Author version of paper published as:
Ahmed, W., Sawant, S., Huygens, F., Goonetilleke, A. and Gardner T. (2009) ***Prevalence and occurrence of zoonotic bacterial pathogens in surface waters determined by quantitative PCR.*** Water Research, Vol. 43, pp. 4918-4928.

Prevalence and Occurrence of Zoonotic Bacterial Pathogens in Surface Waters Determined by Quantitative PCR

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ABSTRACT

The prevalence and concentrations of *Campylobacter jejuni*, *Salmonella* spp. and enterohaemorrhagic *E. coli* (EHEC) were investigated in surface waters in Brisbane, Australia using quantitative PCR (qPCR) based methodologies. Water samples were collected from Brisbane City Botanic Gardens (CBG) Pond, and two urban tidal creeks (i.e., Oxley Creek and Blunder Creek). Of the 32 water samples collected, 8 (25%), 1 (3%), 9 (28%), 14 (44%), and 15 (47%) were positive for *C. jejuni mapA*, *Salmonella invA*, EHEC O157 LPS, EHEC VT1, and EHEC VT2 genes, respectively. The presence/absence of the potential pathogens did not correlate with either *E. coli* or enterococci concentrations as determined by binary logistic regression. In conclusion, the high prevalence, and concentrations of potential zoonotic pathogens along with the concentrations of one or more fecal indicators in surface water samples indicate a poor level of microbial quality of surface water, and could represent a significant health risk to users. The results from the current study would provide valuable information to the water quality managers in terms of minimizing the risk from pathogens in surface waters.

Keywords: Surface water; Fecal pollution; Zoonotic pathogens; Fecal indicators; Health risk

1. Introduction

Fecal pollution of surface waters in coastal areas results in the degradation of recreational and commercial waterways in many parts of the world. Both non-point and point sources are regarded as contributors of such pollution. Various human enteric pathogens such as *Salmonella* spp., and *Shigella* spp., (Savichtcheva et al., 2007), and enteric viruses such as adenoviruses, noroviruses (Fong et al., 2005; Haramoto et al., 2005) have been found in surface waters due to human fecal pollution. Wastewater from domestic and/or farm animals such as cattle, horses, and poultry may further contribute pathogens such as *Escherichia coli* O157:H7 (Ibekwe and Grieve, 2003), *Salmonella* spp. (Lemarchand and Lebaron, 2003), *Cryptosporidium* spp., and *Giardia* spp. (Hörman et al., 2004). Surface waters are commonly used for recreational and commercial use, and therefore, unintended ingestion of fecally contaminated water could pose public health risks.

Fecal pollution is traditionally assessed by monitoring fecal indicator bacteria such as fecal coliforms, *E. coli* and enterococci (US EPA 2000). These indicators are abundant in the intestine of warm-blooded animals, and their presence in environmental waters indicate fecal pollution, and the presence of potential pathogenic microorganisms. However, it has been reported that fecal indicator bacteria such as *E. coli* may replicate in the environment (Anderson et al., 2005; Byappanahalli et al., 2006; Desmarais et al., 2002; Fujioka et al., 1999), and certain encapsulated strains of *E. coli* can cause blooms in surface waters even in the absence of fecal sources (Power et al., 2005). One major limitation of fecal indicators is their inability to predict the presence of pathogenic microorganisms in environmental waters, especially protozoans and enteric viruses (Hörman et al., 2004; McQuaig et al., 2006). Another limitation of traditional fecal indicator bacteria is that they cannot provide information regarding the sources of fecal pollution (see reviews Field and Samadpour, 2007; Scott et al., 2002; Stoeckel and Harwood, 2007).

Limited data is available on the microbiological quality of surface water in Brisbane, Australia. A few studies have reported high levels *E. coli* and enterococci in coastal lakes, rivers and creeks (Ahmed et al., 2005; Carroll et al., 2005; Mill et al., 2006). According to these studies, surface waters in this region tend to have high level of fecal pollution. Various microbial source tracking (MST) methods have also been applied to such areas in order to distinguish the sources of fecal pollution (Ahmed et al., 2005; Ahmed et al., 2008a; Ahmed et al., 2008b). Microbial source tracking methodologies can be used to predict the sources of

fecal pollution (i.e., human vs. animals; human vs. domestic animals vs. wild animals etc) so that it can be managed (Field and Samadpour, 2007). However, it has to be noted that MST methodologies do not necessarily provide information regarding the public health risks associated with polluted water. Furthermore, none of the studies reported the presence or prevalence of zoonotic pathogens in surface waters. Without knowing the prevalence and concentrations of such pathogens, it is difficult to assess the quality of water in terms of public health risks. In addition to information on the prevalence and concentrations of various zoonotic pathogens, it is important to gain insight on their correlation with traditional fecal indicators. In recent times PCR based methodologies have been widely used for the quantitative detection of various pathogenic microorganisms in environmental waters (Guy et al., 2003; Hörman et al., 2004; Sails et al., 2002). An important feature of the PCR based methods is that it can be used to detect and quantify pathogens which are difficult to culture using traditional culture-based methods. PCR based methods also circumvent the need for culturing microorganisms, and enable rapid detection/quantification of pathogens in a sample.

The study investigated the prevalence and concentrations of various zoonotic pathogens belonging to bacterial groups, including *Campylobacter jejuni (mapA* gene), *Salmonella* spp. (*invA* gene), and enterohaemorrhagic *E. coli* (EHEC) virulence genes [i.e., O157 lipopolysaccharides (LPS), verocytotoxin 1 (VT1), and verocytotoxin 2 (VT2)] in surface waters in Brisbane, Australia using PCR/quantitative PCR (qPCR) based methodologies. Secondly, the correlation between traditional fecal indicator bacteria (i.e., *E. coli* and enterococci) and the selected zoonotic bacterial pathogens that are also commonly found in human sewage were investigated.

