Distribution and Diversity of Coliform Bacteria in Estuary of Jiahe River, China

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ABSTRACT: Waterborne outbreaks of pathogenic bacteria from contaminated water are serious threats for public health. Coliform bacteria have been regarded as one of the most important indicators for monitoring pathogenic bacteria. To address potential pathogenic bacterial outbreaks, the distribution and diversity of coliform bacteria in Jiahe river, which flows through densely-populated urban area in China, were detected. *Escherichia Coli* and other coliform bacteria were counted using the membrane filter technique to describe the distribution of the coliform bacteria. Phylogenetic analysis was applied to investigate the diversity of the coliform bacteria. The results suggested that the quantities of coliform bacteria varied greatly between five sampling sites with the highest value at site YT4 and the lowest value at site YT2. Highest concentrations of *E. coli* and other coliform bacteria were also observed at YT4, while the lowest value was detected at sites YT3 and YT2, respectively. Various coliform bacteria were classified by phylogenetic analysis, including *Citrobacter*, *Klebsiella*, *Enterobacter*, and *Raoultella*. Components of coliform bacteria affiliated into these four genera were various in all sampling sites. The statistical analysis suggested that the distribution of coliform bacteria were remarkably influenced by total bacteria amount. Multiple environmental parameters were proved to affect the diversity of coliform bacteria. The results of this study revealed the correlation between coliform bacteria and the environmental parameters, which is important for predicting and preventing waterborne transmission of pathogenic bacteria.

Key words: Pathogenic indicator, Coliform, *Escherichia Coli*, Phylogenetic diversity

INTRODUCTION

Concerning about public health risks, coliform pathogens from fecal contamination have received public and scientific attentions for more than a century. Fecal pathogens widely spread in different environment, not only living with their own host, but also in non-host habits, including freshwater (Barcina *et al.,* 1992), sediments/soils (Anderson *et al.,* 2005; Stenstroem and Carlander 2001), and even epilithic periphyton (Ksoll *et al.,* 2007). Non-host habits could be important intermediaries for pathogens by harboring and enhancing the survival of pathogenic bacteria released into the environment (Ksoll *et al.,* 2007; Byappanahalli *et al.,* 2003). Among these habits, aquatic environment including both freshwater and marine water has been proved to be important sources of pathogenic bacteria outbreaks. This is induced by i, dependence of human beings on water (drinking, fishery, irrigation, entertainment, *et al.*); ii, arbitrary discharge of wastewater, especially sewage disposal with fecal material contaminated by human and warm-blood animals; iii, runoff after rain and hurricane (Nigro *et al.,*

2011), and direct contamination by wild animals (Haley *et al.,* 2009). Waterborne outbreaks caused by *Salmonella* (Haley *et al.,* 2009), *Campylobacter* (Hänninen *et al.,* 2003; Bopp *et al.,* 2003), *Vibrio* (Nigro *et al.,* 2011), *Escherichia* (Bopp *et al.,* 2003) and other coliform bacteria (Eckner 1998) have been well documented all over the world. In August 1999, 775 people were suspected to be infected by the outbreak of waterborne *E. coli* O157:H7. Hospitalized children in that case even showed worsening kidney function (Bopp *et al.,* 2003).

Determination of pathogenic indicator organisms has been well-acknowledged for monitoring the microbiological safety of aquatic environment. The coliform group especially *E. coli* has been widely used as an important indicator of fecal pollution worldwide. In China, total coliforms and *E. coli* are essential inspection items of the Environmental Quality Standard for Surface Water (Ministry of Environmental Protection 2002) and the Environmental Quality Standard for Drinking Water (Ministry of

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Environmental Protection 2006). The U. S. Environmental Protection Agency (EPA) also deems total coliforms as standard indicators of pollution for drinking water (U.S.EPA, 2001), while regards *E. coli* as a standard indicator of pollution for freshwater (U.S. EPA, 2003).Various methods have been developed to detect the distribution of coliforms in aquatic environment (Rompré et al. 2002). Membrane filter (MF) technique, a culture-dependent method, is the most widely used method in all of these techniques. It is based on enzymatic hydrolysis of fluorogenic or chromogenic substrates for β-d-galactosidase and β-d-glucuronidase, two markers for coliforms and *E. coli*, respectively (Tryland and Fiksdal 1998).

