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* (<8.6×10^2 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 frequence 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.

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℃±2℃. Red, maroon or pink color colonies are considered to be presumptive EC. After incubation, membranes were transferred onto plates of bile-aessculin-azide agar, incubating at

44℃±0.5℃ 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℃±2℃. 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'-CGTTACCCCGCCTACTATCTAATG-3'. The optimum reaction system performed as previously described with some modifications [11]. PCR started with 94℃ for 5 min, followed by 25 cycles at 94℃ for 1 min, and 56℃ for 45 s, 72℃ for 1 min. Then 72℃ extends 7 min. The corresponding amplicon product was cloned into the pMD19-T Vector (TaKaRa). QPCR was performed using a ABI 7500 sequence detection system. Cycling parameters involved: pre-denaturation at 95℃ 30 s, 95℃ 5 s, 60℃ 34 s, followed by 40 cycles collecting signals, then denaturation at 95℃ for 15 s and annealing/extension step at 60℃ for 1 min, 95℃ 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.

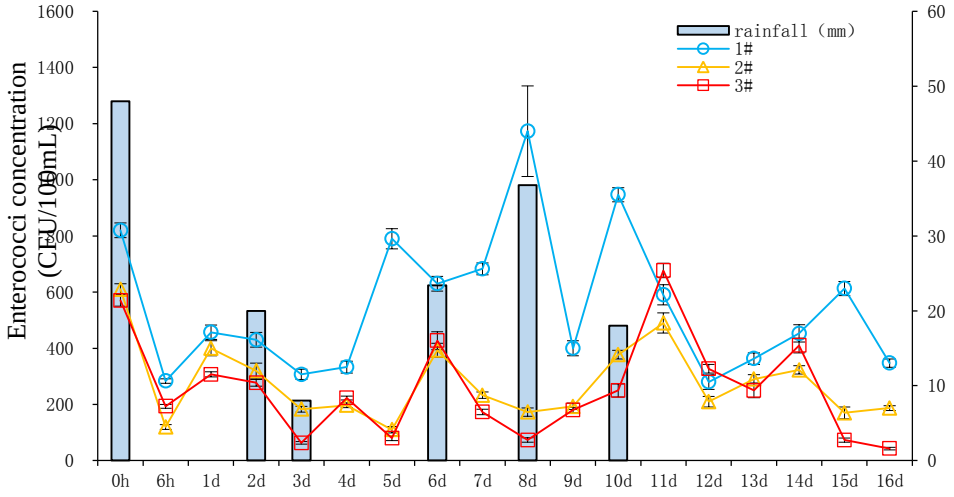

Figure 2. Relationship between enterococcus and rainfall in seawater of FBB.

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.

