazole has been previously documented in Central American and Costa Rica (Critchley *et al.*, 2020; Gupta *et al.*, 1999; Jimenez Pearson *et al.*, 2018; Murray *et al.*, 1985), therefore the high frequency of resistance of *E. coli* to trimethoprim- sulfamethoxazole found in this study is not surprising.

Ampicillin-resistant *E. coli* isolated from hospital wastewater were more likely to be resistant to most of the other antibiotics tested compared to all other sampling sites. For example, resistance of *E. coli* to cefotaxime, a third-generation cephalosporin, was ~50% in hospital wastewater and less than 25% in residential, treated effluent and the estuary. Resistance to cefotaxime is not surprising, as *E. coli* resistance to third generation cephalosporins has been increasing in hospital and community settings in all European countries. For example, the European Antimicrobial Resistance Surveillance Network (EARS-Net) reported an increase in resistance (~15%) of *E. coli* to third generation cephalosporins in bacteraemias in the hospital setting during 2017 (ECDC, 2019). The WHO reported that resistance to third generation cephalosporines in *Escherichia coli* ranged from 2-70% in Africa, 16-68% in South-East Asia, and 0-77% in Western Pacific region in 2015. Resistance to third generation cephalosporins is considered a high community and health-care burden by the World Health Organization (WHO, 2017). For example during 2015, worldwide median estimates of the incidence of infections with *E. coli* resistant to third generation cephalosporins was 297,416, with 9,066 attributable deaths (Cassini *et al.*, 2019).

Multidrug resistance of E. coli and Enterococcus in wastewater, effluent, and the Puntarenas estuary

Although *E. coli* and *Enterococcus* isolates from the hospital wastewater were more likely to be multidrug-resistant compared to the residential wastewater, treated effluent and the estuary, the explanatory power of the logistic regression model is weak (6%), therefore unmeasured variables have a greater effect on the frequency of multidrug resistance. Multidrug-resistance of FIB in untreated hospital and residential wastewater is expected, as wastewater is considered a hotspot for antibiotic resistant bacteria and their genes (Che *et al.*, 2019; Guo *et al.*,

2017; Hultman *et al.*, 2018; Pazda *et al.*, 2019; Rizzo *et al.*, 2013). The prevalence of multidrug resistant bacteria after treatment in the effluent is not surprising as primary and secondary treatments of conventional WWTPs are not designed for the reduction of resistant microbes, and the effluent is not disinfected prior to its release into the estuary. However, the discharge of Puntarenas WWTP is probably not the only contributor to the high frequency of antibiotic resistance in the estuary. Deterioration of the water quality of Puntarenas estuary has been well described over the years (Marín Alpízar, 2006; Mora, 2011). For example, Puntarenas estuary not only receives the treated effluent of El Roble wastewater treatment plant, but it receives untreated wastewater from household onsite wastewater treatment systems (Anonymous, 2008).

Conclusions

Wastewater treatment reduces bacteria in wastewater, yet some antibiotic-resistant bacteria remain in the effluent, which is not disinfected (i.e., chlorine, ozone, UV light) prior to its release, therefore removal of bacteria may not prevent the spreading of antibiotic resistant bacteria into the aquatic environment. Our results indicate that wastewater from hospital and residential water can be important sources of antibiotic resistance bacteria, those bacteria are also present in high frequency in the treated effluent and the estuary. Identifying regionally specific patterns of resistance in wastewater and the environment in Costa Rica is likely to improve our understanding of antibiotic resistance to inform decision and policy makers to develop locally relevant interventions.

Antimicrobial stewardship is defined by the WHO as a “coherent set of actions which promote the responsible use of antimicrobials. This definition can be applied to actions at the individual level as well as the national and global level, and across human health, animal health

and the environment" (WHO, 2019, s. p.). Costa Rica has antibiotic hospital stewardship initiatives (Hegewisch-Taylor *et al.*, 2020). In 2019, the government of Costa Rica passed an executive decree with the officialization and declaration of public interest and national "Action Plan to tackle resistance to antimicrobial resistance. Costa Rica 2018-2025" (Diario Oficial La Gaceta, 2019, s. p.). Nevertheless, the scope of this plan focuses efforts to optimize the use of antibiotics and presents strategies to improve antibiotic stewardship in human health, animal health and plant health. The action plan does not address antibiotic resistant bacteria in wastewater effluent or surface waters as potential contributing factors to the rise in antibiotic resistance, nor does it include the environmental fate of antibiotics. Results from our study highlight the importance of treating wastewater effluent prior to its release into the environment. Antibiotic stewardship initiatives in the country should also consider the fate and consequences of antibiotic resistant bacteria in the environment and specifically address the environmental release of antibiotic resistant bacteria in wastewater into superficial waters.

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Tables

Table 6: Comparison of analysis of deviance results and calculated p-value to test if the independent variables provide a statistically significant improvement on the null model for: A) Logistic regression models used separately to predict *E. coli* and *Enterococcus* frequency of resistance with sampling sites, antibiotics tested and their interaction (sites*antibiotic) and B) a linear regression model used to predict FIB (*E. coli* and *Enterococcus*) frequency of resistance to three or more antibiotic classes with sampling site and FIB type

(A)

Model	FIB	Chisq (χ2)	p-value
Site	*E. coli*	29.076	**2.16E-06**
	Enterococcus	*9.862*	**0.01977**
Antibiotic	*E. coli*	141.95	**< 2.2e-16**
	Enterococcus	*71.301*	**2.25E-15**
Site: Antibiotic	*E. coli*	30.312	**0.002506**
	Enterococcus	*7.495*	0.58573

(B)

Model	Chisq (χ2)	p-value
Site	7.8561	**0.04928**
FIB	2.0838	0.14887

Table 7: Influence of site and antibiotics on the log odds of *E. coli* resistance frequency to additional antibiotics. The model's intercept corresponds to site [residential wastewater], antibiotic [GEN] or their interaction [residential wastewater] * [GEN]. Significant relationships are bolded (P-values <0.05)

Resistance			
Predictors	*Log-Odds*	*CI*	*p*
(Intercept)	-2.56 ***	-4.39 – -1.36	**<0.001**
Site [Hospital]	2.50 **	1.06 – 4.43	**0.002**
Site [Effluent]	0.82	-0.91 – 2.86	0.371
Site [Estuary]	0.44	-1.43 – 2.53	0.641
Antibiotic [TET]	3.48 ***	2.01 – 5.46	**<0.001**
Antibiotic [CTX]	1.04	-0.60 – 3.05	0.24
Antibiotic [SXT]	2.71 **	1.27 – 4.65	**0.001**
Antibiotic [CIP]	0.44	-1.43 – 2.53	0.641
Site [Hospital] *Antibiotic [TET]	-2.92 **	-5.11 – -1.08	**0.003**
Site [Effluent] *Antibiotic [TET]	-0.25	-2.61 – 1.91	0.823
Site [Estuary] *Antibiotic [TET]	0.43	-2.00 – 2.75	0.714
Site [Hospital] *Antibiotic [CTX]	-1.04	-3.26 – 0.90	0.313
Site [Effluent] *Antibiotic [CTX]	-1.82	-4.54 – 0.58	0.153
Site [Estuary] *Antibiotic [CTX]	-1.04	-3.62 – 1.36	0.401
Site [Hospital] *Antibiotic [SXT]	-1.49	-3.69 – 0.37	0.137

Site [Effluent] *Antibiotic [SXT]	-0.74	-3.00 – 1.29	0.488
Site [Estuary] *Antibiotic [SXT]	-0.59	-2.90 – 1.55	0.591
Site [Hospital] *Antibiotic [CIP]	-0.72	-3.03 – 1.41	0.507
Site [Effluent] *Antibiotic [CIP]	-1.95	-5.42 – 0.80	0.192
Site [Estuary] *Antibiotic [CIP]	-0.12	-2.69 – 2.37	0.926
Observations	560		
R^2 Tjur	0.314		

* p<0.05 ** p<0.01 *** p<0.001

Table 8: Influence of site and antibiotic on the log odds of *Enterococcus* resistance frequency to additional antibiotics. The model's intercept corresponds to site [residential wastewater] and antibiotic [GEN]. Significant relationships are bolded (P-values <0.05)

Resistance			
Predictors	*Log Odds*	*CI*	*p*
(Intercept)	-3.68***	-5.12 – -2.48	**<0.001**
Site [Hospital]	1.06	-0.00 – 2.17	0.055
Site [Effluent]	1.13*	0.18 – 2.15	**0.024**
Site [Estuary]	1.50**	0.53 – 2.54	**0.003**
Antibiotic [TET]	3.93***	2.81 – 5.29	**<0.001**
Antibiotic [CIP]	1.59	0.48 – 2.91	**0.009**
Antibiotic [ERY]	1.94**	0.86 – 3.24	**0.001**
Observations	232		
R^2 Tjur	0.310		

* p<0.05 ** p<0.01 *** p<0.001

Table 9: Influence of site and FIB on the log odds of multidrug resistance frequency. The model's intercept corresponds to site [residential wastewater] or FIB [*E. coli*]. Significant relationships are bolded (P-values <0.05)

Multidrug Resistance			
Predictors	*Log Odds*	*CI*	*p*
(Intercept)	0.74	0.38 – 1.42	0.373
Site [Hospital]	3.63 **	1.46 – 9.43	**0.006**
Site [Effluent]	1.71	0.72 – 4.12	0.224
Site [Estuary]	1.76	0.74 – 4.26	0.201
FIB [Enterococcus]	0.62	0.32 – 1.19	0.150
Observations	170		
R^2 Nagelkerke	0.078		

* p<0.05 ** p<0.01 *** p<0.001

Figures

Figure 8: Map of the study site. El Roble Wastewater Treatment Plant (WWTP) and Monseñor Sanabria Hospital appear on map. Sampling stations are designated: A) hospital wastewater, B) residential wastewater, C) treated effluent, and D) Puntarenas estuary where the WWTP effluent is discharged