The Jiahe river flows through Yantai Economic Development Zone surrounded by dense population and various industries which conduces the river to high risk of pathogenic contamination originated from fecal pollution. The objectives of this study were to: 1, investigate the distribution and diversity of coliform bacteria along the Jiahe river; 2, reveal the impacts of environmental factors on distribution and diversity of coliform bacteria.

MATERIAL & METHODS

Water samples were collected from four sampling sites along the Jiahe river, surrounded by the Economic Developing Zone with dense population and various industries. Another sampling site was located at the entrance of Jiahe river, with limited human activities. Water samples were collected from the surface (ca. 5 to 10 cm depth) at each site using autoclaved, five-litter, polycarbonate barrels. Aliquots (9 ml) of water samples were fixed with formalin (final concentration, 5%) immediately after sampling for the total bacterial count. All the samples were collected between 10:00 am and 11:00 am and transported to the lab in two hours in coolers with insulation and ice packs. Further analysis were immediately processed and finished in three hours.

In situ measurements of temperature, salinity, conductivity, oxidation-reduction potential (ORP) and dissolved oxygen concentration (DO) were conducted by using YSI 556 Multiparameter Handheld Water Quality Meter (YSI Environmental, Yellow Springs, OH). Total carbon (TC), total inorganic carbon (TIC), total organic carbon (TOC) and total nitrogen (TN) were measured by Analysis and Test centering of Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences using TOC-VCPH Total Organic Carbon Analyzer (Shimadu, Japan).

Total bacterial amount (TBA) was counted by fluorescence microscope after DAPI staining. Briefly, samples were serially diluted 10-fold or 100-fold in 0.9%

salt solution and stained with DAPI (final concentration, 5μg/ml) in the dark for 30 min. Stained samples were then filtered through 0.22 um pore-size membrane (Whatman, Stanford, ME, USA). The filters were mounted on microscope slides and imaged using a Leica DM 5000 fluorescence microscope (Wetzlar, Germany).Ten fields were randomly chosen on each filter section to get the mean abundances of total bacteria.

Enrichment for coliform bacteria and visible counting were performed by the membrane filtration technique. Briefly, aliquots (100 ml, 10 ml and 5 ml) of each water sample were filtered through 0.45 um pore-size GN-6 filters (Pall corporation, Port Washington, NY) in triplicate. All the filters were then placed onto CHROMagar™ ECC medium (CHROMagar Microbiology, Paris) and incubated at 37°C for 24 hours. CHROMagar™ ECC is a selective medium for the simultaneous detection and enumeration of *E. coli* and other coliforms. *E. coli* could form blue colonies on the medium while other coliform bacterial colonies appear red. Colonies of different colors were recorded and inoculated in LB broth (BD Bioscience, Franklin Lakes, NJ) and incubated overnight with shaking at 37°C. One milliliter liquid culture of each colony was cryopreserved at -80°C in LB broth supplemented with 30% glycerol, while 1.8 ml of the culture was used for further analysis.

One hundred and thirty isolates including 50 blue colonies and 80 red colonies were selected for sequencing of the 16S rRNA gene in order to confirm identifications based on the chromogenic media. Genomic DNA was extracted from liquid cultures (mentioned above) using an Ultra-Clean microbial DNA isolation kit (MoBio Laboratories, Carlsbad, CA).The 16S rRNA gene from each isolate was PCR amplified using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TGACTGACTGAGGYTACCTTGTTACGACTT-3') under the following conditions: 1 × PCR Buffer, 0.2 mM of each dNTP, 0.2 mM of each primer and 1 U Taq polymerase (Invitrogen Life Technologies, Carlsbad, CA). An initial denaturing period of 5 min at 94°C was followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension (72°C) for 10 min (Wang et al., 2009). 16S rRNA gene sequences from isolates were analyzed using the BLASTn tool at the National Center for Biotechnology Information website. Isolates were presumptively identified according to the identity of the closest cultured relative in the top BLAST hits.

16S rRNA gene sequences of all the 130 isolates and their closest reference sequences were applied for phylogenetic analysis using the Mega 4 software

package (Tamura *et al.,* 2007), and sequences were aligned using the positional tree server with a data set containing the nearest relative matches. Trees were constructed using the neighbor-joining (Jukes-Cantor correction) algorithms. The robustness of the inferred tree topologies was evaluated after 1,000 bootstrap replicates of the neighbor-joining data.