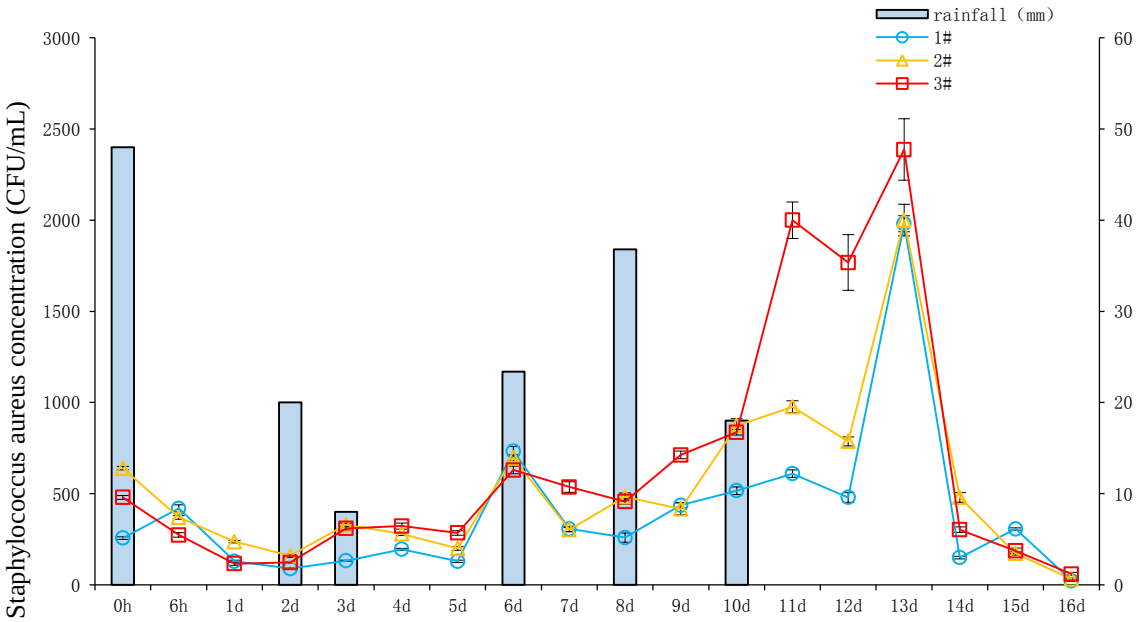

Figure 3. Relationship between *staphylococcus aureus* and rainfall in seawater of FBB.

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 2.63×10^{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.

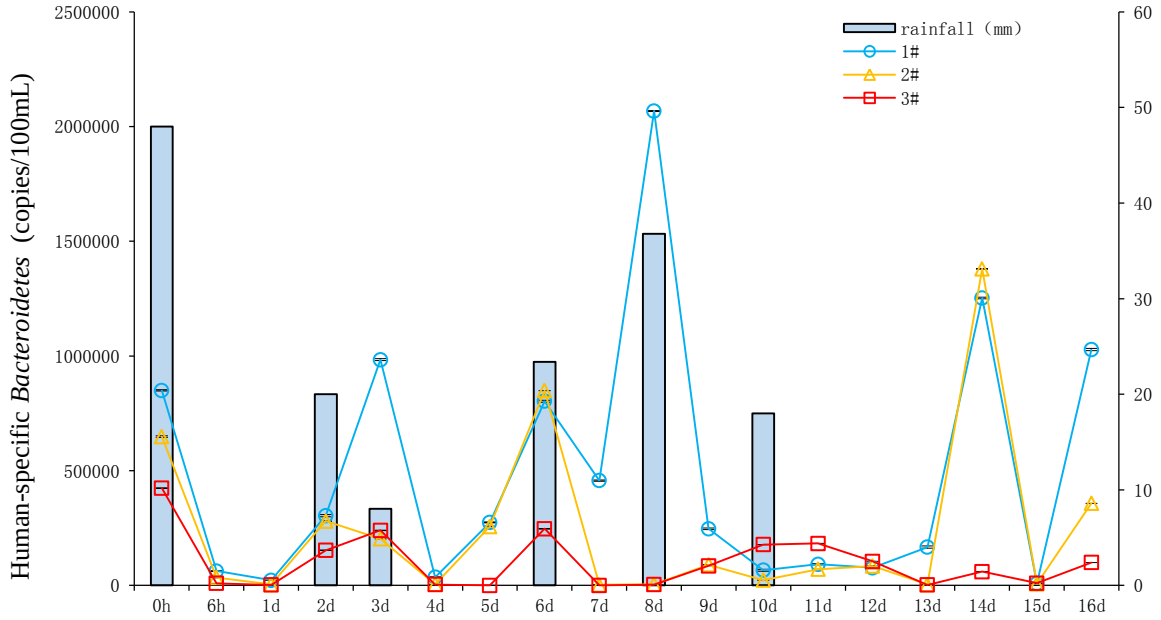

Figure 4. Relationship between human *bacteroides* and rainfall in seawater of FBB.

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. Correlationship 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 6×10^5 CFU enterococci, 6×10^6 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.

Acknowledgments
This work was supported by the National Key R & D Program of China (Grant 2017 YFC1404500), and Natural science foundation of China (No. 41406129).

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