2. Materials and methods

2.1 Study area and sampling sites

Surface water samples were collected from Brisbane City Botanic Gardens (CBG) Pond, and two creeks (Oxley Creek and Blunder Creek) in Brisbane, Australia. Oxley Creek is a tributary of the Brisbane River, and Blunder Creek is a major tributary of Oxley Creek (Fig.1). Oxley Creek is tidally influenced up to sample site OC1. The main water source of CBG Pond (location is not shown) is surface runoff during the wet season. When the water level is low during the dry season, the pond is topped up with chlorinated and UV treated human wastewater. A large number of waterfowls are present in the vicinity. The upper Oxley Creek catchment is sparsely populated with forested hills and grazing land. The middle and

lower catchment is highly populated, and the catchment is characterized by industrial areas, as well as a wastewater treatment plant (WTP). Blunder Creek is characterized by rural residential areas and a WTP. The wastewater at the WTPs receives tertiary treatment and treated wastewater is discharged (i.e., approximately 74 mega litre/day) into Brisbane River. Samples were collected from three sites (i.e., CBGP1-CBGP3) from the CBG Pond, four sites (i.e., OC1-OC4) from Oxley Creek, and one site (i.e., BC1) from Blunder Creek between September 2008 and November 2008 on four separate occasions. A total of 32 samples were collected for bacteriological analysis. Water samples were collected in 5-l sterile plastic containers at 30 cm below the water surface, and transported on ice to the laboratory and tested within 4 h.

2.2 Isolation and enumeration of fecal indicators

The membrane filtration method was used to process the water samples for *E. coli* and enterococci enumeration. Sample serial dilutions were made, and filtered through 0.45-μm pore size (47-mm-diameter) nitrocellulose membranes (Advantec, Tokyo, Japan), and placed on modified membrane-thermotolerant *Escherichia coli* agar (modified mTEC agar) (Difco, Detroit, MI, USA) and membrane-Enterococcus indoxyl-D-glucoside (mEI) agar (Difco) for the isolation of *E. coli* and enterococci respectively. Modified mTEC agar plates were incubated at 35°C for 2 h to recover stressed cells, followed by incubation at 44°C for 22 h (USEPA, 2002), and mEI agar plates were incubated at 41°C for 48 h (US EPA, 1997). For bacterial enumeration, all water samples were tested in triplicate.

2.3 DNA extraction

The following positive control strains were purchased from the American Type Culture Collection (ATCC): *C. jejuni* ATCC 33560, and *Salmonella enterica* serovar Typhimurium ATCC 14028. *Escherichia coli* NCTC 12079 (serotype O157:H7) strain was kindly donated by Mr. Jack Tucker from the University of the Sunshine Coast, Queensland, Australia. A pure colony was isolated for each target and was inoculated into a flask containing 15 ml of Tryptic Soy Broth (TSB). The flasks were kept in an incubator shaker overnight at 37°C. After incubation, *C. jejuni*, *Salmonella enterica* serovar Typhimurium, and *E. coli* DNA were extracted from broth culture using DNeasy blood and tissue kit (Qiagen, Inc., Valencia, CA). For qPCR quantification of *C. jejuni mapA* and *Salmonella invA* genes, 500 ml of each water sample was filtered through 0.45-μm pore size membrane (Advantec). In case of membrane clogging during filtration, multiple membranes were used. The membranes were immediately

transferred into 15-ml screw cap tube containing 10-ml of sterile STE buffer (0.1 M NaCl, 10 mM Tris, and 1 mM EDTA [pH 7.6]. The tubes were vortexed vigorously for 8-10 min to detach the bacteria from the membranes followed by centrifugation at 15,000 rpm for 30 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 2-ml of sterile distilled water. DNA was extracted using DNeasy blood and tissue kit (Qiagen), serially diluted, and stored at -20°C until use. For qPCR detection of EHEC virulence genes (i.e., O157 LPS, VT1, and VT2), 500 ml of water sample was filtered through 0.45-µm pore size membrane (Advantec), and *E. coli* were isolated according to the USEPA method described above. The estimated number of *E. coli* isolated from each water samples ranged between 5.0 X 10^0 and 6.0 X 10^4 CFU/500 ml. After isolation, the membranes containing various range of *E. coli* were transferred to sterile tubes containing 10-ml of Luria-Bertani (LB) broth (Oxoid, London, UK), and incubated at 44ºC for 24 h to obtain enriched bacterial culture. The enrichment step was performed to detect EHEC virulence genes as their concentrations could be low in environmental waters. DNA was extracted from 1 ml culture using DNeasy blood and tissue kit (Qiagen), and stored at -20ºC until use.

2.4 Specificity of the PCR primers

Quantitative PCR detection and quantification of pathogenic bacteria was done using previously published primers (Ahmed et al., 2007; Chiu and Ou, 1996; Inglis and Kalischuk, 2004). The primer sequence and annealing temperature for corresponding targets are shown in Table 1. Primer specificity was determined by searching for similar sequences in microbial genomes using the Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/BLAST/). This ensured that no homology was observed with known gene sequences of other pathogenic microorganism commonly found in environmental waters. The cross reactivity of each primer set was also evaluated by testing DNA isolated from other non-target species of bacteria commonly found in environmental waters (Table 2).

2.5 Generation of Quantitative PCR standards for C. jejuni and Salmonella

For quantitative detection, the standards were prepared from the genomic DNA of *C. jejuni* (ATCC 33560), and *S.* Typhimurium (ATCC 14028). Standard was also prepared from *E. coli* (NCTC 12079) and used for limit of detection assay. The concentration of genomic DNA was determined by measuring the absorbance at A_{260} using Beckman Coulter DU® 730 spectrophotometer. The gene copies were calculated using the mean mass of the *C. jejuni*, and *S.* Typhimurium genome which were assumed to be approximately 1.9 and 4.7 Mb,

respectively. A tenfold dilution was prepared from the genomic DNA, ranging from 10^6 to 10^0 copies/µl of DNA extract using CAS-1200™ precision liquid handling system (Corbett Life Sciences, Brisbane, Australia), and stored at -20°C until use. For each standard, the concentration was plotted against the cycle number at which the fluorescence signal crossed the threshold value (C_T value). The amplification efficiency (E) of the PCR standard was estimated from the slope of the standard curve by the formula $E = (10^{-1/slope}) - 1$. A reaction with 100% efficiency generates a slope of -3.32.

