Occurrence of fecal and non-fecal sources bacteria during several overlap rainfall events at Fujiazhuang bathing beach in Dalian, China

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Abstract. With an aim to provide guidance for public health swimming in the beach, as well as provide a scientific basis for beach safety management, a continuous 16-day monitoring after the first rainfall and 6-day monitoring after the last rainfall in the swimming season in 2015 was carried out at Fujiazhuang bathing beach in Dalian to evaluate the effects of rainfall on typical fecal sources (enterococci, human specific bacteroides) and non-fecal bacteria (staphylococcus aureus) contamination. The results showed that the concentration of enterococci using culture dependent method and human bacteroides using real-time PCR always fluctuated with six overlap rainfalls, which had a severe impact on fecal bacteria indicators. During sampling period, the water quality exceeded the single sample standard for enterococci (<35 cfu/100 mL) and human specific bacteroides (<8.6 × 10² copies/100 mL) in 100% and 96% of the samples, respectively, higher concentrations were consistently distributed near the drain outlet, indicating that most of the stormwater flowed to the drainage system discharging into the marine. The fecal sources bacteria, enterococci and human specific bacteroides were significantly correlated with each other, both of them have a strong relationship with rainfall (p<0.01), while non-fecal contaminated bacterial concentration showed no correlation with rainfall (p>0.05). Swimmer density is another major factor affecting the concentration of both fecal and non-fecal sources bacteria in seawater. Considering from the public health, it is recommended to reduce the swimming frequency during continuous rainfall period, as well as to increase periodic surveying of highly frequented beaches, especially during periods of peak bather density.

1. Introduction
It has been reported that rainfall can affect the microbial quality of coastal bathing beach seawater [1-3]. Zhang et al. showed that the concentration of fecal indicators can reach to the peak post-rainfall 6 hours [1]. Fan et al. showed that small rainfall events impact the water quality for 24 h, while storm events (>20 mm) may influence water quality for 72 h. If there is an overlap of rainfall events the pollution would be severe, swimming post-rainfall can increase human health risk [2]. Given the importance of rainfall events to the microbial contamination, rainfall should be considered when performing routine monitoring survey [4]. In China, beach environment monitoring program pointed that, when high-intensity rainfall occurs (6 hours of rainfall over 6 mm or over 24 hours of rainfall...
over 25 mm), supplementary monitoring of the microorganism should be carried out until water quality is repaired. However, there is currently no relevant criterion, especially after several rainfalls overlapped.

Previous report has showed that rainfall can impact fecal origin bacteria, but whether affect non-fecal sources pathogenic bacteria is still unclear. World Health Organization (WHO) and U.S. EPA all use enterococci (EC) as health-risk indicator for marine recreational water [5,6]. Human Bacteroides is proposed as alternative fecal indicator to characterize human fecal pollution [7]. However, these organisms may not be capable of accurately assessing the risk of disease from non-enteric pathogens. Some of these infections have been attributed to staphylococcus aureus [8], is considered to be opportunistically pathogenic. Therefore, in this study, traditional fecal bacteria EC, alternative fecal indicator (human specific Bacteroides), non-fecal pathogenic bacteria (staphylococcus aureus) were chosen to assess the impact of rainfall on the water quality.

2. Methodology

2.1. Seawater sampling

Fujiazhuang bathing beach (FBB), a famous and the biggest bathing beach in Dalian China, carries 50,000 people per day in peak season. A small urban sewage treatment plant is located near the beach, which processes 10,000 tons of sewage every day. Samples for microbiological analyses were taken at 3 sites, evenly distributed along the 450 meters beach, which is about 20 m away from the shore (corresponding to knee-depth) (figure 1). Site SW1 located near the drain outlet (DO) of the sewage treatment plant. Site SW2 and SW3 were located on the area of the beach where bathers present is higher. 3-L of surface seawater was collected from a depth of 0.3 m on incoming waves. On July 31 morning, large-scale rainfall happened (48 mm), half an hour later (9:00 am), samples were collected and marked as 0 h post-rainfall, 6 h samples were collected at 3 p.m. Then 1 d, 2 d, until 16 d samples were collected at 9 a.m. every day. There were six overlaps of rainfall during sampling period, and sampling ended on the sixth day of the last rainfall (16 August). A total of 54 samples were collected.