The 16S rRNA gene sequences from isolates newly determined in this work have been deposited in GenBank under accession numbers KF418610-KF418632.

To detect the correlation between environmental parameters and coliform bacteria, statistical software R (R studio, Boston, Massachusetts, USA) was applied to calculate the correlation coefficients. All the environmental parameters were firstly evaluated by "cor" to compare the correlation and selected representative parameters. RDA analysis was then used for identifying representative parameters that could relate to the diversity and distribution of *E. coli* and other coliform bacteria.

RESULTS & DISCUSSION
Temperatures at all five sampling sites ranged from 26.2°C to 27.55°C with the highest temperature of 27.55°C at YT1 (Table 1). Little variety of pH was detected in all the five samples which ranged from 7.86 to 8.92. Salinity, conductivity, TC, TIC and TOC showed the same variation trend. Low salinity was observed at sites YT1, YT2, YT3, YT4 ranged from 0.24‰ to 0.31‰, while dramatic change was found at the sampling site YT5 with a salinity of 7.1‰. Conductivity at YT1, YT2, YT3, and YT4 ranged from 0.484 µS/cm to 0.657 µS/cm, while a higher rate (12.68 µS/cm) was detected at YT5, which is consistent with the salinity distribution. Parameters of TC, TIC, and TOC at sites YT1, YT2, YT3, YT4 also showed slight changes, which ranged from 23.76, 19.82 and 3.66 to 28.13, 23.89 and 4.50 mg/L, respectively. Higher values of TC, TIC, and TOC were also detected at site YT5 (32.59, 25.30 and 7.29 mg/L, respectively). The results suggested opposite trend of TN. YT5 showed the lowest value in all five sampling sites, which was 3.75 mg/L, while the highest values were detected at site YT1 (12.25 mg/L). TN ranged from 3.75 mg/L to 6.25 mg/L for the other four sites. The highest ORP was detected at site YT2 (158.4 mV), while values in other four sampling sites ranged from 117.5 mV to 151.4 mV. DO ranged from 6.37 to 8.31 mg/L at sampling sites YT2, YT3, YT4, and YT5, while site YT1 showed the highest DO value of 13.71 mg/L.

This is the first district study of fecal indicators in water samples of Yantai area, which could provide important information about the water quality. Concentrations of total bacteria at five sampling sites

ranged from 1.29×10^8/ml to 3.35×10^9/ml. Bacteria amounts at sites YT2 and YT3 reached up to 10^9/ml, while values at other three sampling sites were tenfolder lower (Table 1). Total CFU of coliform bacteria including *E. coli* (blue) and other coliform bacteria (red) varied greatly among five sampling sites, which ranged from 2.08×10^4 CFU/L to 2.49×10^5 CFU/L (Fig. 1). The highest value of total coliform bacteria was detected at site YT4, while the lowest value was measured at site YT2. Concentration of other coliform bacteria (exclude of *E. coli*) was consistent with the total CFU of coliform. Site YT4 also showed the highest value of 1.36×10^4 CFU/L, while site YT2 had the lowest value of 6.4×10^3 CFU/L. Site YT4 was at the downstream of Jiahe river, which was more densely populated and easily polluted by sewages. Amounts of total coliform bacteria, *E. coli* and other coliform bacteria all showed the highest value at the site YT4. This supported previous conclusions that reservoirs surrounded by densely-populated urban areas are more likely contaminated by fecal bacteria (Mehaffey et al. 2005; Hong et al. 2010). The high concentration of coliform bacteria raises the risk of infection, when people are exposed in the contaminated water. The concentration of coliform bacteria was not consistent with that of the total bacteria. All the tested sites were impacted by human being's activities.

For the concentration of *E. coli*, the highest value was consistently seen at YT4 (1.12×10^5 CFU/L), while the lowest value was detected at site YT3 (7.9×10^3 CFU/L), but not site YT2. The relative proportion of *E. coli* and other coliform bacteria at sites YT1, YT4, and YT5 remained equitable. 35%, 45% and 30% of total CFU at theses three sites were classified as *E. coli*. Sites YT2 and YT3 showed dramatic differences from the other three sampling sites. CFU of *E. coli* counted up to 69% of total coliform at site YT2. In contrast, only 19% of CFU was classed as *E. coli*, while CFU of other coliform bacteria could count up to 91% at site YT3.