2.6 Quantitative PCR

Amplification was performed in 25-µl reaction mixtures using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA). The PCR mixture contained 12.5-µl SuperMix, 300 nM of each primer, 6.75-µl of DNase and RNase free deionised water and 5-µl of template DNA. For each PCR experiment, corresponding positive DNA and negative controls (sterile water) were included. The qPCR amplification was done using the Rotor-Gene 6000 real-time cycler (Corbett Life Sciences). PCR sample preparation was done using the CAS-1200 liquid handling system (Corbett Life Sciences). Cycling parameters for the *Salmonella invA* gene were 2 min at 50 ºC, 5 min at 94ºC for initial denaturation, and 45 cycles of 94ºC for 30 s, 59ºC for 35 s for annealing, and 72ºC for 2 min, followed by a final extension step of 72 ºC for 10 min; for *C. jejuni mapA* gene, 2 min at 50 ºC, 15 min at 95ºC for initial denaturation, and 50 cycles of 94ºC for 15 s, 58ºC for 30 s for annealing, and 72ºC for 30 s followed by a final extension step of 72 ºC for 5 min; for *E. coli* O157 LPS, VT1 and VT2 genes 2 min at 50 ºC, 10 min at 95ºC for initial denaturation, and 40 cycles of 95ºC for 30 s, 59ºC for 30 s for annealing and 72ºC for 30 s, followed by a final extension step of 72 ºC for 5 min. To separate the specific product from non-specific products (if any), DNA melting curve analysis was performed for each PCR experiment. During melting curve analysis, the temperature was increased from 62 to 95°C at approximately 2°C/min. Amplified products were also visualized by electrophoresis through 2% E-gel® (Invitrogen), and exposure to UV light for further confirmation. Samples were considered to be positive when the visible band was the same size as that of the positive control DNA, and had the same melting temperature as the positive control.

2.7 PCR optimization and quality control

During setting up the PCR assays, the PCR conditions for annealing temperature were optimized by performing gradient analysis (i.e., temperature ranged from 53°C to 63°) for

each target. The primer concentrations (100 nM to 500 nM) were also optimized to reduce the level of primer dimer for each target. In addition, non-specific products were not observed with melting curve and gel analysis. To minimize PCR contamination, DNA extraction, PCR set up, and gel electrophoresis were performed in separate laboratories. To prevent false positive results for surface water samples, a method blank was included for each batch of water samples. In brief, 500 ml of distilled water sample was filtered through 0.45-μm pore size membrane (Advantec). The filter paper was washed with sterile STE buffer followed by centrifugation as described above. The supernatant was discarded, and the pellet was resuspended in sterile distilled water. DNA was extracted using DNeasy blood and tissue kit (Qiagen).

2.8 Quantitative PCR reproducibility

The reproducibility of the qPCR was assessed by determining intra-assay repeatability and inter-assay reproducibility. The Coefficient of Variation (CV) was calculated using six dilutions (10^6 to 10^1 gene copies) of the *C. jejuni*, and *S.* Typhimurium genomic DNA. Each dilution was quantified in replicates of three. The CV for evaluation of intra-assay repeatability was calculated based on the C_T value by testing the six dilutions six times in the same experiment. The CV for inter-assay reproducibility was calculated based on the C_T value of six dilutions on six different days.

2.9 PCR limit of detection

The LOD assays were performed by analysing purified genomic DNA isolated from pure cultures of *C. jejuni* ATCC 33560 (for *mapA* gene), *S.* Typhimurium ATCC 14028 (for *invA* gene), and *E. coli* NCTC 12079 (for O157 LPS, VT1 and VT2 genes) strains containing target genes. To determine the qPCR lower limits of the detection (LOD), known gene copies (i.e., 10^3 -10^0) of each target gene were tested by qPCR. The lowest concentration of gene copies detected consistently in replicate assays was considered as qPCR LOD.

2.10 Effects of PCR inhibitors

An experiment was conducted to determine the potential presence of PCR inhibitory substances in surface water samples. For this purpose surface water samples (n=3) were collected from the Brisbane River, Australia because the study pond and Creeks were located within the Brisbane River catchment. Each sample (i.e., 400-500 ml) was concentrated using membrane filtration technique as described earlier. DNA was extracted using DNeasy blood

and tissue kit (Qiagen), serially diluted, and tested by qPCR. DNA was also extracted from 500 ml of ultra pure DNase and RNase free sterile distilled water (Invitrogen) in the same manner. All samples were spiked with 10^3 gene copies of *S.* Typhimurium DNA. The threshold cycle (C_T) values obtained for the DNA samples from spiked river water samples were compared to the DNA samples from spiked distilled water. The C_T value reflects the PCR cycle number at which the fluorescence generated crosses the threshold. It is inversely correlated to the logarithm of the initial copy number. All DNA samples were tested in triplicate.

2.11 Quantitative PCR limit of detection in surface water samples

Quantitative PCR LOD in environmental waters was only performed for *Salmonella* spp. as a representative of other bacterial pathogens, and it was postulated that qPCR LOD of other bacterial pathogens would be similar to that of *Salmonella* spp. To determine LOD, surface water samples (n=3) from Brisbane River were spiked with a known concentration (i.e., 8.3 X 10^7) of *S.* Typhimurium cells. Water samples were autoclaved to kill the existing microorganisms, and tested for the presence of *Salmonella invA* gene using PCR. This was done to ensure that environmental water samples that were used for spiking did not contain *Salmonella* spp. Each spiked sample was serially diluted, and filtered through membranes according to the method described earlier. DNA was extracted from each filter paper, and tested by qPCR. The lowest concentration of cells detected consistently in replicate assays was considered as qPCR LOD.