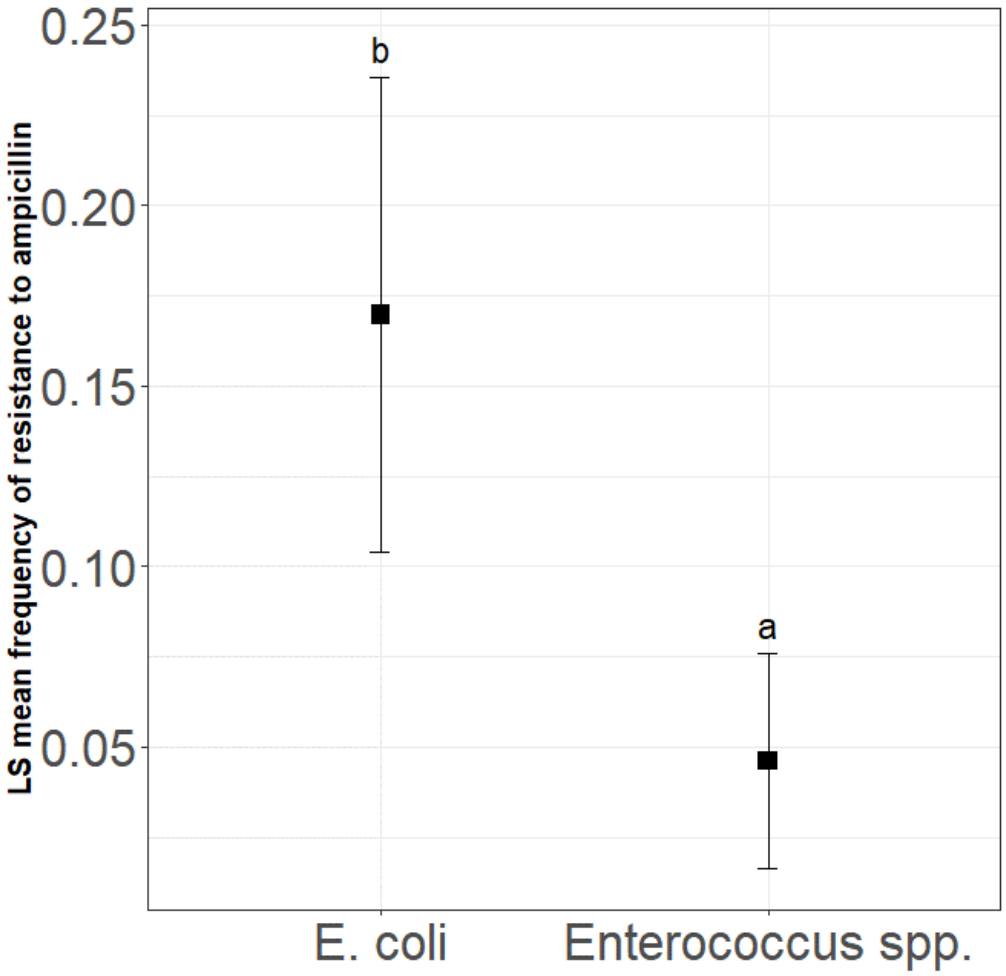

Figure 9: Comparison of the frequency of ampicillin-resistant *E. coli* and *Enterococcus* spp. (proportion of total population) with data combined from all sites. The figure shows estimated least-square (LS) means derived from the fitted beta regression model. Whiskers are the 95% confidence intervals (CIs). The means of groups that do not share a letter are significantly different (p-value <0.05). Comparison among sampling sites is not shown in figure as sampling site was not a significant predictor

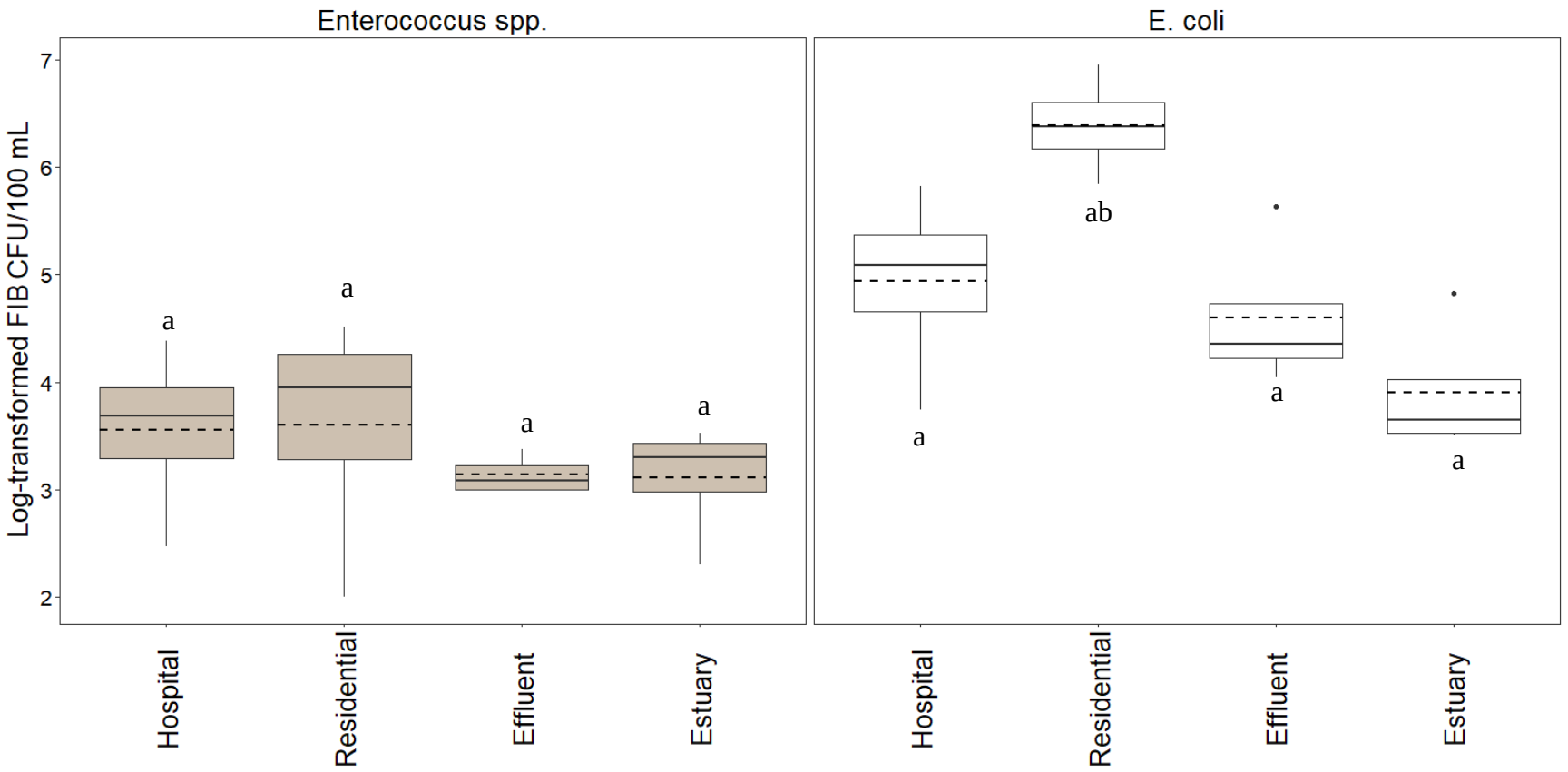

Figure 10: Comparison of $\log_{10}$ mean concentrations ($\log_{10}$ CFU/100 mL) of ampicillin resistant *Enterococcus* spp. and *E. coli* among sampling sites. Boxplots represent 1st, median and 3rd quartile, and the dotted line represents the mean. Whiskers are the minimum and maximum and dots are outliers. The means of groups that share a letter are not significantly different

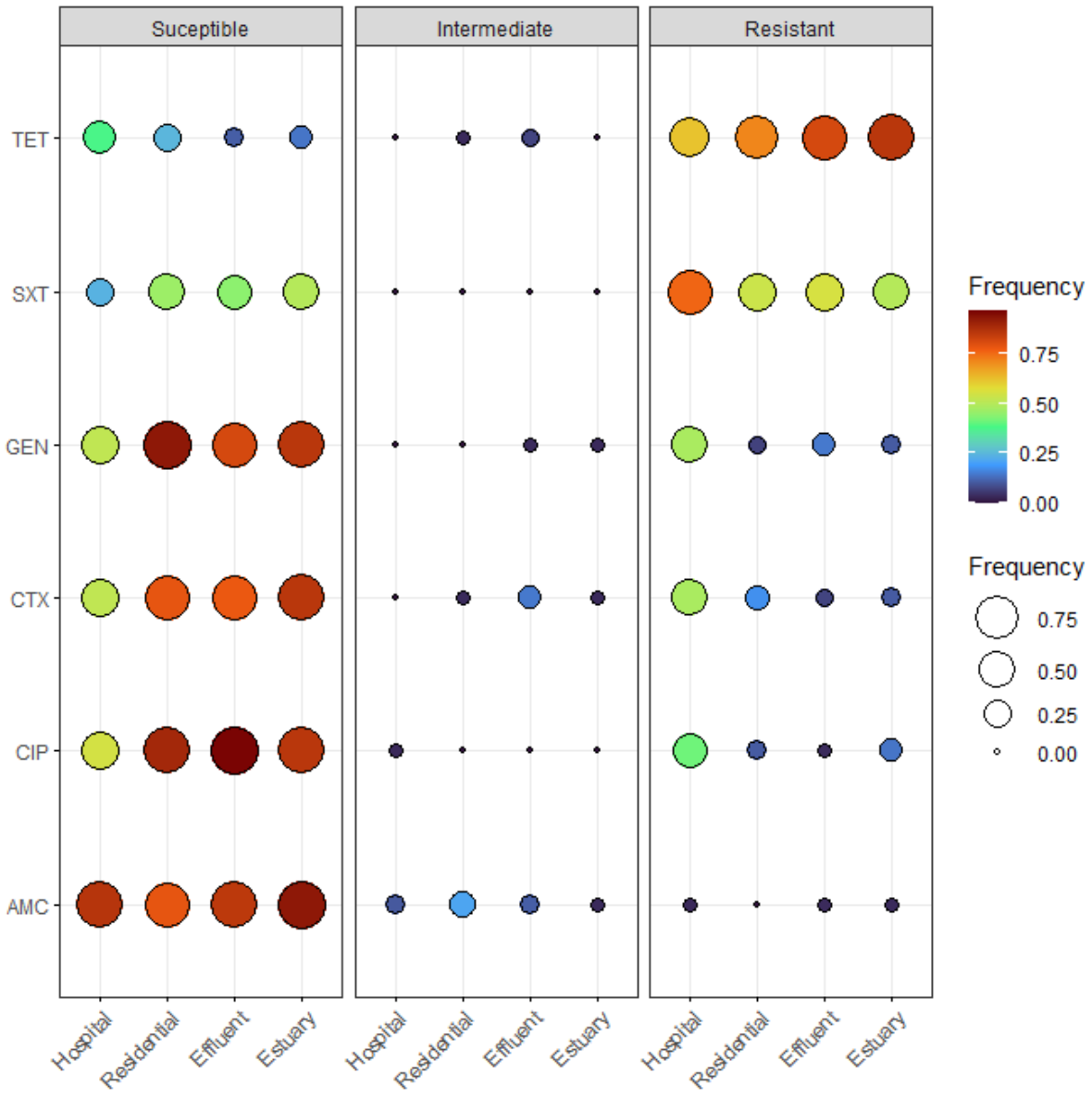

Figure 11: Frequency of susceptibility of ampicillin-resistant *E. coli* isolates at each site to the following antibiotics: cefotaxime (CTX), ciprofloxacin (CIP), amoxicillin with clavulanic acid (AMC), gentamicin (GEN), tetracycline (TET), and trimethoprim sulfamethoxazole (SXT). Ampicillin is not shown in the plot as all isolates tested were fully resistant to ampicillin, the selective antibiotic in the isolation procedure. Larger circles denote higher frequency of isolates and smaller circles denote lower frequency. Additionally, the color scale from blue to red demotes increasing frequency of isolates