![Figure 1. Schematic diagram of the survey stations at FBB.](image)

2.2. Environmental parameters

Rainfall was recorded, and physical–chemical parameters were analyzed using multi-parameter water analyzer (YSI6600), including seawater temperature, conductivity, salinity, transparency, pH value, oxidation-reduction potential (PRP), turbidimetry, dissolved oxygen, average air temperature, wave height, and so on.

2.3. Biological analysis

2.3.1. Microbial counting. Method for enumeration of EC was referred to ISO 7899-2: 2000 [9]. Briefly, samples were ten-folder gradient diluted, a 100 mL aliquot of diluted seawater were filtered through 0.45 μm pore filter. The membranes were placed on the Slanetz & Bartely agar, incubating for 44 h±4 h at 36°C±2°C. Red, maroon or pink color colonies are considered to be presumptive EC. After incubation, membranes were transferred onto plates of bile-aesculin-azide agar, incubating at
44°C ±0.5°C for 2 hours. Typical colonies showing a tan or black color in the surrounding medium is considered to be EC. Verification of colonies may be required in evidence, referred to verification procedure of ISO 7899-2: 2000. Each sample was done in 3 replicates.  

3M Petrifilm plate was used for enumeration of *staphylococcus aureus* [10]. Briefly, pipette 1 mL each seawater sample with appropriate gradient dilution was aseptically added; then, slowly rolled down the top film, incubating for 24 hours at 36°C ±2°C. Records dark purple colonies as *staphylococcus aureus*. Pink clones were further determined by confirmed piece, until dark purple colonies were observed.

2.3.2. Microbial molecular testing. To quantify human bacteroides, SYBR Green I real-time PCR (qPCR) method was used. A plasmid containing the target gene was constructed to create standard curve. Target fragment was amplified using a pair of primers HF183F: 5'<ATCATGAGTTCACATGTCCG-3' and BacHum241R: 5'-CGTTACCCTGCCTACTATCTAATG-3'. The optimum reaction system performed as previously described with some modifications [11]. PCR started with 94°C for 5 min, followed by 25 cycles at 94°C for 1 min, and 56°C for 45 s, then 72°C extends 7 min. The corresponding amplicon product was cloned into the pMD19-T Vector (TaKaRa). QPCR was performed using ABI 7500 sequence detection system. Cycling parameters involved: pre-denaturation at 95°C for 30 s, 95°C for 5 s, 60°C for 34 s, followed by 40 cycles collecting signals, then denaturation at 95°C for 15 s and annealing/extension step at 60°C for 1 min, 95°C for 15 s, and each reaction was prepared in triplicate.

1 L seawater samples were filtered through 0.45 μm pore filter; two-parallel samples were treated using two filters. DNA extraction from the membranes was carried out according to the manufacturer’s instructions of nucleic acid extraction (MOBIO, Power Soil™ DNA Isolation Kit). It should be noted that the membrane should be properly cut into small pieces before extraction. Then, qPCR analysis was implemented as above.

2.4. Statistical analysis

Statistical analysis was performed using SPSS 19.0. Microbial concentrations, physical-chemical parameter values and rainfall were utilized for calculation of Pearson correlation coefficients (p). “p” values less than 0.05 were considered to represent significant differences. Statistical analyses were conducted based on the Alpha 95% credible intervals for regression parameters.

3. Results

3.1. Influence of rainfall on the concentration of enterococci

The distribution of EC during sampling period is shown in figure 2. The concentration of EC ranged from 43 CFU/100 mL to 1173 CFU/100 mL. All samples exceeded the single sample marine water quality standard, geometric mean criterion value of 30 or 35 CFU/100 mL for EC based on U.S. EPA directive standard [6]. The concentration of EC fluctuated with rainfall, although it reduced quickly, even happened within 6 h, the concentration still exceeded the single sample marine water quality standard. The highest EC concentration distributed is near the area of drain outlet. Among the 18 batch samples collected, except the thirteenth and fourteenth sampling events, 16 batches of samples showed that the concentration of EC on site SW1 was higher than that of site SW3.
3.2. Influence of rainfall on the concentration of *staphylococcus aureus*

From 31 July to 16 August, 2015, the distribution of *staphylococcus aureus* in Fujiazhuang bathing beach showed an upward trend (figure 3), ranged from 23 CFU/mL to 2389 CFU/mL. Concentration dynamic of *staphylococcus aureus* did not change with the rain. A 100-fold difference was occurred between the highest and lowest concentrations. High concentrations mainly distributed from 11 d to 13 d, which happened after an overlap of six rainfalls.