2.12 Quantitative PCR detection efficiency

The recovery efficiency was determined by spiking distilled water (n=3), and surface waters (n=3) with known concentrations of *S.* Typhimurium cells. Surface water samples were collected from the Brisbane River, and autoclaved to remove the existing microorganism followed by exposure under UV light for 1 h in order to minimize the background DNA level of *Salmonella* spp. (if any). PCR assay was performed to ensure none of the environmental samples contained *Salmonella spp.* The *S.* Typhimurium strain was cultured overnight in LB broth and cell concentrations were enumerated using microscope. The enumeration was performed at 10 different microscopic fields. Ten-fold serial dilutions (i.e., 4.4 X 10^8, 4.4 X 10^7 and 4.4 X 10^6) were prepared, and added to 400 ml of distilled and surface water samples. These samples were subsequently filtered through membranes, and. DNA extraction was performed according to the method described earlier. Samples were tested in triplicate for

each concentration, and the recovery efficiency (%) was calculated using the following equation: Recovery (%) = (No. of cells after filtration/No. of cells before filtration) X 100.

2.13 DNA sequencing

To verify the identity of the PCR products obtained from surface water samples, up to three PCR-amplified products from each target were purified using the QIAquick PCR purification kit (Qiagen) as recommended by the manufacturer's instructions (Qiagen), and cloned, in duplicate, into the pGEM®-T Easy Vector system (Promega, Madison, WI, USA) as recommended by the manufacturer. Plasmids were extracted using the QIAprep Spin-Miniprep kit (Qiagen). Bidirectional sequences were obtained using T7 and SP6 long sequencing primer targeting sites on either side of the insert. DNA sequencing was carried out at the Australian genome Research Facility (St Lucia, Queensland, Australia). The sequences were analysed using Bioware Jellyfish Software, and were verified to the published sequence.

2.14 Statistical analysis

Analysis of variance (ANOVA) was performed to determine the differences between C_T values obtained for distilled water, and those obtained for surface water samples. GraphPad Prism (GraphPad Software, Inc., San Diego, CA) was used to perform the ANOVA. A binary logistic regression analysis was also performed to obtain correlations between the presence/absence of pathogen detection by PCR, and the concentrations of fecal indicators. Logistic regression is the technique most commonly used to model such a binary (i.e., presence/absence) response. The presence/absence of pathogens was treated as the dependent variable (i.e., a binary variable). When a target organism was present, it was assigned the value 1, and when a target organism was absent, it was assigned the value 0. Minitab Release version 11.12 (State College, Pa.) software was used for logistic regression analysis. In all cases, a difference was considered significant if the *P* value for the model chi square was 0.05.

3. Results

3.1 Cross-reactivity of PCR primers

The cross-reactivity of each primer set for each target was assessed by testing a panel of other microorganisms commonly found in environmental waters (Table 2). The primers used in this study did not amplify any PCR products other than those products that were expected.

3.2 Quantitative PCR standards and melting curves

DNA from 10-fold dilutions of quantified *C. jejuni* and *S.* Typhimurium strains were analysed in order to determine the reaction efficiencies. The standard curves had a linear range of quantification from 10^6 to 10^1 gene copies/µl of DNA extracts. The amplification efficiencies were > 98% for each PCR as determined by the Rotor-Gene software (Corbett Research). The correlation coefficient (r^2) was > 0.99 for both q PCR assays. The amplification of the correct PCR products was verified by analysing the melting curves, which showed a peak at melting temperature 76.2 ± 0.2°C (for *C. jejuni mapA* gene), and 80.2 ± 0.2°C for (*Salmonella invA* gene), indicating a positive and correct amplification. The melting curves for *E. coli* O157 LPS, VT1, and VT2 genes were 77.3 ± 0.2°C, 80.9 ± 0.2°C, and 78.3 ± 0.2°C, respectively.

3.3 Quantitative PCR reproducibility

The reproducibility of the qPCR assays was determined by assessing intra-assay and inter-assay CV of the standards. These values were less than 3% and 5% for both *C. jejuni mapA* gene and *Salmonella invA* gene, respectively, indicating high reproducibility (Table 3).

3.4 Lower detection limits of the Quantitative PCR

The LOD assays were performed by analysing purified genomic DNA isolated from pure cultures of bacterial strains containing target genes. To determine the reproducibility of the assay, several replicates (n = 10) were tested. The q PCR detection limits were as low as one gene copy for *Salmonella invA* gene. For *C. jejuni mapA*, and *E. coli* O157 LPS, VT1 and VT2 genes, the detection limits were 10 gene copies. Lower levels (i.e., one copy) were tested for these targets, but the results were not reproducible for all replicates.

3.5 PCR inhibitors

To detect the presence of inhibitors, surface water samples (n = 3) were spiked with 10^3 gene copies of *S.* Typhimurium DNA containing the *invA* gene. The qPCR C_T values were compared to those obtained from the same concentrations of DNA that was used to spike 500-ml of distilled water. For the spiked distilled water, the mean C_T value for *Salmonella invA* gene was 21.6 ± 0.4. For surface water samples, the mean C_T values for undiluted DNA, ten-fold, 100-fold and 1000-fold are shown in Table 4. One-way ANOVA was performed to determine the differences between the C_T values obtained for distilled water and those obtained for surface water samples. Significant (P <0.001) differences were observed between the C_T values for spiked distilled water and undiluted DNA from surface water samples,

indicating that the undiluted DNA extracted from surface water samples contained PCR inhibitory substances. However, no significant differences ($P > 0.05$) were observed between the C_T values for spiked distilled water and serially diluted DNA (i.e., ten-fold, 100-fold, and 1000-fold) indicating that ten to 100 fold dilution of DNA is required to remove the effects of PCR inhibitory substances from surface water samples.