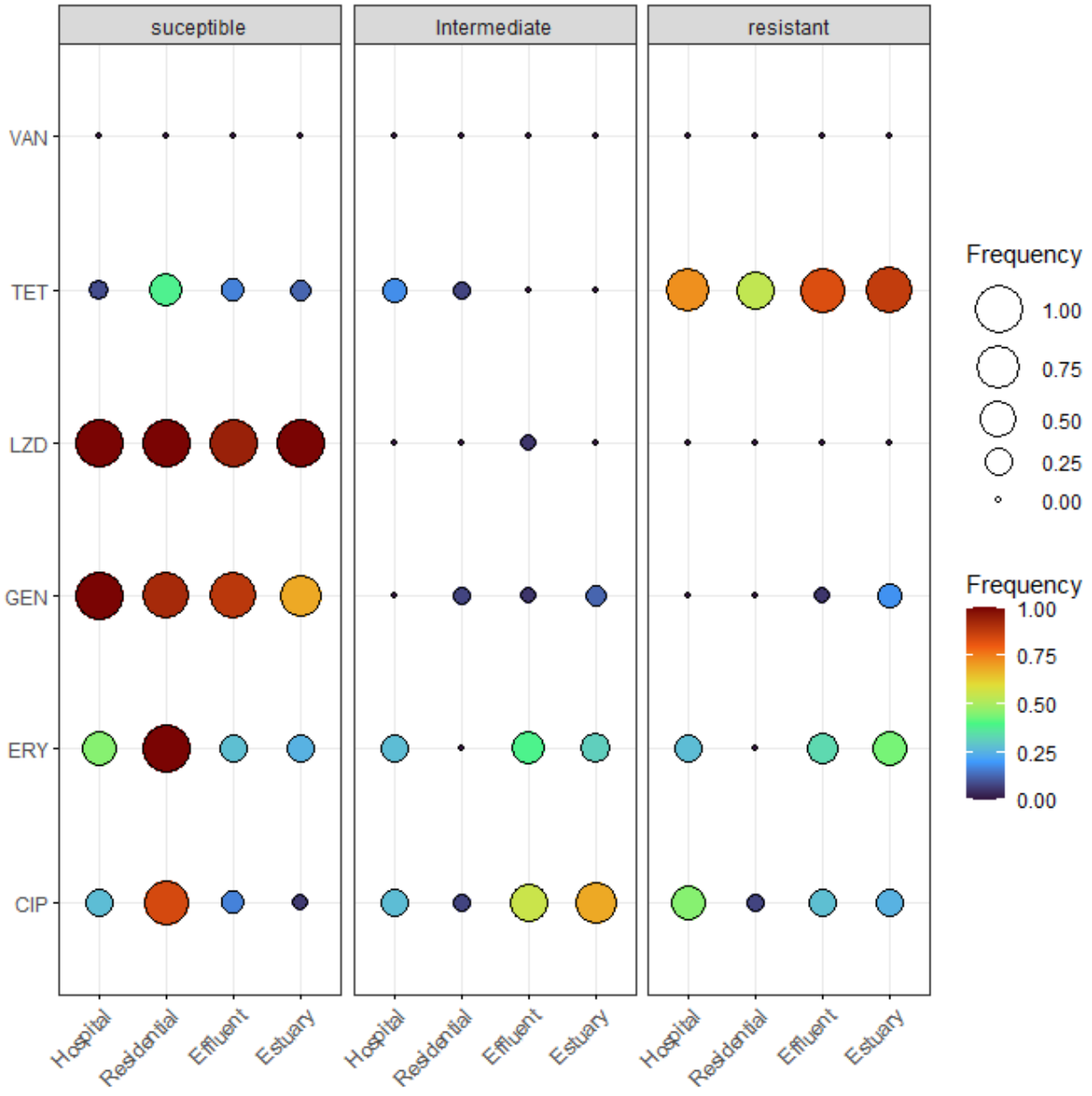

Figure 12: Frequency of susceptibility of ampicillin-resistant *Enterococcus* isolates to the following antibiotics: ciprofloxacin (CIP), erythromycin (ERY), gentamycin (GEN), linezolid (LZD), tetracycline (TET), and vancomycin (VAN). Ampicillin is not shown in the plot as all isolates tested were fully resistant to ampicillin, the selective in the isolation procedure. Larger circles denote higher frequency of isolates and smaller circles denote lower frequency. Additionally, the color scale from blue to red demotes increasing frequency of isolates

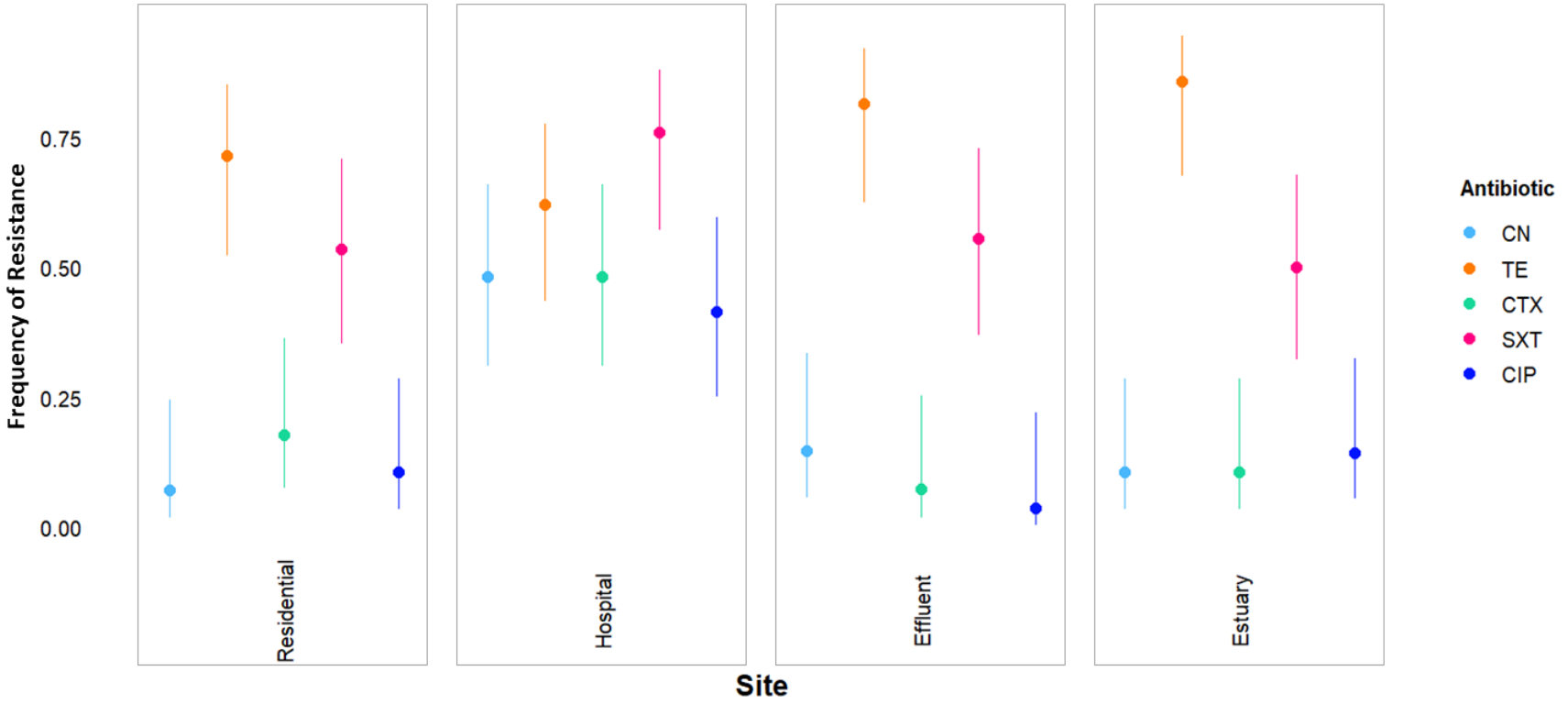

Figure 13: Frequency of additional resistance phenotypes in ampicillin-resistant *E. coli* isolates by site. CN (gentamicin), TE (tetracycline), CTX (cefotaxime), trimethoprim sulfamethazole (SXT) and ciprofloxacin (CIP). We fitted a logistic model to predict resistance (yes/no) with site, antibiotic, and the interaction effect between both factors (site* antibiotic). Predicted frequency of resistance (dots) and confidence intervals (CI) are shown in figure

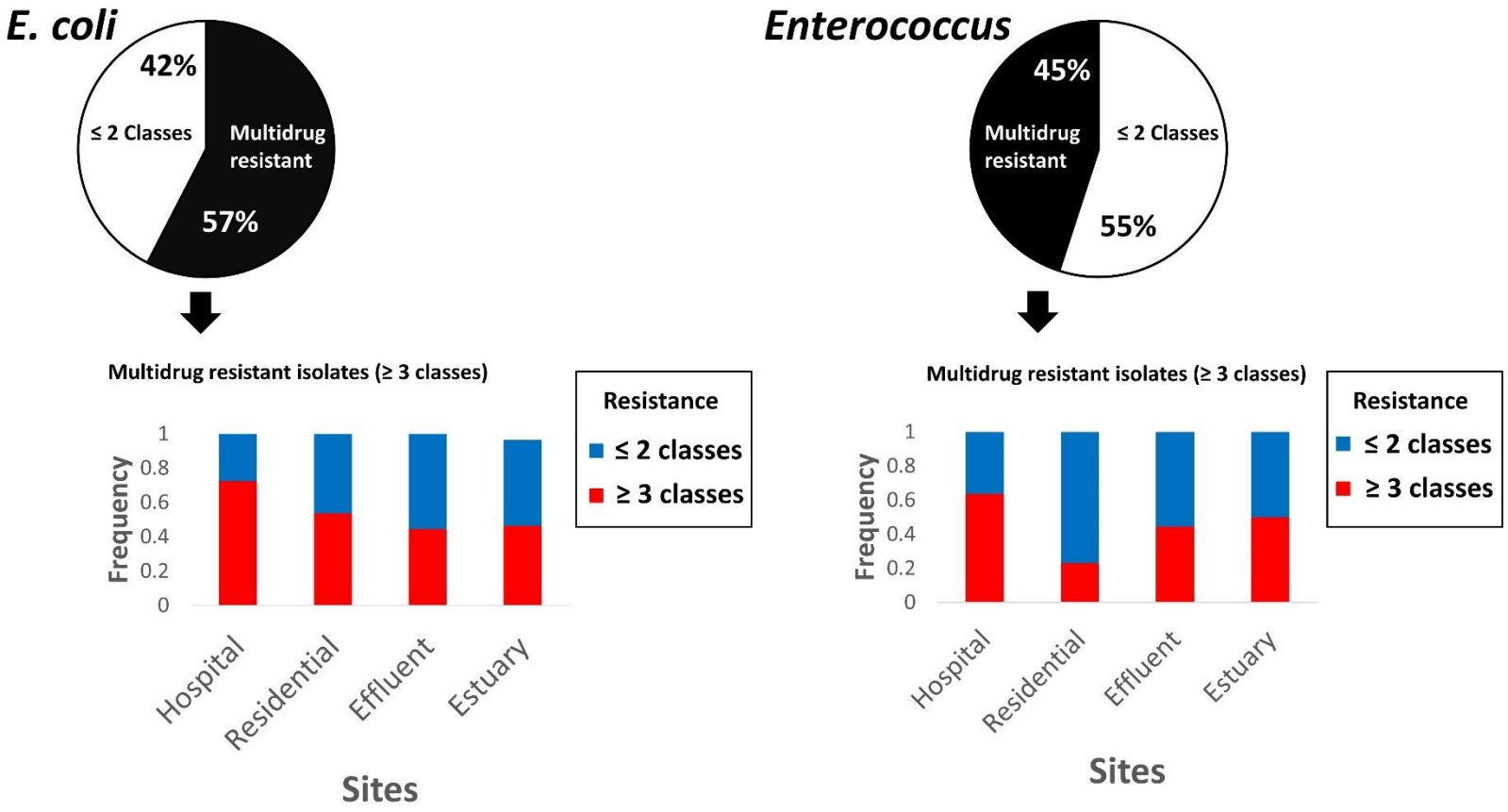

Figure 14: Comparison of frequency of multidrug resistance of *E. coli* and *Enterococcus* isolates per sampling site. The percent of multidrug-resistant isolates (≥ 3 antibiotic classes) is shown in a pie chart for *E. coli* (overall n= 112) (left) and *Enterococcus* (overall n = 58) (right). Sample size per site for *E. coli* and *Enterococcus* is as follows: Hospital influent (n = 27 and 11), residential influent (n=29 and 13), effluent (n = 28 and 18) and estuary (n=28 and 16)