3.3. Influence of rainfall on the contamination of human specific bacteroides

Human bacteroides was detected using qPCR method. Seven serial dilutions of plasmid DNA were made, 1 μL aliquots of each dilution were used as templates. Between \(2.63 \times 10^7\) copies/μL and
2.63×10^2 copies/μL of genome equivalent, a good correlation was found between Ct values and copy numbers that were logarithmically transformed. The linear regression equation calculated by ABI 7500 software was y = -3.45x + 41.216, the correlation coefficient R^2 = 0.997, and the amplification efficiency E = 94.94%. The dissolution curve is a single peak, which indicated that the specificity of real-time PCR is good.

![Graph showing the relationship between human bacteroides and rainfall in seawater of FBB.](image)

**Figure 4.** Relationship between human *bacteroides* and rainfall in seawater of FBB.

The results showed that the detection rate of human *bacteroides* by qPCR method was 100% in Fujiazhuang bathing beach, and the concentration ranged from 8.66 × 10^1 copies/100 mL to 2.07 × 10^6 copies/100 mL (figure 4). At least 96% of the samples exceeded Water Quality Standards for Coastal Recreation Waters of 8.6 × 10^2 copies/100 mL, which posed heavy risk to public health [12]. The concentration of human *bacteroides* showed an increasing trend after rain, and the pollution was also severe near the area of outlet, which were the same as enterococci. Of the 18 batch samples collected, 14 batches of samples showed that the concentration of human *bacteroides* on sampling site SW1 was higher than that of SW3.

3.4. Correlation analysis
The bacterial concentration and physical–chemical parameter values, rainfall was analyzed by SPSS. Results showed that EC was significantly correlated with human *bacteroides* (r^2=0.526, p=0), both of these two indicators showed a significant relationship with rainfall (r^2=0.384, p=0.004; r^2=0.433, p=0.01), but *staphylococcus aureus* was poorly correlated with rainfall (r^2=-0.076, p=0.587). *Staphylococcus aureus* was correlated with transparency (r^2=0.382, p=0.004) and wave height (r^2=0.434, p=0.001), but other bacterial indicators showed a poorly relationship with physical–chemical parameter (r^2=-0.007–0.230, p= 0.62–1). Besides, correlation analysis also revealed a particularly strong effect of rainfall on pH value (r^2=0.306, p=0.024), oxidation-reduction potential (r^2=0.293, p=0.032), and dissolved oxygen (r^2=-0.352, p=0.009).

4. Discussions
The purpose of this study was to elucidate the influence of rainfall on fecal and non-fecal contamination of bacteria. The results showed that rainfall can significant influence the concentration
of fecal bacteria indicators, but not on the non-fecal bacteria (*staphylococcus aureus*). Until now, there were no reports about the impact of rainfall on non-fecal bacteria, one report about the impact of rainfall on the abundance dynamic of *vibrio spp.*, marine indigenous bacteria, which showed that vibrio cells preferentially colonized during the period of excess rainfall [3]. The result is different from ours, mainly because vibrio maybe not typical non-fecal source bacteria, one of their ways into water is through human fecal contamination. Another reason maybe because their different mechanism, heavy rainfall causes an abrupt decrease in salinity and major flooding notably favored growth of *vibrio spp.*, was linked to their rapid proliferation [13]. While *staphylococcus aureus* is frequently found in the nose, respiratory track and on the skin, and proposed as an indicator for non-enteric diseases [8]. Our results suggested that it may not be necessary to monitor the contamination of typical non-fecal bacteria after rainfalls.

Post-rainfall was defined as the period between 0 and 72 h after a rainfall event of more than 20 mm. During the first 10 consecutive days, there was six overlap rainfalls, marked as post-rainfall days. The average concentration of enterococci post-rainfall was 1.22 times higher than that of the sunny days in 2014 [14]. Besides, analyzing the concentration dynamic of human specific *bacteroides* also showed that rainfall brought more sewage through runoff. Our results further confirmed that if there is the overlap of rainfall events, the concentration of bacteria would increase [2]. Numerous studies have demonstrated a relationship between EC, human specific *bacteroides* and swimming-related illnesses in marine beach using qPCR methods [7]. Therefore, the overlap of rainfall event can increase human health risk induced by fecal sources pathogens.