Based on the sequencing results, 46 among 47 tested blue colonies selected on CHROMagar™ ECC medium (excluding three of the total 50 tested colonies got incorrect sequences) were identical to the cultured *E. coli* strain RRL36 (JQ398845.1). The rate of false negative identifications was as low as 2%. The CHROMagar™ ECC medium has been widely used in previous studies and successfully evaluated the concentrations of *E. coli* in food and water samples (Jong et al. 2010; Kim et al. 2009; Selma et al. 2008). The results here also showed low false identification rate which proved that it is reliable to distinguish *E. coli* from other coliform bacteria using CHROMagar™ ECC medium. 16S rRNA gene sequencing of 80 randomly selected red colonies generated 72 available sequences,

Table 1. Physicochemical parameters and total bacterial amounts in five sampling sites. ORP, oxidation-reduction potential; DO, dissolved oxygen concentration; TC, total carbon; TIC, total inorganic carbon; TOC, total organic carbon; TN, total nitrogen; TBA, total bacteria amount

| Sampling site | Latitude /Longitude | *In situ* tested parameters | | | | | | TN (mg/L) | TC (mg/L) | TIC (mg/L) | TOC (mg/L) | TBA (10^8/ml) |
		Temperature (°C)	Salinity (‰)	pH	conductivity (µS/cm)	OPR (mV)	DO (mg/L)					
YT1	N: 37°30.698 E: 121°16.650	27.55	0.31	8.92	0.657	117.5	13.71	12.25	23.76	19.82	3.94	1.99
YT2	N: 37°30.674 E: 121°17.075	26.2	0.24	7.86	0.492	158.4	7.08	6.00	25.66	21.16	4.50	33.5
YT3	N: 37°31.607 E: 121°17.263	26.91	0.23	8.22	0.484	142.7	8.31	6.25	24.63	20.97	3.66	25.8
YT4	N: 37°32.112 E: 121°16.933	26.95	0.29	7.99	0.610	127	7.41	4.75	28.13	23.89	4.24	1.72
YT5	N: 37°33.476 E: 121°17.055	26.8	7.1	8.02	12.68	151.4	6.37	3.75	32.59	25.30	7.29	1.29

while Chimeric sequences were identified by using the CHECK_CHIMERA program of the Ribosomal Database Project (Cole *et al.,* 2003). Sequences of coliform bacteria excluding *E. coli* were applied for further phylogenetic analysis.

A total of 72 sequences from all five sampling sites and a reference *Pseudomonas aeruginosa* sequence (NCBI accession no. X06684.1) were used to construct a phylogenetic tree to study the distribution of coliform bacteria along the Jiahe river (Fig. 2). The 72 sequences consist of 16 sequences from site YT1, 16 sequences from site YT2, 12 sequences from site YT3, 13 sequences from site YT4, and 15 sequences from site YT5.

Since there is no taxonomically definition for coliform bacteria, different species from genus *Citrobacter*, *Klebsiella*, *Enterobacter*, *Hafnia*, *Serratia*, *et al.* were classified as coliform bacteria including both fecal originated and non-fecal originated (Alonso et al. 1999). All the 72 sequences measured in this study fell into eight bacteria species in four different genera (*Citrobacter*, *Klebsiella*, *Enterobacter*, *Raoultella*) of the order Enterobacteriales and Gammaproteobacteria. Members of the *Enterobacter* dominated the bacterial group in four sampling sites including YT1, YT2, and YT3. Fifty-eight percent of sequenced isolates from site YT3 affiliated into *Enterobacter* and showed high similarity (99%) to previously cultured bacteria *Enterobacter hormaechei* strain M. D. NA2-2 and *Enterobacter coli* strain KNUC5006. Sequenced isolates from both sites YT1 and YT2 affiliated into *Enterobacter* counted up

to 50%. Fifty-four percent of sequences from site YT4 showed highest similarity with two cultured species of the genus *Klebsiella*, while sequences affiliated into *Enterobacter* only counted for 15% of the total sequenced isolates. Sequenced isolates from site YT5 mainly affiliated into the genera *Citrobacter* and *Enterobacter* with with the values of 41% and 30%, respectively. Genus *Klebsiella* was identified in all the sites, which is classified as human being's opportunistic pathogen.