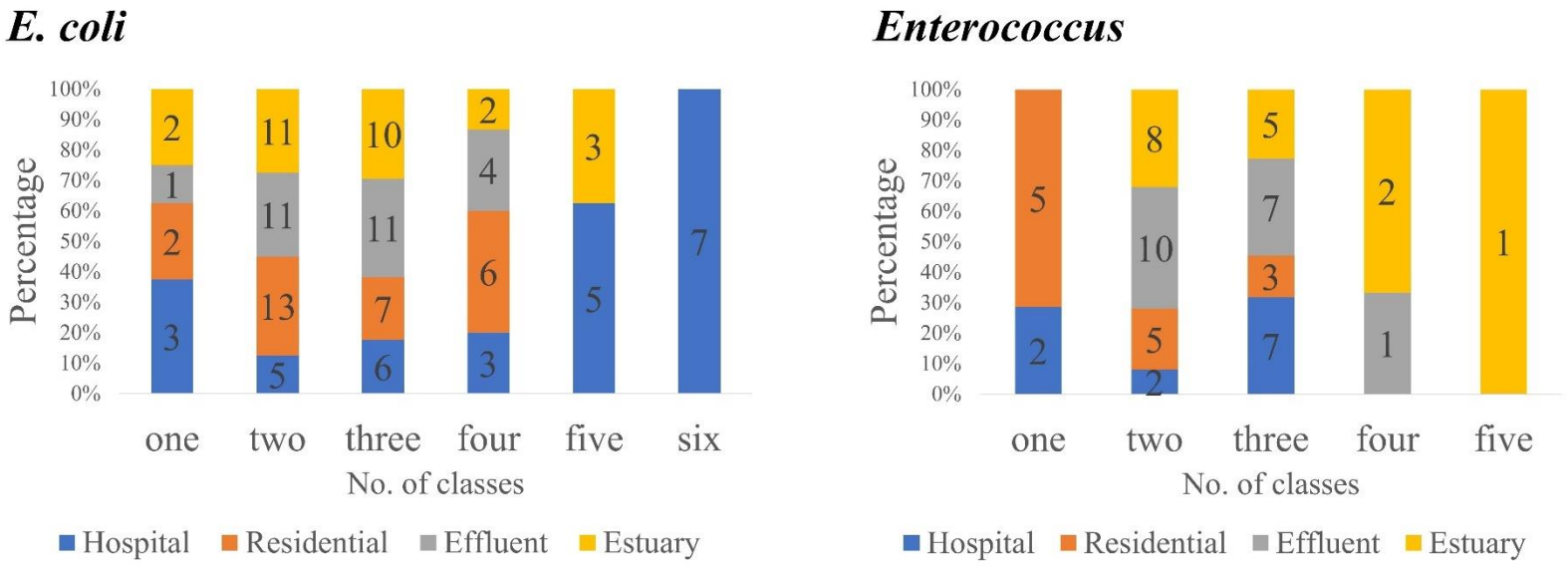

Figure 15: Proportion of isolates to multiple classes of antibiotics among *E. coli* and *Enterococcus* isolates at each site. Sample size per site for *E. coli* and *Enterococcus* is as follows: Hospital influent (n = 27 and 11), residential influent (n=29 and 13), effluent (n = 28 and 18) and estuary (n=28 and 16)

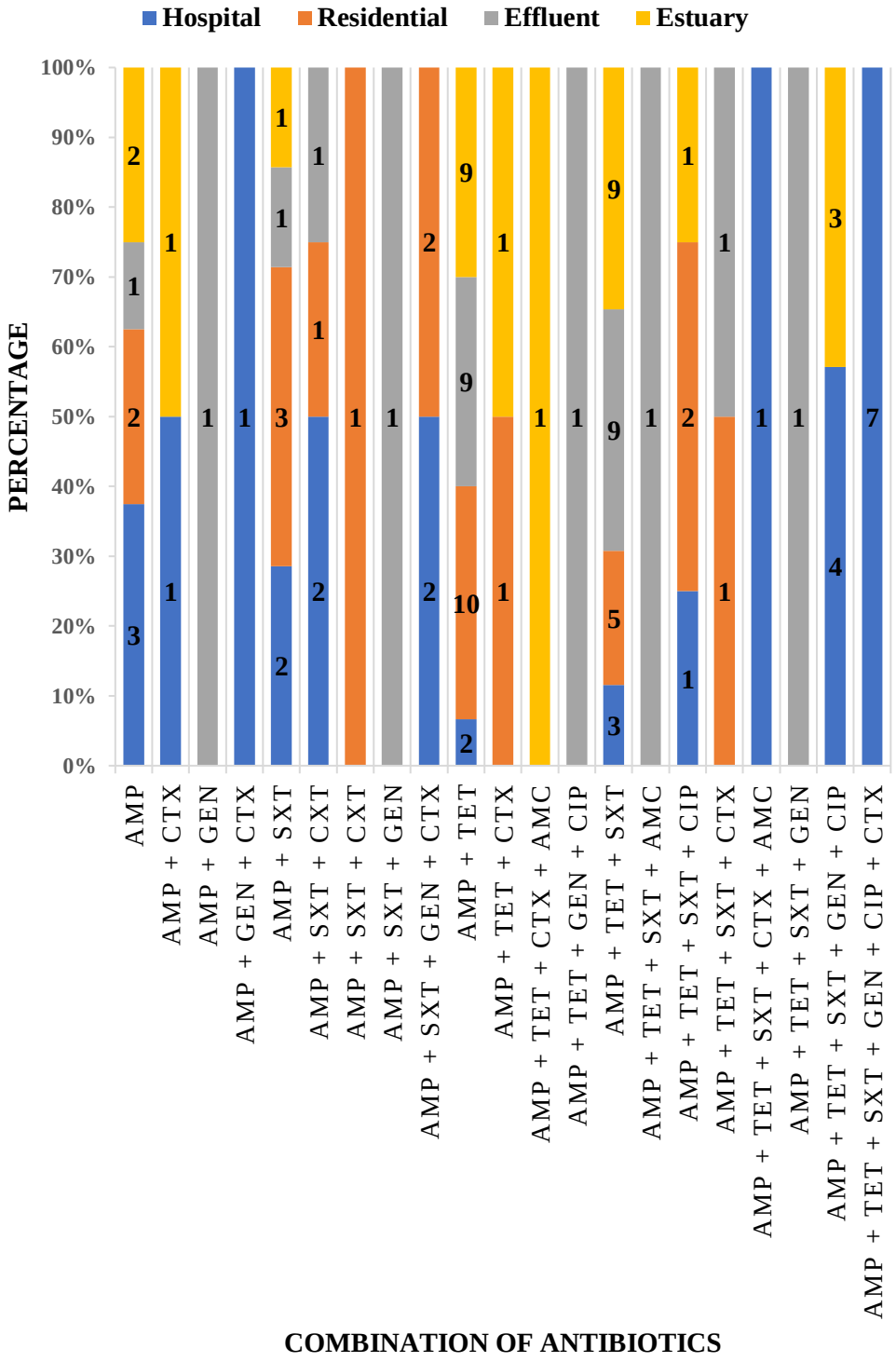

Figure 16: Proportion of different combinations of antibiotics found in sampling sites for *E. coli* isolates. Sample size per site is as follows: Hospital influent (n = 27), residential influent (n=29), effluent (n = 28) and estuary (n=28)

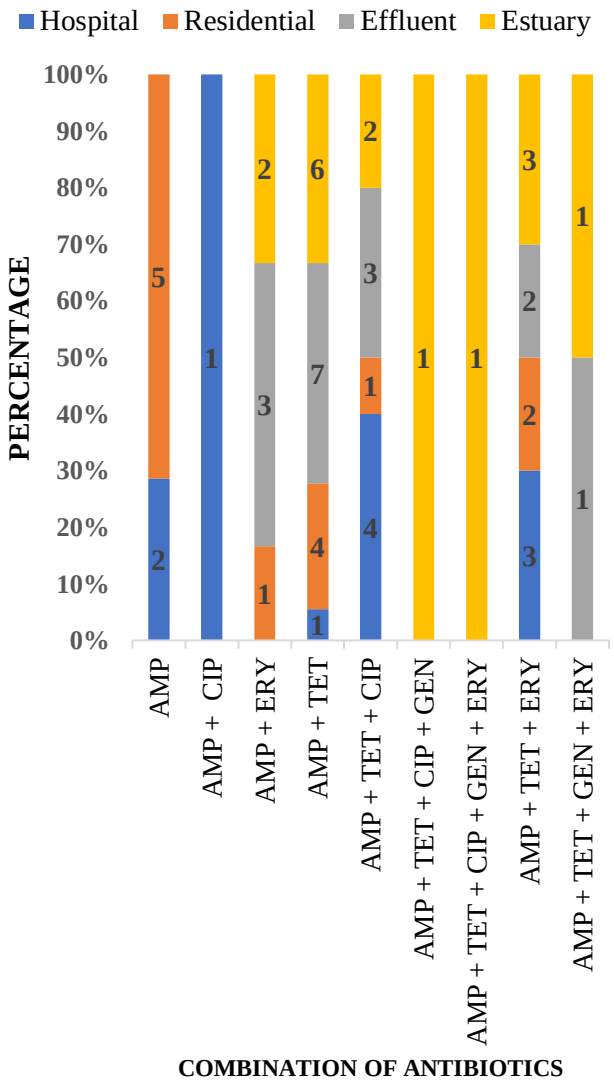

Figure 17: Proportion of different combinations of antibiotics found in sampling sites for *Enterococcus* spp. isolates. Sample size is as follows: Hospital influent (n = 11), residential influent (n= 13), effluent (n =18) and estuary (n=16)

AFTERWORD

Summary

This research provides valuable information that can be used to inform and help evaluate future beach management strategies in Costa Rica, raise awareness on the risk of swimming in tropical polluted waters, and protect public health.

My research demonstrated that there is high risk of gastrointestinal illness associated with swimming in polluted rivers in tropical Costa Rica, primarily due to the presence of human enteric viruses in the rivers. These rivers are a year-round source of pollution to the beach, and this pollution persists to a lesser extent in the ocean. My results also reaffirm that hospital and residential wastewater are an import source of antibiotic resistant bacteria, and highlight the importance of disinfection (i.e., chlorine, UV, ozone) of the treated effluent prior to its release to the environment.

Future directions

Effective monitoring strategies are essential to characterize water quality and support management strategies to protect human health and the environment. This research provides data that can be used as a baseline when establishing future strategies for the surveillance of microorganisms of public health concern (i.e., pathogens and antibiotic resistant bacteria) in tropical regions at a localized scale.