3.6 Quantitative PCR limit of detection (LOD) in freshwater samples

To determine the limit of detection of the qPCR assay in freshwater samples, known concentrations of *S.* Typhimurium were spiked into autoclaved surface water samples. Serial dilutions resulted in the detection of 8.3 CFU/500 ml of *S.* Typhimurium.

3.7 Recovery efficiency

The recovery efficiency was determined by spiking autoclaved distilled water and surface water with known concentrations of *S.* Typhimurium cells. The estimated detection efficiency in autoclave distilled water samples ranged between 90% and 49% with the greatest variability occurring at lower cell counts. The mean detection efficiency was 72% ± 10% (Table 5). The estimated detection efficiency in autoclaved freshwater samples ranged between 89% and 46% with the greatest variability occurring at lower cell counts. The mean detection efficiency was 67% ± 12%.

3.8 Concentrations of fecal indicators

The concentrations of *E. coli* in water samples collected from the CBG Pond were high, ranging from 3.7 X 10^2 to 3.5 X 10^4 colony forming units (CFU)/100 ml of water sample (Table 6). For enterococci, this figure ranged from 8.0 X 10^2 to 6.3 X 10^4 CFU/100 ml. However, the concentrations of both fecal indicators were generally much higher in samples collected during the first sampling occasion compared to other sampling occasions. The concentrations of fecal indicator bacteria in all water samples collected from the CBG Pond exceeded the Australian and New Zealand Environment and Conservation Council (ANZECC) recreational water quality guidelines for fresh and marine waters of 150 faecal coliforms/100 ml (data not shown), and 35 enterococci/100 ml for primary contact (ANZECC, 2000). The concentrations of *E. coli* in water samples from Oxley Creek ranged from 5.0 X 10^1 to 4.7 X 10^3 CFU/100 ml of water. For enterococci, this figure ranged from 9.0 X 10^1 to 2.0 X 10^3 CFU/100 ml. The concentrations of both *E. coli* and enterococci were high at the OC1 site which is located upstream of Oxley Creek wastewater treatment plant

(WTP). Reduced levels of *E. coli* and enterococci were found at downstream sites of Oxley WTP (see Table 6). The concentrations of fecal indicator bacteria in all water samples collected from Oxley Creek exceeded the ANZECC recreational water quality guidelines (ANZECC, 2000). The concentrations of *E. coli* and enterococci in samples from Blunder Creek ranged from 1.0×10^0 to 1.1×10^2 CFU/100 ml (for *E. coli*) and 5.5×10^1 to 2.5×10^3 CFU/100 ml (for enterococci). The concentrations of indicator bacteria in water samples from Blunder Creek also exceeded the ANZECC water quality guidelines for primary contact except one sample which had 1.0×10^0 *E. coli*/100 ml.

3.9 Prevalence and concentrations of zoonotic bacterial pathogens

Of the 12 samples tested from the CBG Pond, five (42%) were positive for *C. jejuni mapA* gene. Quantitative PCR detected 3.0×10^1 to 7.0×10^1 gene copies/100ml of *C. jejuni mapA* gene in these positively identified samples. Two samples (i.e., 12%) from Oxley Creek were positive for *C. jejuni mapA* gene. However, the concentrations were below PCR LOD, and the results were not reproducible for replicate assays. Among the four samples tested from Blunder Creek, one (25%) sample was positive for *C. jejuni mapA* gene. Similarly of the 12 samples tested from the CBG Pond, only one (8%) was positive for *Salmonella invA* gene, and the concentration was 1.2×10^2 gene copies/100 ml of water sample. However, the *Salmonella invA* could not be detected in any samples from Oxley Creek or Blunder Creek. Among the 12 samples tested from the CBG Pond, five (42%), three (25%), and five (42%) were positive for EHEC O157 LPS, VT1 and VT2 genes, respectively. Three samples (25%) were positive for all three EHEC virulence genes, and six (50%) samples were positive for at least one of the EHEC virulence genes tested.

Among the 16 samples tested from Oxley creek, three (19%), nine (56%) and eight (50%) were positive for O157 LPS, VT1 and VT2 genes, respectively. Two samples from Blunder Creek were positive for EHEC virulence genes. Overall, of the 32 samples tested, eight (25%), one (3%), nine (28%), 14 (44%) and 15 (47%) were positive for *C. jejuni mapA* gene, *Salmonella invA* gene, *E. coli* O157 LPS, VT1, and VT2 genes, respectively. For the 32 samples, one (3%) was positive for all five target genes, three (9%) were positive for at least four target genes, four (13%) were positive for at least three target genes, seven (22%) were positive for at least two target genes, and four (13%) were positive for at least one target gene. In contrast, none of these potential pathogens were detected in 13 (41%) samples. Binary logistic regressions were used to identify whether any correlation existed between the

concentrations of fecal indicators and the presence/absence results for potential target pathogens. The presence/absence of the potential pathogens did not correlate with either *E. coli* or enterococci concentrations. Nagelkerke's *R* square (range from 0.0 to 1.0) was used to indicate the association between dependent and independent variables. Stronger association has values close to 1.0. However, Nagelkerke's *R* square values for each indicator and pathogen were less than 0.001. The significance level for variable evaluation was alpha = 0.05.