In this study, the value of 90% was applied as the criterion to evaluate the correlation rate between two environmental parameters (Fig. 3). Four different parameters were selected as the representative parameters, including temperature (tightly correlated with ORP), salinity (tightly correlated with conductivity, TC, TIC and TOC), DO (tightly correlated with pH and TN) and TBA. The statistical analysis suggested that the distribution of *E. coli* and other coliform bacteria at the five sampling sites were mainly influenced by temperature, ORP and TBA. The amount of *E. coli* and other coliform bacteria showed positive correlations with temperature, ORP, while showed obvious negative correlation with total bacteria amount. All the other environmental parameters showed unremarkable correlation with distribution of coliform bacteria.

All the components of other coliform bacteria including *Citrobacter*, *Enterobacter*, *Klebsiella* and *Raoultela* were applied to detect their correlation with environmental parameters. The results indicated that *Citrobacter* and *Raoultela* were negatively correlated with total bacteria amount, while positively correlated
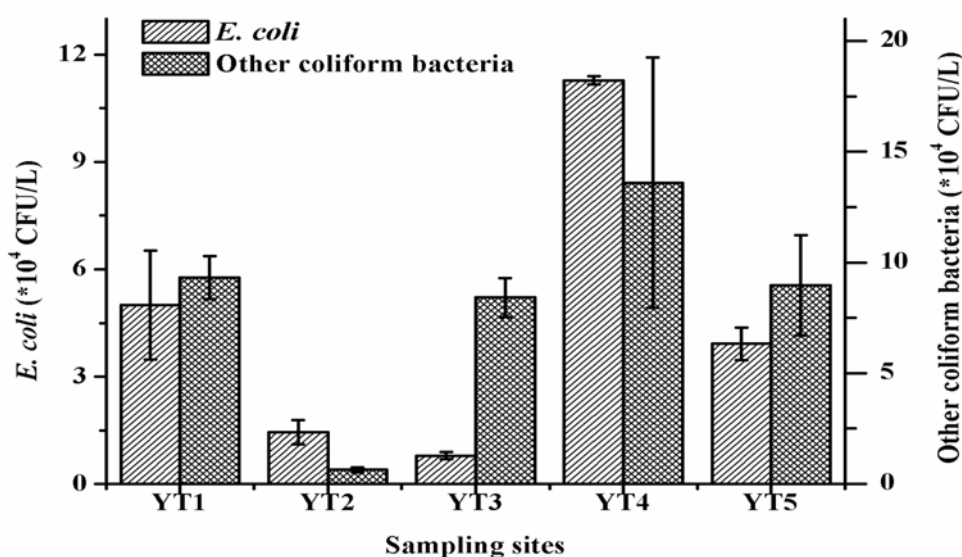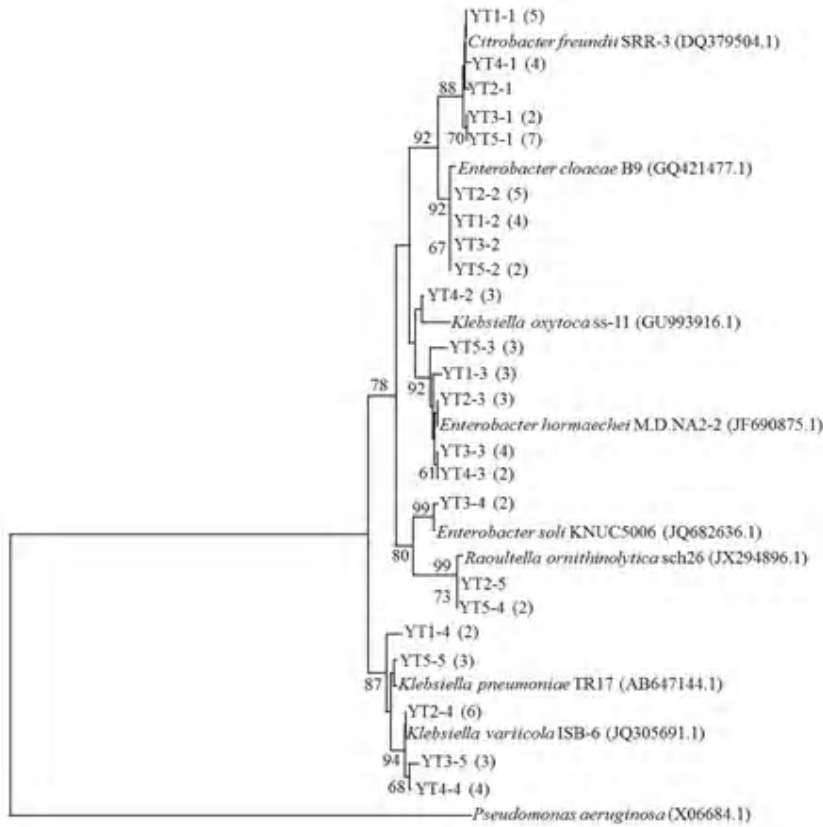