APPENDICES

Appendix A. Chapter two

Note to reader:

This chapter has been previously published in *Water Research* (2021) doi: https://doi.org/10.1016/j.watres.2020.116507 and has been reprinted with permission from © 2021 Elsevier, which allows authors to include their full articles in a thesis or dissertation for non-commercial purposes.

Appendix B. Chapter three

Note to Reader.

This chapter contains information used for a manuscript that has been submitted to *Applied and environmental microbiology* (2022) and is under revision. It is included with permission from *Journal of Applied Microbiology* © 2022 Society for Applied Microbiology, which allows authors to include their full articles in a thesis or dissertation for non-commercial purposes.

Appendix C. Chapter three supplementary materials

Risk of Gastroenteritis from Swimming at a Wastewater-Impacted Tropical Beach Varies across Localized Scales.

Supplementary Material Submitted to Applied and Environmental Microbiology.

Supplementary material contains Table S1 through S11 and Figure S1 through S5.

Table S1: Descriptive statistics for rivers (CP= Copey, NJ=Naranjal and MD= Madrigal) sampled (n=13) during the rainy season (n=39). A) bacterial indicator microorganisms, B) viral indicator microorganisms, C) microbial source tracking (MST) markers and D) pathogens. Percent censored refers to the percentage of observations that were not quantifiable, arranged in order of non-detects (below the assay LOD)/ detected but not quantifiable (above assay LOD and below the assay LOQ)/combined non-detect and detected but not quantifiable. SD = standard deviation

A)

Microbe	Enterococci CFU/L			Fecal coliforms MPN/L			*C. perfringens* CFU/L		
River	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**
% censored	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
Mean	$1.9x10^4$	$4.3x10^4$	$1.4x10^4$	$2.1x10^5$	$6.4x10^5$	$4.1x10^5$	$2.2x10^2$	$4.0x10^2$	$2.4x10^2$
SD	$1.8x10^4$	$5.2x10^4$	$9.9x10^3$	$2.1x10^5$	$6.2x10^5$	$5.4x10^5$	$1.3x10^2$	$6.5x10^2$	$1.3x10^2$

Table S1 (continued)

B)

Microbe	Somatic coliphage PFU/L			F+ coliphage PFU/L		
River	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**
% censored	0/0/0	0/0/0	0/0/0	0/23/23	0/8/8	0/15/15
Mean	$10.0x10^{2}$	$1.2x10^{3}$	$6.6x10^{2}$	$4.6x10^{1}$	$2.3x10^{2}$	$1.9x10^{2}$
SD	$1.2x10^{3}$	$8.9x10^{2}$	$8.0x10^{2}$	$7.9x10^{1}$	$5.8x10^{2}$	$5.1x10^{2}$

C)

Microbe	HF183 GC/L			PMMoV GC/L		
River	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**
% censored	0/0/0	8/0/8	0/0/0	0/0/0	0/0/0	0/0/0
Mean	$2.6x10^{4}$	$7.4x10^{3}$	$7.4x10^{3}$	$5.4x10^{4}$	$6.4x10^{4}$	$3.1x10^{5}$
SD	$3.4x10^{4}$	$9.8x10^{3}$	$1.0x10^{2}$	$5.5x10^{4}$	$4.3x10^{4}$	$9.0x10^{5}$

Table S1 (continued)

D)

Mi-crobe	*Giardia* Cysts/L			*Cryptosporidium* Oocysts/L			*Salmonella* CFU/L			NoVGI GC/L			AdV GC/L		
River	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**
% cen-sored	23/0/23	15/0/15	15/0/15	85/0/85	100/0/100	92/0/92	23/0/23	31/0/31	24/0/24	62/38/100	15/69/84	85/8/92	62/38/100	31/38/69	92/0/92
Mean	$2.4x10^{0}$	$2.4x10^{0}$	$1.1x10^{0}$	NA[a]	NA	NA	$7.4x10^{0}$	$5.8x10^{0}$	$2.6x10^{1}$	NA	NA	NA	NA	$1.5x10^{3}$	NA
SD	$1.7x10^{0}$	$3.7x10^{0}$	$1.3x10^{0}$	NA	NA	NA	$6.4x10^{0}$	$4.7x10^{0}$	$2.0x10^{1}$	NA	NA	NA	NA	$3.1x10^{3}$	NA

NA[a]= Does not apply, mean and standard deviation not calculated because percent of left censored observations >80%

Table S2: Descriptive statistics per river (CP= Copey, NJ=Naranjal and MD= Madrigal) sampled (n=13) during the dry season (n=39). A) bacterial indicator microorganisms, B) viral indicator microorganisms, C) MST markers and D) pathogens. Percent censored refers to the percentage of observations that were not quantifiable, arranged in order of non-detects (below the assay LOD)/ detected but not quantifiable (above assay LOD and below the assay LOQ)/combined non-detect and detected but not quantifiable. SD = Standard deviation

A)

Microbe	Enterococci CFU/L			Fecal coliforms MPN/L			*C. perfringens* CFU/L		
River	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**
% censored	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
Mean	$1.4x10^4$	$6.4x10^3$	$8.8x10^3$	$7.5x10^5$	$2.2x10^5$	$1.5x10^5$	$8.9x10^2$	$7.4x10^2$	$4.5x10^2$
SD	$1.2x10^4$	$5.7x10^3$	$6.8x10^3$	$6.3x10^5$	$2.3x10^5$	$2.4x10^5$	$8.4x10^2$	$6.5x10^2$	$4.8x10^2$

Table S2 (continued)

B)

Microbe	Somatic coliphage PFU/L			F+ coliphage PFU/L		
River	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**
% censored	0/0/0	0/0/0	0/0/0	0/0/0	15/0/0	0/0/0
Mean	$2.0x10^3$	$1.4x10^3$	$6.9x10^3$	$8.8x10^1$	$1.7x10^2$	$7.9x10^1$
SD	$2.0x10^3$	$1.6x10^3$	$2.6x10^2$	$1.1x10^2$	$2.8x10^2$	$9.0x10^1$

C)

Microbe	HF183 GC/L			PMMoV GC/L		
River	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**
% censored	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
Mean	$6.3x10^4$	$4.2x10^4$	$2.7x10^4$	$1.6x10^5$	$1.7x\ 0^5$	$1.4x10^5$
SD	$5.7x10^4$	$5.2x10^4$	$4.0x10^4$	$1.6x10^5$	$1.3x10^5$	$1.9x10^5$

Table S2 (continued)

D)

Microbe	*Giardia* Cysts/L			*Cryptosporidium* Oocysts/L			*Salmonella* CFU/L			NoVGI GC/L			AdV GC/L		
River	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**
% censored	0/0/0	8/0/8	54/0/54	85/0/85	100/0/100	100/0/100	0/0/0	0/0/0	0/0/0	0/23/23	0/23/23	0/38/38	38/38/76	54/8/62	92/8/100
Mean	$1.5x10^{0}$	$1.4x10^{0^{0}}$	$2.7x10^{-1}$	NA[a]	NA	NA	$3.4x10^{1}$	$1.3x10^{2}$	$1.7x10^{2}$	$1.6x10^{3}$	$2.4x10^{3}$	$4.5x10^{3}$	$7.0x10^{2}$	$9.8x10^{2}$	NA
SD	$2.3x10^{0}$	$2.9x10^{0^{0}}$	$4.1x10^{-1}$	NA	NA	NA	$2.6x10^{1}$	$2.4x10^{2}$	$1.4x10^{2}$	$1.7x10^{3}$	$4.6x10^{3}$	$9.0x10^{3}$	$4.5x10^{2}$	$1.1x10^{3}$	NA

NA[a]= Does not apply, mean standard deviation not calculated because percent of left censored observations >80%

Table S3: Descriptive statistics for microbial variables at ocean sites impacted by each river regardless of season (n=26 samples per ocean site). Percent censored refers to the percentage of observations that were not quantifiable, arranged in order of non-detects (below the assay LOD)/ detected but not quantifiable (above assay LOD and below the assay LOQ)/combined non-detect and detected but not quantifiable. SD = standard deviation.

Microbe	Enterococci CFU/L			Fecal coliforms MPN/L			HF183 GC/L		
River	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**	**CP**	**NJ**	**MD**
% censored	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	8/12/19	8/24/32	8/42/50
Mean	$1.4x10^3$	$9.2x10^2$	$3.3 x 10^2$	$8.7x10^3$	$3.8x10^3$	$2.9x10^3$	$5.1x10^4$	$3.8x10^4$	$4.0x10^3$
SD	$2.5x10^3$	$2.1x10^3$	$6.1x10^2$	$6.2x10^3$	$6.2x10^3$	$4.4x10^3$	$1.6x10^5$	$1.7x10^5$	$8.4x10^3$

Table S4: Statistical output of A) Multiple regression of species (microorganisms) with ordination axes in data. P-values <0.05 are bolded, B) Permutational multivariate analysis of variance (PERMANOVA) to test for differences in microorganisms between seasons, subwatersheds and the interaction effect between season and subwatershed in river and ocean data, C) Pairwise PERMANOVA with Holm p-value adjustment in river data, D) Pairwise PERMANOVA with Holm p-value adjustment in ocean data, E) Redundancy analysis (RDA) in river data, F) RDA analysis in ocean data

A)

Microorganism	**r**	**P-value**
Giardia	0.11	**0.02**
Cryptosporidium	0.00	0.95
Salmonella	0.61	**0.01**
NoVGI	0.59	**0.01**
AdV	0.07	0.09
enterococci	0.22	**0.01**
fecal coliforms	0.08	0.07
C. perfringens	0.40	**0.01**
somatic coliphage	0.09	**0.03**
F+ coliphage	0.43	**0.01**
HF183	0.38	**0.01**
PMMoV	0.77	**0.01**

Table S4 (continued)

B)

Groups	**River**		**Ocean**	
	F statistic	p-value	F statistic	p-value
seasons (rainy vs. dry)	3.82	**0.001**	2.36	0.09
river areas (Copey, Naranjal and Madrigal)	16.94	**0.001**	7.85	**0.001**
Interaction season:river areas	16.94	**0.001**	0.25	0.921

Table S4 (continued)

C)

Combined factor subwatershed/season	Copey_rainy	Naranjal_rainy	Madrigal _rainy	Copey _dry	Naranjal _dry
Naranjal_rainy	**0.02**				
Madrigal_rainy	0.176	**0.015**			
Copey_dry	**0.015**	**0.015**	**0.015**		
Naranjal_dry	**0.015**	**0.015**	**0.015**	0.176	
Madrigal_dry	**0.015**	**0.015**	**0.015**	**0.015**	0.063

D)

Factor	Copey	Naranjal
Naranjal	**0.003**	
Madrigal	**0.003**	0.747

Table S4 (continued)

E)

River RDA Analysis		
constrained proportion	9.40%	
Pseudo F statistic	3.93	
p-value	0.001	
Adjusted R2	7.10%	
Optimal environmental variable	Pseudo F statistic	P-value
Cummulative rainfall 48 h	5.8	**0.001**
Water temperature	2.2	**0.029**

Table S4 (continued)

F)

Ocean RDA Analysis		
constrained proportion	12.20%	
Pseudo F statistic	3.6	
p-value	0.002	
Adjusted R2	8.00%	
Optimal environmental variable	Pseudo F statistic	P-value
Water temperature	4.00	**0.026**
Cummulative rainfall 48 h	3.79	**0.030**
Cummulative rainfall 96 h	2.60	0.095

Table S5: Quantitative microbial risk assessment (QMRA) model parameter assumptions and equations