4. Discussion

In this study, qPCR assays using SYBR Green I dye was used to detect bacterial zoonotic pathogens in surface water samples collected in Brisbane, Australia. For the 12 samples tested from the CBG Pond, a significant number of samples were positive for *C. jejuni mapA* gene. However, only one sample was positive for *Salmonella invA* gene. Quantitative PCR detected 3.0×10^1 to 7.0×10^1 gene copies of *C. jejuni mapA* gene, and 1.2×10^2 gene copies of *Salmonella invA* gene/100 ml of water sample. Both *C. jejuni mapA* and *Salmonella invA* are single copy genes which allow the conversion of the gene copies into cell counts. The concentration of *Salmonella invA* gene possibly does not pose a significant threat to humans because the minimum infectious dose is 10^5 for *S. typhi* and 10^9 cells for *S.* Typhimurium required to initiate disease (Le Minor, 1981). However, it has to be noted that infectious dose may vary from person to person.

However, the concentrations of *C. jejuni* in the CBG Pond water samples could pose significant health risks due to their low infectious dose (i.e., 500 organisms can cause illness) (Kothary and Babu, 2007). The *C. jejuni* and *Salmonella* spp. could originate from the feces of ducks and wild birds found within the vicinity of the pond. It has been reported that birds are *C. jejuni* carriers (Kakoyiannis et al., 1988; Waldenstrom et al., 2002). In 2003, New Zealand had a higher notifiable rate of campylobacteriosis. Birds including ducks have been implicated as vectors of transmission (Murphy et al., 2005). Several water samples from the CBG Pond were also positive for EHEC virulence genes. The presence of EHEC virulence genes such as VT1 and VT2 in birds (i.e., pigeon and crows) has been reported elsewhere (Fukuyama et al., 2003). Most of the potential pathogens were detected in samples which were collected during the first and second sampling occasions. Prior to first sampling occasion, the study area (i.e., CBG Pond and surrounding areas) had received > 45 mm rainfall, and prior to second sampling occasion, the study area further received >25 mm

rainfall. Samples were collected 24 h after rainfall events. It is hypothesized that deposited bird and duck feces on the banks of the pond would have washed into the water, thereby introducing pathogenic microorganisms. The concentrations of *E. coli* and enterococci were also high during the sampling indicating the occurrence of significant fecal pollution. However, none of the EHEC virulence genes were detected in samples collected during the third and fourth sampling occasions when the study area received no rainfall.

A significant number of samples from Oxley Creek were positive for the EHEC VT1, VT2 and O157 LPS genes Two samples were also positive for *C. jejuni mapA* gene. Enterohaemorrhagic *E. coli* O157 LPS, VT1, VT2, and *C jejuni mapA* genes were also detected in Blunder Creek. Blunder Creek site (i.e., BC1) is characterized by agricultural practices including cattle farming. Site BC1 is located within a cattle farm, and grazing cattle have free access to the creek water. It is postulated that cattle fecal matter is the source of EHEC virulence genes detected at this site. This is in accordance with Chapman et al., (1997) who suggested that cattle are a principal reservoir of EHEC. The high prevalence of EHEC in water samples from Oxley Creek could have originated from Blunder Creek as it is a major tributary of Oxley Creek (see Fig. 1). It has to be noted that all water samples were collected during low tide, and probably for this reason, EHEC virulence genes were more frequently detected in downstream sites. Nonetheless, the high prevalence of EHEC positive samples from Oxley Creek, especially in the downstream sites (i.e., OC3 and OC4) which are used for recreational activities, indicates potential public health risks. Outbreak of EHEC O157:H7 infection involving recreation water has been reported in the USA (Rangel et al., 2005). The infectious dose of EHEC O157:H7 bacteria could be as low as ten to 100 cells (Paton and Paton, 1998).

In this study, the prevalence of EHEC virulence genes was higher than *C. jejuni mapA* and *Salmonella invA* genes. This could be due to the cultural enrichment step that was performed prior to PCR assays to promote growth of injured and stressed cells. This technique is often used to detect pathogenic bacteria that generally occur at low concentrations in environmental waters (Savitcheva et al., 2007). In contrast, to obtain quantitative data for *C. jejuni mapA* and *Salmonella invA* genes, the samples were processed without the enrichment step which may have reduced the sensitivity of the qPCR detection (Myint et al., 2006). It is also possible that the prevalence of EHEC genes is higher than *C. jejuni* and *Salmonella* spp. in surface waters in Brisbane Australia. The presence of these potential pathogens did not correlate with either

E. coli or enterococcal concentrations. It has been reported previously that *E. coli* and enterococci do not correlate well with pathogenic *Salmonella* spp. (Lemarchand and Lebaron, 2003), and *Campylobacter* spp. (Hörman et al., 2004). The use of fecal indicator bacteria alone to assess the microbial quality of surface water has been questioned (Bonadonna et al., 2002; Hörman et al., 2004; Lemarchand and Lebaron, 2003; Pusch et al., 2005), and the results of the study also suggest that the monitoring of traditional fecal indicator concentrations alone cannot be used to assess the water quality and/or safety.

We also investigated the presence of human fecal pollution in the study creeks as the sampling sites were located downstream of two WTPs. In pervious studies, we have shown the presence of human fecal pollution in surface waters in Australia (Ahmed et al., 2008a; Ahmed et al., 2008b). It has been reported that WTPs overflow can contribute fecal pollution in receiving waters (Haramoto et al., 2005). All water samples from Oxley and Blunder Creeks were tested for two human-specific molecular markers namely *Bacteroides* HF183 (Bernhard and Field, 2000), and the enterococci surface protein (*esp*) marker found in *Enterococci faecium* (Scott et al., 2005). Sample processing and experimental procedures have been described elsewhere (Ahmed et al., 2008a; Ahmed et al., 2008b). In the 20 samples tested, only one (5%) sample from site BC1 was positive for the HF83 marker. However, the PCR product was faint, indicating a low level of human fecal pollution in that particular sample. On the other hand, none of the samples were positive for the *esp* marker. These results suggest that human fecal pollution may not be a major concern in the study creeks.