Except rainfall, swimmers as the pathogens carrier are another important potential non-point source in recreational seawaters [15]. Studies have found that swimmers shed microbes via their excrement, urine, skins into the water column, and swimming related illnesses appear to be associated with the microbial water quality. When swimmers exposed to seawater for 15 min, they can shed $6 \times 10^5$ CFU enterococci, $6 \times 10^6$ CFU of *staphylococcus aureus* into seawater [16]. Previous study also found significant correlation between concentration of EC and bather density [14]. This phenomenon is much obvious in the distribution of *staphylococcus aureus*. During the last few monitoring days, namely sunny days, there was a continuous peak of *staphylococcus aureus* with high density of bathers we observed. Therefore, it is essential to monitor fecal source indicator post rainfall other than non-fecal bacteria, as well as monitor the recreational bathing water during the number of bathers is high, which can provide information for the public or administrative department to take effective measures to avoid pathogen risks.

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**References**
[1] Zhang W W, Wang J Y, Fan J F, *et al* 2013 Effects of rainfall on microbial, water quality on Qingdao No. 1 bathing beach, China *Mar. Pollut. Bull.* 66 185-90
[2] Fan J, Ming H, Li L, *et al* 2015 Evaluating spatial-temporal variations and correlation between fecal indicator bacteria (FIB) in marine bathing beaches *Journal of Water & Health* 13 1029
[3] Böer S I, Heinemeyer E A, Luden K, *et al* 2013 Temporal and spatial distribution patterns of potentially pathogenic vibrio, spp. at recreational beaches of the German north sea *Microbial Ecology* 65 1052-67
[4] Hsu B M and Huang Y L 2008 Intensive water quality monitoring in a Taiwan bathing beach *Environ. Monit. Assess.* 144 463-8
[5] WHO 2003 Guidelines for safe recreational water environments *Coastal and Fresh Waters* 1
[6] U.S. EPA. Environmental Protection Agency 2012 Recreational Water Quality Criteria EPA -820-F-12-061. U.S. EPA, Washington, DC
[7] Staley C, Gordon K V, Schoen M E, *et al* 2012 Performance of two quantitative PCR methods
for microbial source tracking of human sewage and implications for microbial risk assessment in recreational waters *Applied & Environmental Microbiology* **78** 7317-26

[8] Enns A A, Vogel L J, Abdelzaher A M, *et al* 2012 Spatial and temporal variation in indicator microbe sampling is influential in beach management decisions *Water Research** **46** 2237

[9] ISO. International Organization for Standardization 2000 Membrane filtration method *Water Quality Detection and Enumeration of Intestinal Enterococci—Part 2* pp 7899-2 (Geneva, Switzerland)

[10] Ingham S C, Losinski J A, Dropp B K, *et al* 2004 Evaluation of *Staphylococcus aureus* growth potential in ham during a slow-cooking process: use of predictions derived from the U.S. Department of Agriculture Pathogen Modeling Program 6.1 predictive model and an inoculation study *J Food Prot* **67** 1512-6

[11] Sauer E P, Vandewalle J L, Bootsma M J, *et al* 2011 Detection of the human specific Bacteroides genetic marker provides evidence of widespread sewage contamination of storm water in the urban environment *Water Research* **45** 4081-91

[12] Soller J A, Schoen M E, Bartrand T, *et al* 2010 Estimated human health risks from exposure to recreational waters impacted by human and non-human sources of faecal contamination *Water Research* **44** 4674-91

[13] Esteves K, Hervio-Heath D, Mosser T, *et al* 2015 Rapid proliferation of *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio cholerae* during freshwater flash floods in French Mediterranean coastal lagoons *Applied & Environmental Microbiology* **81** 7600-9

[14] Shi Y, Ming H X, Ma YJ, *et al* 2016 Evaluating human health risk of Dalian marine bathing beaches by Enterococcus *Chin J Appl Environ Biol* **22** 1161-6

[15] Ashbolt N J, Schoen M E, Soller J A, *et al* 2010 Predicting pathogen risks to aid beach management: the real value of quantitative microbial risk assessment (QMRA) *Water Research* **44** 4692-703

[16] Yahamara K M, Sassoubre L M, Goodwin K D, *et al* 2012 Occurrence and persistence of bacterial pathogens and indicator organisms in beach sand along the California coast *Appl Environ Microbiol*. **78** 1733-45