Parameter	Units	Value or distribution	Reference
Volume ingestion rate for general population	ml min^{-1}	log_{10}normal(1.267, 0.628)	(US EPA, 2010)
Human Adenovirus Harmonization factor	$TCID_{50}$ $copies^{-1}$	uniform (0.00143, 0.1)	(Flint *et al.*, 2009; Heider and Metzner, 2014; Kundu *et al.*, 2013)
Pathogen dose-Response models to calculate probability of Infection (P_{inf}), where d = dose			
Human Adenovirus (oral)	Exact Beta-Poisson	$P_{inf} = 1 - {}_1F_1(\alpha, \alpha + \beta, -d)$ α = 5.11; β = 2.80	(Teunis *et al.*, 2016)
Cryptosporidium spp.	Fractional Poisson	$P_{inf} = P \times [1 - e^{\frac{-d}{\mu}}]$ P = 0.737, μ = 1	(Messner & Berger, 2016)
Giardia lamblia	Exponential	$P_{inf} = 1 - e^{\frac{-d}{k}}$ k = 50	(Rose *et al.*, 1991)
Norovirus genotype I	Fractional Poisson	$P_{inf} = P \times [1 - e^{\frac{-d}{\mu}}]$ P = 0.87, μ = 1106 (aggregate), 1 (disaggregate)	(Messner *et al.*, 2014; Morera *et al.*, 2003; Van Abel *et al.*, 2017)
Salmonella enteritidis *Salmonella typhimurium*	Beta Function (simplified conditional function, originally Hypergeometric Beta Poisson)	$P_{inf} = \frac{B(\alpha, \beta + d)}{B(\alpha, \beta)}$ α = 0.0085, β = 3.14	(Teunis *et al.*, 2010)

Table S5 (continued)

Probability of Illness (P_{ill}) is calculated by $\boldsymbol{P_{ill} = P_{inf} \times M}$		(Sunger *et al.*, 2019)
Morbidity Ratio (*M*)	proportion	
$M_{Adenovirus}$	0.5	(Haas *et al.*, 2014)
$M_{Cryptosporidium}$	0.39	(DuPont *et al.*, 1995)
$M_{Giardia}$	0.5	(Rose *et al.*, 1991)
$M_{Norovirus}$	0.6	(Eftim *et al.*, 2017)
$M_{Salmonella}$	0.2	(Haas *et al.*, 2014)

Table S6: Estimated distribution of pathogen concentrations in rivers calculated for quantitative microbial risk assessment (QMRA). CFU = colony forming units, rROS = robust regression on order statistics

Parameter	Units	Log_{10}normal Distribution (mean, standard deviation)	Estimation Method
Copey River – Rainy Season			
Adenovirus	Copies L^{-1}	(1.94, 0.70)	95^{th} percentile estimation
Cryptosporidium	Oocysts L^{-1}	(-1.52, 0.30)	95^{th} percentile estimation
Giardia	Cysts L^{-1}	(0.22, 0.48)	rROS
Norovirus	Copies L^{-1}	(1.92, 0.70)	95^{th} percentile estimation
Salmonella	CFU L^{-1}	(0.66, 0.49)	rROS
Copey River – Dry Season			
Adenovirus	Copies L^{-1}	(2.77, 0.26)	rROS
Cryptosporidium	Oocysts L^{-1}	(-1.67, 0.32)	95^{th} percentile estimation
Giardia	Cysts L^{-1}	(-0.07, 0.45)	N/A; data not censored
Norovirus	Copies L^{-1}	(2.97, 0.45)	rROS
Salmonella	CFU L^{-1}	(1.41, 0.34)	N/A; data not censored
Naranjal River – Rainy Season			
Adenovirus	Copies L^{-1}	(2.33, 0.92)	rROS
Cryptosporidium	Oocysts L^{-1}	(-2.30, 0.36)	95^{th} percentile estimation
Giardia	Cysts L^{-1}	(-0.23, 0.89)	rROS

Table S6 (continued)

Norovirus	Copies L^{-1}	(2.48, 0.35)	95th percentile estimation
Salmonella	CFU L^{-1}	(0.60, 0.42)	rROS
Naranjal River – Dry Season			
Adenovirus	Copies L^{-1}	(2.74, 0.49)	rROS
Cryptosporidium	Oocysts L^{-1}	(-2.30, 0.36)	95th percentile estimation
Giardia	Cysts L^{-1}	(-0.39, 0.72)	rROS
Norovirus	Copies L^{-1}	(2.99, 0.54)	rROS
Salmonella	CFU L^{-1}	(1.64, 0.60)	N/A; data not censored
Madrigal River – Rainy Season			
Adenovirus	Copies L^{-1}	(1.56, 0.66)	95th percentile estimation
Cryptosporidium	Oocysts L^{-1}	(-1.85, 0.23)	95th percentile estimation
Giardia	Cysts L^{-1}	(-0.36, 0.71)	rROS
Norovirus	Copies L^{-1}	(1.69,0.66)	95th percentile estimation
Salmonella	CFU L^{-1}	(1.30, 0.35)	rROS
Madrigal River – Dry Season			
Adenovirus	Copies L^{-1}	(2.04, 0.50)	95th percentile estimation
Cryptosporidium	Oocysts L^{-1}	(-2.27, 0.35)	95th percentile estimation
Giardia	Cysts L^{-1}	(-1.06, 0.74)	rROS
Norovirus	Copies L^{-1}	(3.00, 0.76)	rROS
Salmonella	CFU L^{-1}	(2.06, 0.46)	N/A; data not censored

Table S7: Quantitative microbial risk assessment (QMRA) model parameter assumptions for sensitivity analyses with realistic values from the literature

Parameter	Units	Value or distribution	Explanation	Reference
Pathogen river concentrations				
Human Adenovirus	copies l^{-1}	log_{10}uniform (-3.00, 3.86)	full range from 0.001 to maximum mean wastewater concentrations from literature	(Symonds *et al.*, 2017)
Cryptosporidium spp.	oocysts l^{-1}	log_{10}uniform (-3.00, 4.40)		(Boehm *et al.*, 2018)
Giardia lamblia	cysts l^{-1}	log_{10}uniform (-3.00, 5.02)		(Maier *et al.*, 2009)
Norovirus genotype I	copies l^{-1}	log_{10}uniform (-3.00, 6.73)		(Symonds *et al.*, 2017)
Salmonella enteritidis *Salmonella typhimurium*	CFU l^{-1}	log_{10}uniform (-3.00, 4.70)		(Boehm *et al.*, 2018)
Volume of water ingested	ml	log_{10}uniform (0, 2.08)	Extreme values, 0 ml to 120 ml	(Stone *et al.*, 2008)
Human Adenovirus Harmonization factor	$TCID_{50}$ $copies^{-1}$	uniform (0.00143, 0.1)	Did not change from the original model	(Flint *et al.*, 2009; Heider and Metzner, 2014; Kundu *et al.*, 2013)

Table S7 (continued)

Dose-Response models to calculate probability of infection (P_{inf})				
Human Adenovirus (oral)		α = 5.11; β = 2.80	Paired values; could not change	(Teunis *et al.*, 2016)
Cryptosporidium spp.		p: uniform (0.67, 0.08) μ: 1	90% credible interval given one oocyst	(Messner & Berger, 2016)
Giardia lamblia		k: uniform (28, 102)	95th percentile confidence interval estimate	(Rose *et al.*, 1991)
Norovirus genotype I		p: uniform (0.72, 1) μ: uniform (1, 1106)	p: susceptible population could be as high as 100% or as low as 72% μ: disaggregate to aggregate NoVGI	(Messner *et al.*, 2014; Van Abel *et al.*, 2017)
Salmonella enteritidis *Salmonella typhimurium*		α = 0.0085, β = 3.14	Paired values; could not change	(Teunis *et al.*, 2010)
Morbidity ratio (M)				
$M_{Adenovirus}$	proportion	uniform (0.25, 1)	The minimum value reported in the literature to the maximum value possible corresponding to 100% illness as a result of infection.	(Viau *et al.*, 2011)
$M_{Cryptosporidium}$	proportion	uniform (0.2, 1)		(US EPA, 2010)
$M_{Giardia}$	proportion	uniform (0.2, 1)		(US EPA, 2010)
$M_{Norovirus}$	proportion	uniform (0.3, 1)		(Teunis *et al.*, 2008; US EPA, 2010)

Table S7 (continued)

$M_{Salmonella}$	proportion	uniform (0.17, 1)		(Boehm *et al.*, 2018; Haas *et al.*, 2014)

Table S8: Frequency of pathogen detection (percentage of observations >LOD) with data combined from all rivers in the rainy and dry seasons. The theoretical process limit of detection and quantification is included in Table 1

Pathogen	**pLOD**	**pLOQ**	**Units**	**Dry Season % (n) Detected**	**Rainy Season % (n) Detected**
Giardia	<0.04	*NA*	Cysts/ L	79% (31)	82% (32)
Cryptosporidium	<0.04	*NA*	Oocysts/ L	5% (2)	8% (3)
Salmonella	<1.7	*NA*	CFU/ L	100% (39)	74% (29)
NoVGI	<128.2	1210.2	GC/ L	100% (39)	41% (16)
AdV	<168.2	1155.2	GC/ L	39% (15)	39 % (15)

Table S9: Mean and standard deviation of pathogens in the river data (n = 78) per season sampled (n=39). Percent left-censored refers to the percent of observations that are below the LOD of *Giardia*, *Cryptosporidium* and *Salmonella* assays and below the LOD and LOQ of NoVGI and AdV assays

Microorganism	**Units**	**Season**	
		Rainy	**Dry**
Giardia	Cysts/ L	$1.97 \times 10^{0} \pm 2.51$	$1.08 \times 10^{0} \pm 2.19$
Cryptosporidium	Oocysts/ L	NA[a]	NA
Salmonella	CFU/ L	$1.27 \times 10^{1} \pm 15.45$	$1.12 \times 10^{2} \pm 166.24$
NoVGI	GC/ L	NA	$2.85 \times 10^{3} \pm 5.88 \times 10^{3}$
AdV	GC/ L	NA	$5.87 \times 10^{2} \pm 7.69 \times 10^{2}$

NA[a] Does not apply, mean standard deviation not calculated because percent of left censored observations >80%

Table S10: Descriptive statistics in the ocean data (n = 78) per season sampled (n=39). Percent censored refers to the percentage of observations that were not quantifiable, arranged in order of non-detects (below the assay LOD)/ detected but not quantifiable (above assay LOD and below the assay LOQ)/combined non-detect and detected but not quantifiable. SD = standard deviation

Microbe	Enterococci CFU/L		Fecal coliforms MPN/L		HF183 GC/L	
Season	Rainy	Dry	Rainy	Dry	Rainy	Dry
% censored	0/0/0	0/0/0	0/0/0	0/0/0	8/26/34	8/26/34
Mean	1.48×10^3	2.77×10^2	5.50×10^3	4.79×10^3	3.33×10^4	2.78×10^4
SD	2.61×10^3	3.19×10^2	6.10×10^3	1.39×10^3	1.31×10^5	1.39×10^5

Table S11: Viral log10 reduction values (LRVs) needed to ensure safe swimming conditions at Jacó beach by river, and season. The LRV range represented was based upon the cumulative viral risk of GI illness to swimmers calculated using the norovirus genogroup I (NoVGI) aggregate (agg) model