We also validated the PCR positive results obtained in this study by analysing DNA melting curves. The melting of the PCR amplicons at the correct temperature indicated true and positive amplification. The presence of positive amplicons was further confirmed by visualization on agarose gels. Finally, up to two amplicons were sequenced for each target, and verified they were >97% identical to the published sequences (data not shown). It is acknowledged that the PCR results are expressed as the presence/absence for EHEC virulence genes, and do not provide information regarding the degree of fecal pollution. Another limitation of the current PCR assays is that they do not provide information regarding the pathogenicity of the target organisms. The inability of PCR assays to distinguish between viable and nonviable pathogenic microorganisms is another concern. Therefore, in this study, it cannot be ruled out that in some cases, the PCR assays may have detected DNA from nonviable pathogenic microorganisms. Methods have been developed to distinguish between

viable and non-viable cells using ethidium monoazide (Rudi et al., 2005). Such a method could provide valuable information regarding the viability of cells in environmental samples.

5. Conclusions

- Quantitative PCR detection of pathogens is rapid, and results can be obtained within a day, compared to the number of days required for using conventional culture-based methods. The results from the current study provide valuable information to the water quality managers in terms of minimizing the risk from zoonotic pathogens in surface waters.
- The high prevalence and concentrations of potential zoonotic pathogens along with the concentrations of one or more fecal indicators in surface water samples indicate a poor level of microbial quality of surface water especially after rainfall events, and could represent a significant health risk to users. This underlines the need to undertake appropriate mitigation measures to protect public health risks.
- This study also indicated a poor correlation between fecal indicators and potential zoonotic pathogens tested. Therefore, testing fecal indicators alone may not be adequate to assess the microbiological quality of surface water and consequent health risks. The need to undertake a suite of tests to assess the microbiological quality is recommended.
- The study undertaken was limited in terms of the geographica area. Additionally, the results derived were based on four sampling episodes. It is recommended that more widespread sampling is undertaken to determine the geographical and temporal stability of the methods adopted and to assess the prevalence of the detected pathogens outside the study area within this region.

Acknowledgements

This study was funded by Queensland Department of Natural Resources and Water (DNRW). This was a joint project between DNRW and Queensland University of Technology (QUT). We thank Charles Wan from QUT and Barry Hood from DNRW for assisting in water sampling. We also thank Joe Lane from DNRW for Geographical Information System (GIS) mapping.

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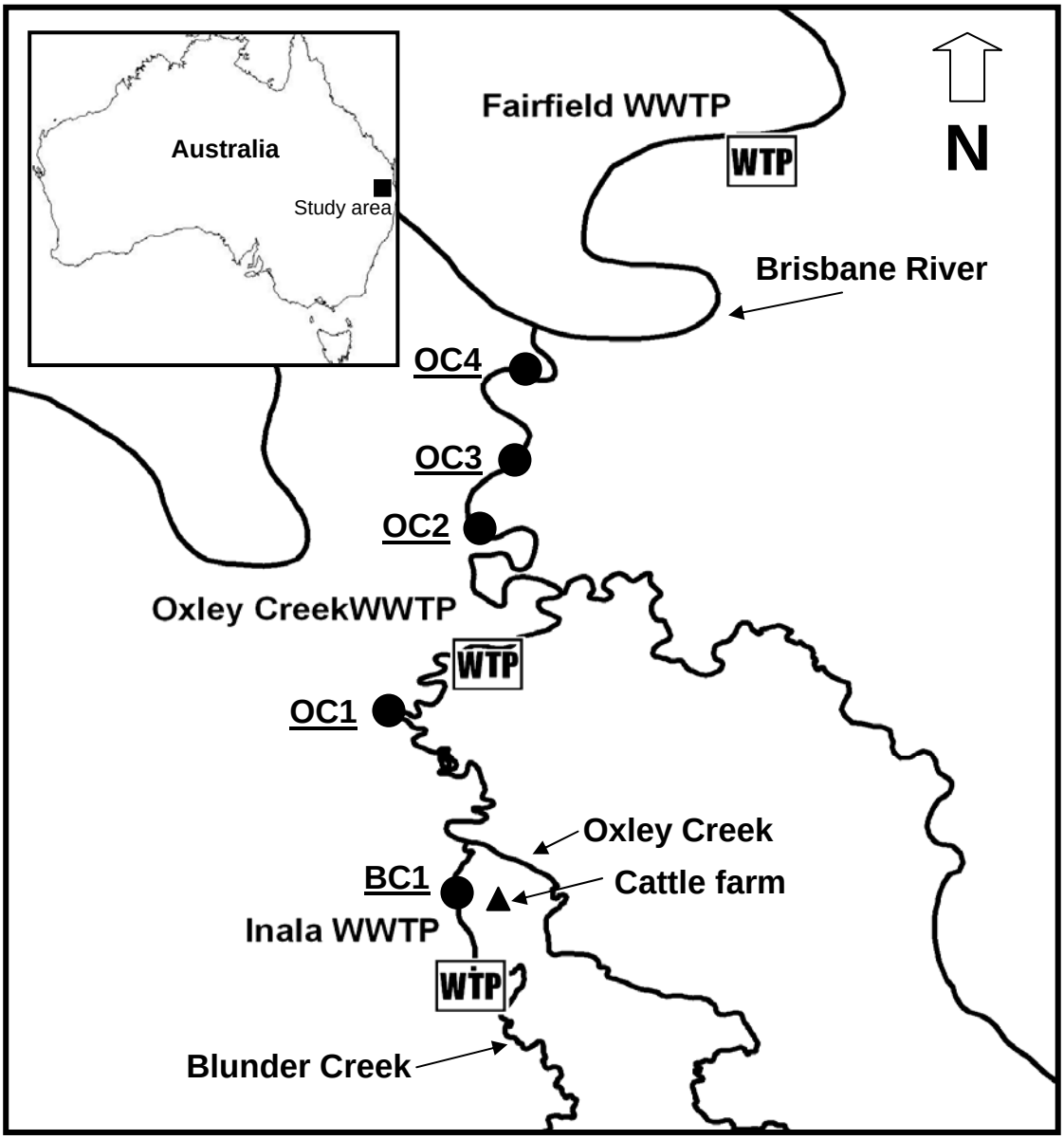

Fig.1 - Map of Oxley Creek and Blunder Creek showing sampling sites (●) and a cattle farm (▲).