Fig. 1. Total concentrations of *E. coli* and other coliform bacteria at each sampling sites

Fig. 2. Rooted neighbor-joining tree of partial 16S rRNA gene (>1000 bp) sequences from each sampling sites and their reference strains. The tree was constructed using Mega 5 software. *Pseudomonas aeruginosa* (NCBI accession no. X06684.1) was used as the out-group. Bootstrap confidence values >60% are shown at the nodes. The scale bar indicates 0.02 substitutions per nucleotide position. Reference sequences are shown with GenBank accession numbers listed after each sequence name
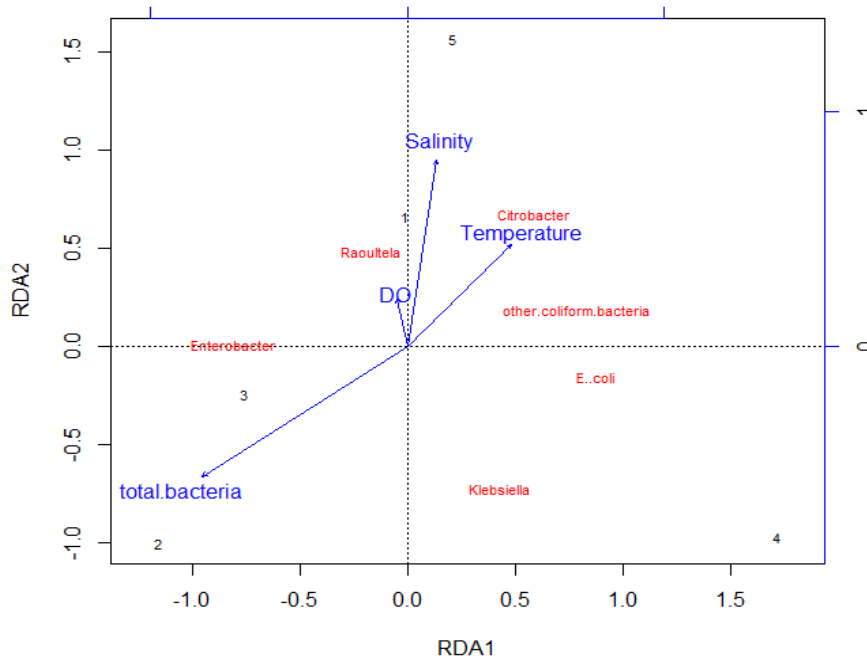
Fig. 3. Statistical analysis of the correlation between environmental parameters and coliform bacteria

with all the other environmental parameters. Interestingly, *Klebsiella* showed completely opposite correlation. *Enterobacter* was positively correlated with TBA, while negatively correlated mainly with temperature and ORP.

Overall, the distribution of total coliform bacteria including *E. coli* and other coliform bacteria were consistent with TBA with a negative correlation. Diversity of other coliform bacteria was affected by the multiple environmental parameters. Dissolved oxygen showed no obvious correlation with diversity and distribution of coliform bacteria.

CONCLUSION

This study has led to the first investigation of distribution and diversity of waterborne pathogenic bacteria along Jiahe river, China, which flows through densely-populated urban area. The results suggested that reservoirs surrounded by densely-populated urban areas are more likely contaminated by fecal bacteria. Phylogenetic analysis indicated that various coliform bacteria including *Citrobacter*, *Klebsiella*, *Enterobacter*, *Raoultella* as well as *E. coli* were detected at all sampling sites along the river. The statistical analysis suggested that temperature, ORP and TBA were three main parameters impacting the distribution and diversity of coliform bacteria. This study provided a deep understanding of the distribution and diversity of coliform bacteria along Jiahe river, which could be helpful to predict and prevent waterborne transmission of pathogenic bacteria in this area.

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