River	**Viral log_{10} Reduction Value**	
	Dry Season	Rainy Season
Copey	1.9	1.5
Naranjal	2.1	2.2

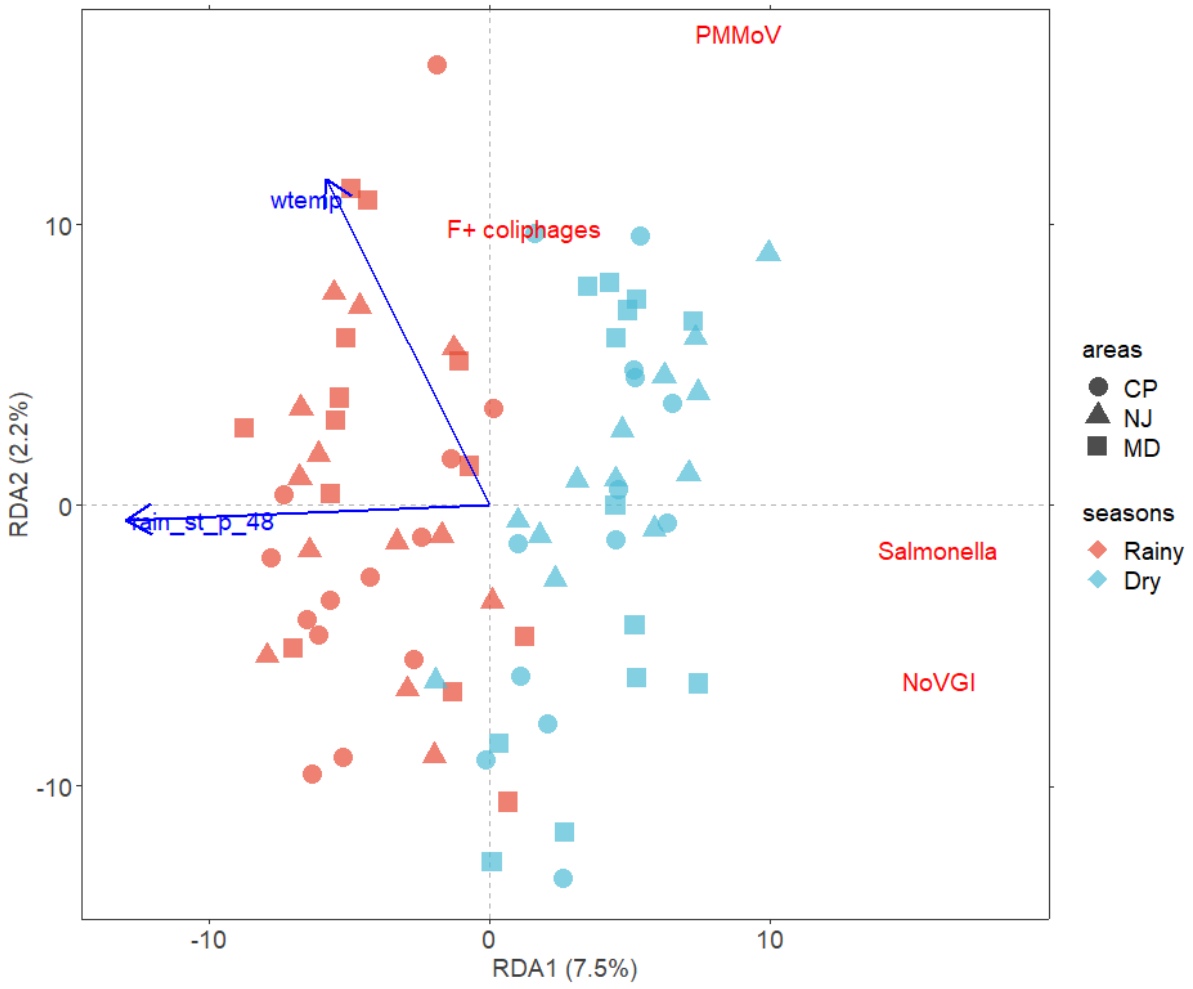

Figure S1: Redundancy analysis (RDA) ordination plot showing that water temperature and rainfall 48 h prior to sampling (blue arrows) were the environmental variables that optimized the relationship between microorganisms in rivers. Species (red) plotted were selected based on goodness of fit (r^2=0.4)

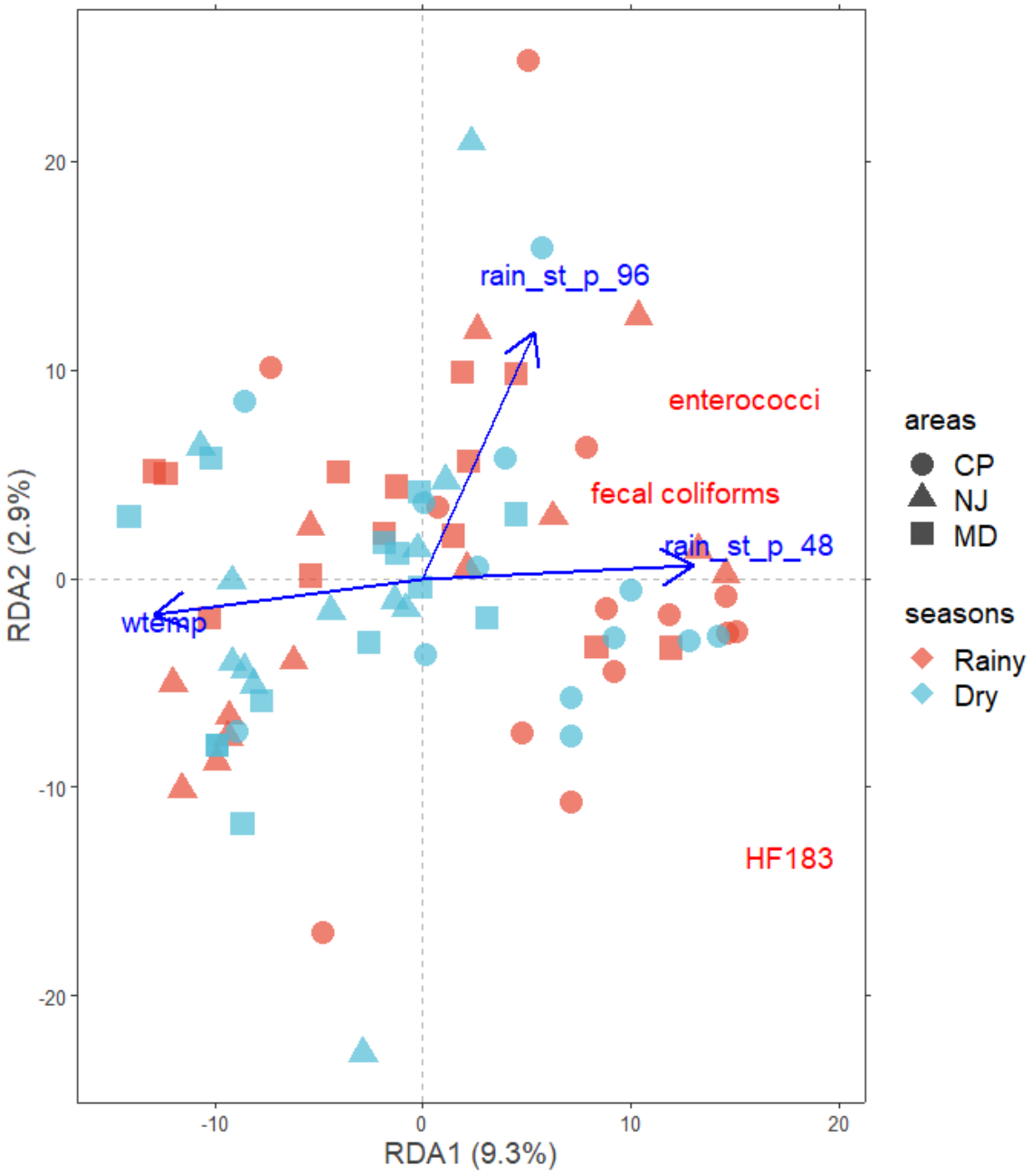

Figure S2: Redundancy analysis (RDA) ordination plot showing that water temperature and rainfall 48 h and 96 h prior of sampling were the environmental variables that optimized the relationship between microorganisms in the ocean

Figure S3: Median risk of gastrointestinal (GI) illness to swimmers for each river and season by pathogen, as well as cumulatively. Estimates for NoVGI and cumulative risk were calculated with the aggregate NoVGI model. Color scales indicate how the median value of risk compared to the US EPA Recreational Water Quality health benchmark (recommendation 1: 36 illness per 1000)

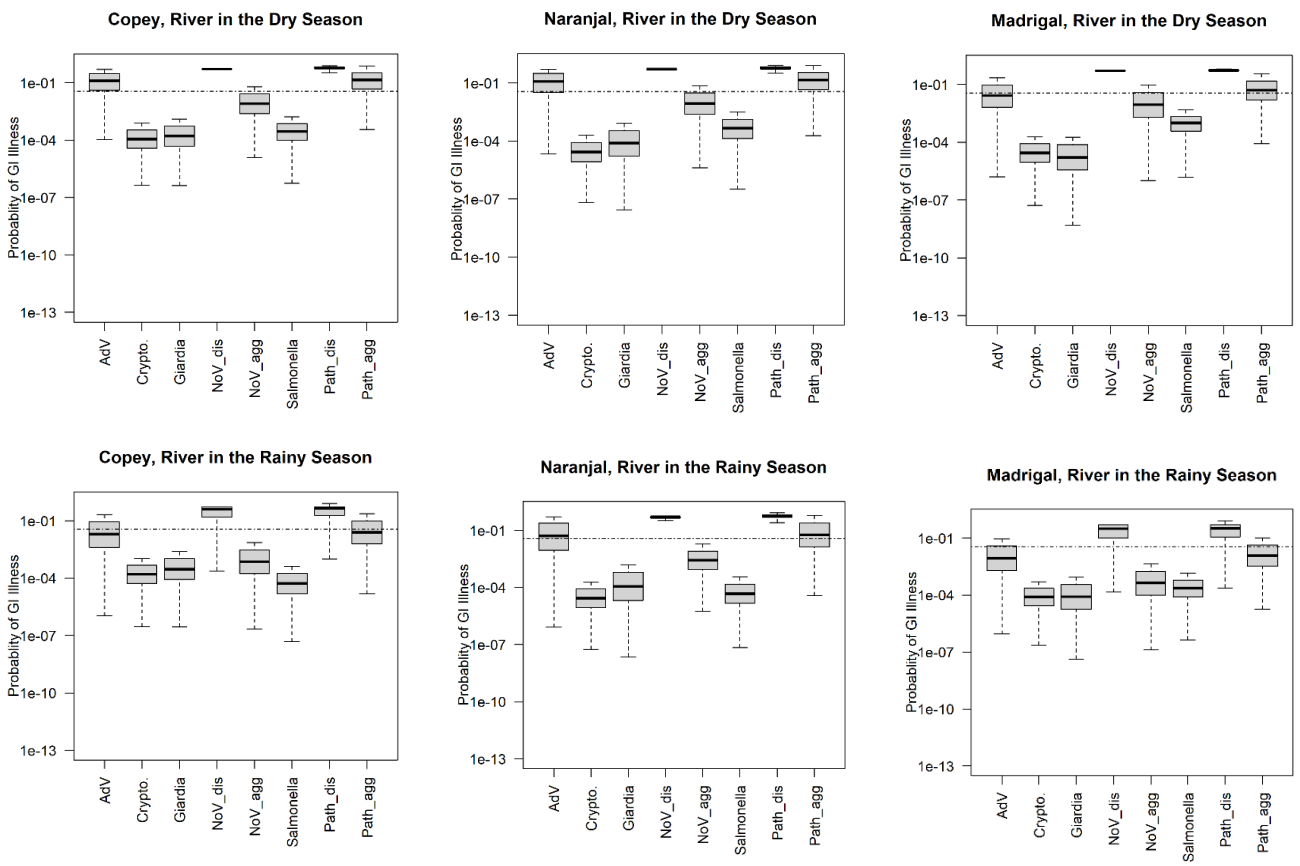

Figure S4: Risk of gastrointestinal (GI) illness for swimmers recreating in the rivers during the dry and rainy seasons by pathogen, as well as cumulatively for all pathogens, in comparison to the US EPA Recreational Water Quality benchmark of 36 GI illness per 1000 swimmers (Recommendation 1, 0.036). Adenovirus (AdV), *Cryptosporidium* (Crypto), norovirus aggregate model (NoV_agg), norovirus dissagregate model (NoV_dis), all pathogens with NoVGI aggregate model (Path_agg), all pathogens with NoVGI dissagregate model (Path_dis). Boxes represent 25th, 50th, 75th quartiles and whiskers represent minimum and maximum

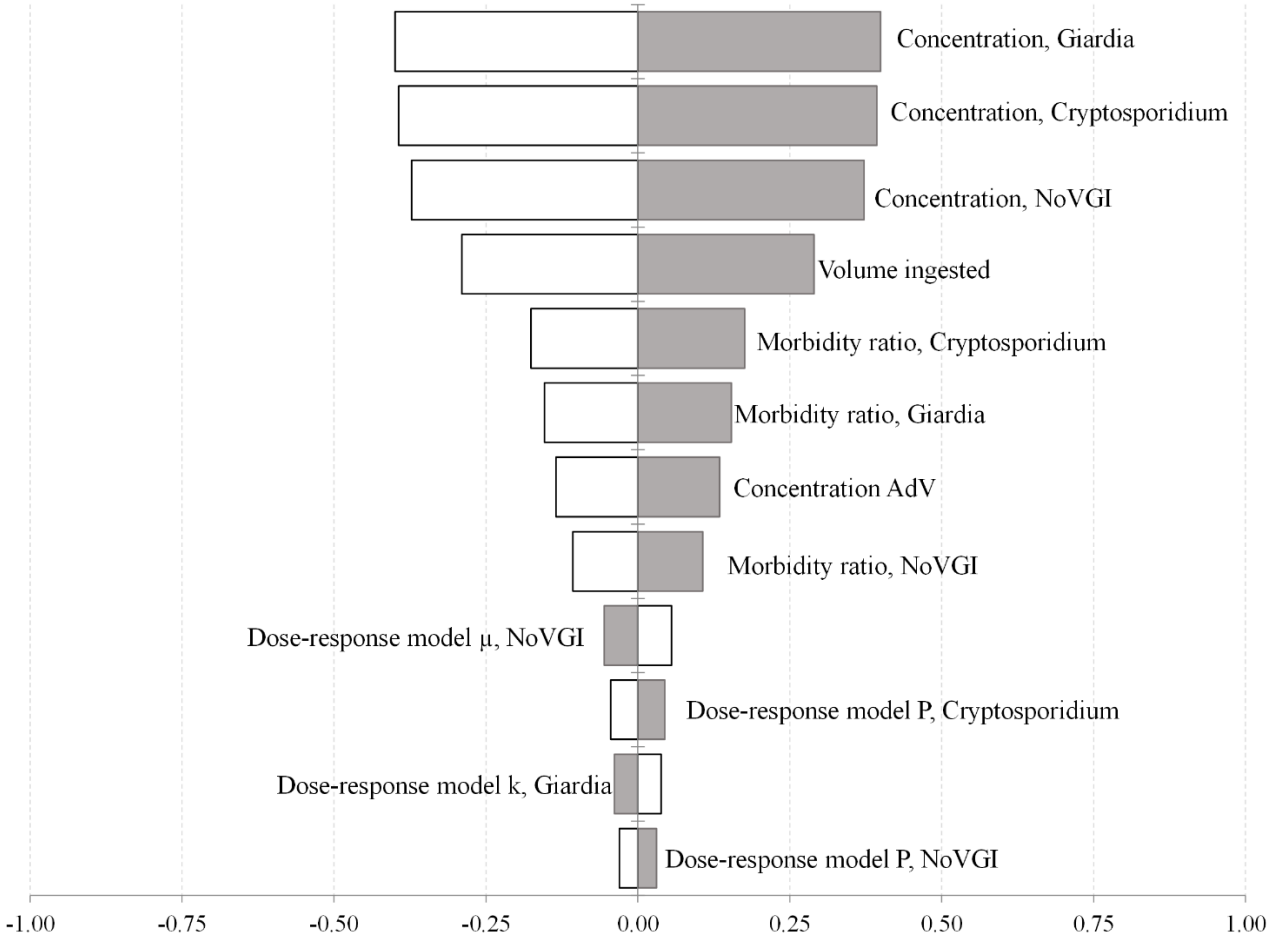

Figure S5: Sensitivity analysis of the quantitative microbial risk assessment (QMRA) for swimmers in the rivers, demonstrating the sensitivity of each model parameter (y axis) on the cumulative pathogen illness based upon Spearman rank order correlations (x axis) depicted in a tornado plot

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Appendix D. Chapter four supplementary materials

Multidrug-resistant E. coli and Enterococcus sp. in Costa Rican wastewater and surface waters.

Supplementary material contains Table S1 through S4.

Table S1: Antibiotics chosen for the Kirby-Bauer disc diffusion assay for *E. coli* as well as their relevance to the study

Antibiotic	Test Group	Class	Disk Content	Relevance	Therapeutic use
Ampicillin	A[a]	Penicillins	10 ug	High resistance in Costa Rica (Tzoc *et al.*, 2004)	Treat infection (i.e., throat, sinuses, lungs, reproductive organs, urinary tract, and gastrointestinal tract
Gentamycin	A	Aminoglycosides	10 ug	Used in Costa Rica (Gobierno del Bicentenario Costa Rica, 2018)	Treat infection (i.e., blood, abdomen (stomach area), lungs, skin, bones, joints, and urinary tract).
Ciprofloxacin	B	Fluoroquinolones	5 ug	Used in Costa Rica (Gobierno del Bicentenario Costa Rica, 2018)	Treat infection (i.e., gonorrhea, typhoid fever, infectious diarrhea and infections of the skin, bone, joint, abdomen, and prostate)
Cefotaxime	B	Cephem	30 ug	Used in Costa Rica (Gobierno del Bicentenario Costa Rica, 2018)	To treat infection (i.e., gonorrhea, meningitis, abdominal, female reproductive organs, skin, blood, bone, joint, and urinary tract infections)
Amoxicillin with clavulanate	B	Beta-Lactamase inhibitor combinations	20/10 ug	Used in Costa Rica (Gobierno del Bicentenario Costa Rica, 2018)	To treat infection (i.e., pneumonia, ear infections, bronchitis, urinary tract infections, and infections of the skin)

Table S1 (continued)

Tetracycline	C	Tetracyclines	30 ug	Used in Costa Rica (Gobierno del Bicentenario Costa Rica, 2018; Gutiérrez *et al.*, 2010).	Treat infection (pneumonia and other respiratory tract infections; skin, eye, lymphatic, intestinal, genital and urinary system infections)
Trimethoprim-sulfamethoxazole	B	Folate pathway inhibitors	1.25/23.75 ug	Used in Costa Rica (Gobierno del Bicentenario Costa Rica, 2018)	Treat infection (i.e., prevent urinary tract infections (UTIs), such as cystitis)

[a] Group A appropriate to include in primary testing.
[b] Group B when the microorganism is resistant to antibiotics in Group A.

Table S2: Antibiotics chosen for the Kirby-Bauer disc diffusion assay for *Enterococcus spp.* as well as their relevance to the study

Antibiotic	**CLSI Test Group**	**Class**	**Disk Content**	**Relevance**	**Therapeutical use**
Ampicillin	A	Penicillins	10 ug	High resistance in Costa Rica (Tzoc *et al.*, 2004)	Treat infection (i.e., throat, sinuses, lungs, reproductive organs, urinary tract, and gastrointestinal tract
Gentamycin	A	Aminoglycosides	10 ug	Used in Costa Rica (Gobierno del Bicentenario Costa Rica, 2018)	Treat infection (i.e., blood, abdomen (stomach area), lungs, skin, bones, joints, and urinary
Ciprofloxacin	U	Quinolones and Fluoroquinolones	5 ug	Used in Costa Rica (Gobierno del Bicentenario Costa Rica, 2018)	Treat infection (i.e., sexually transmitted disease, typhoid fever, infectious diarrhea and infections of the skin, bone, joint, abdomen, and prostate)
Vancomycin	B	Glycopeptides	30 ug	Resistance in Costa Rican (INCIENSA, 2011)	Treat infection in intestines and enterocolitis
Tetracycline	C	Tetracyclines	30 ug	Used in Costa Rica (Gobierno del Bicentenario Costa Rica, 2018)	Treat infection (pneumonia and other respiratory tract infections; skin, eye, lymphatic, intestinal, genital and urinary system infections)

Table S2 (continued)

Linezolid	B	Oxazolidones	30 ug	World-wide use (O'Driscoll & Crank, 2015)	Treat infection (i.e., pneumonia, and infections of the skin)
Erythromycin	O	Macrolides	15 ug	Widely used in clinical medicine (Dinos, 2017)	Treat infection (i.e., respiratory tract infections, skin infections, diphtheria, acute pelvic inflammatory disease, Legionnaire's disease, intestinal amebiasis and syphilis)

[a] Group A appropriate to include in primary testing.
[b] Group B when the microorganism is resistant to antibiotics in Group A
[c] Group C when reporting to infection control as an epidemiological aid.
[o] Group O are not tested routinely in the US
[u] Group U for specific urinary pathogens.

Table S3: Statistical results for testing for differences in log_{10} mean total and ampicillin-resistant *E. coli* and *Enterococcus* concentrations among sampling sites. Significant differences (P-values <0.05) are bolded. P- values associated to Tuckey HSD test are shown in the last column

FIB	Total		Ampicillin-resistant	
	F statistic	P- value	F statistic	P- value
E. coli	23.13	**0.0000**	9.4	**0.0018**
Enterococcus spp.	7.01	**0.0056**	0.5	0.687

Table S4: Significance of pairwise comparisons of log_{10} mean concentrations of *E. coli* and *Enterococcus* by site. Significant differences between sites (P-values <0.05) are bolded

Pairwise comparison	*E. coli*	*Enterococcus* spp
	Ampicillin-resistant	Ampicillin-resistant
Residential-Hospital	**0.047**	NA
Residential-Effluent	**0.0137**	NA
Residential-Estuary	**0.0012**	NA
Hospital-Effluent	0.8923	NA
Hospital-Estuary	0.1961	NA
Effluent-Estuary	0.5051	NA

NA- ANOVA (Table S3) found no significant difference among log_{10} mean concentrations of ampicillin-resistant *Enterococcus* by site, therefore a post-hoc test was not necessary.