Table 1 – Primers used for qPCR assays

Target	Primer sequence (5´- 3´)	Length (bp)	Annealing temperature	Amplicon size (bp)	Reference
C. jejuni mapA gene[a]	GGT TTT GAA GCA AAG ATT AAA GG	23	59°C	94	Inglis and Kalischuk, 2004
	AAG CAA TAC CAG TGT CTA AAG TGC	24			
Salmonella invA gene[a]	ACA GTG CTC GTT TAC GAC CTG AAT	24	59°C	244	Chiu and Ou, 1996
	AGA CGA CTG GTA CTG ATC GAT AAT	24			
EHEC O157 LPS gene[b]	CGG ACA TCC ATG TGA TAT GG	20	59°C	259	Ahmed et al., 2007
	TTG CCT ATG TAC AGC TAA TCC	21			
EHEC VT1[b]	ACG TTA CAG CGT GTT GCT GGG ATC	24	59°C	121	Ahmed et al., 2007
	TTG CCA CAG ACT GCG TCA GTT AGG	24			
EHEC VT2[b]	TGT GGC TGG GTT CGT TAA TAC GGC	24	59°C	102	Ahmed et al., 2007
	TTG CCA CAG ACT GCG TCA GTT AGG	24			

[a] qPCR quantification; [b] qPCR detection

Table 2 - Specificities of PCR primers

Target DNA	Primer sets				
	C. jejuni mapA gene	*Salmonella invA* gene	EHEC O157 LPS gene	EHEC VT1 gene	EHEC VT2 gene
A. hydrophila ATCC 7966	-	-	-	-	-
C. coli ATCC 43478	-	-	-	-	-
C. jejuni ATCC 33560	+	-	-	-	-
E. faecalis ATCC 19433	-	-	-	-	-
E. faecium C68	-	-	-	-	-
E. faecium ATCC 27270	-	-	-	-	-
E. coli ATCC 25922	-	-	-	-	-
E. coli NCTC 10418	-	-	-	-	-
E. coli 9602-5069	-	-	+	-	+
E. coli NCTC 8196	-	-	-	-	-
E. coli NCTC 11560	-	-	-	-	-
E. coli NCTC 11603	-	-	+	+	+
E. coli NCTC 12079	-	-	+	+	+
E. coli ED1a	-	-	-	-	-
L. pneumophila ATCC 33152	-	-	-	-	-
S. Typhimurium ATCC 14028	-	-	-	-	-

Table 3 - The intra-assay and inter-assay coefficient of variation (CV) for the qPCR assay of *C. jejuni mapA* and *Salmonella invA* genes

Concentration of gene copies/µl of DNA extract	Coefficient of variation (CV) (%)			
	C. jejuni mapA gene		*Salmonella invA* gene	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
10^6	2.49	0.86	0.97	1.56
10^5	0.69	1.00	1.72	1.28
10^4	0.94	2.03	2.60	1.53
10^3	1.63	1.70	2.65	0.87
10^2	1.05	0.42	1.02	1.93
10^1	3.24	1.26	2.43	4.38

Table 4 - Effects of PCR inhibitors on the qPCR detection of spiked *Salmonella enterica* serovar Typhimurium in surface water samples as opposed to distilled water samples

Samples	Threshold cycle (C_T) value for the qPCR			
	Undiluted DNA	10-fold dilution	100-fold dilution	1000-fold dilution
Surface water 1	37.6 ± 2.6	22.0 ± 0.3	21.7 ± 0.5	21.5 ± 0.1
Surface water 2	34.6 ± 6.1	22.6 ± 1.6	21.6 ± 0.2	21.3 ± 0.5
Surface water 3	31.3 ± 6.5	24.6 ± 3.1	21.6 ± 0.2	21.6 ± 0.1
Mean C_T values	34.5 ± 3.1	23.0 ± 1.3	21.6 ± 0.1	21.4 ± 0.2

Table 5 – Recovery efficiency with the qPCR assay for autoclaved distilled and surface water samples spiked with known concentration of *Salmonella enterica* serovar Typhimurium cells.

Spiked cells/500 ml of water	Detection efficiency ± SD (%)	
	Distilled water	Environmental water
4.4 X 10^8	81% ± 9%	78% ± 11%
4.4 X 10^7	76% ± 4%	74 ± 9%
4.4 X 10^6	60% ± 11%	54% ± 8%

Table 6 – Concentrations of fecal indicator bacteria and zoonotic pathogens in environmental samples

Study area	Sampling sites (occasion)	Geometric mean of fecal indicators and pathogens (CFU/100 ml)		Quantitative PCR quantitative results for bacterial pathogens (gene copies/100 ml) [b]		Quantitative PCR detection for EHEC virulence genes		
		E. coli	Enterococci	*C. jejuni mapA* gene	*Salmonella invA* gene	EHEC O157 LPS gene	EHEC VT1 gene	EHEC VT2 gene
CBG Pond	CBGP1 (1)	3.5×10^4	1.4×10^4	6.0×10^1	1.2×10^2	+	+	+
	CBGP2 (1)	1.8×10^3	6.3×10^4	4.3×10^1	-	+	+	+
	CBGP3 (1)	1.2×10^3	9.5×10^3	-	-	+	-	+
	CBGP1 (2)	4.0×10^3	9.1×10^2	-	-	+	-	-
	CBGP2 (2)	3.8×10^3	3.5×10^3	7.0×10^1	-	+	+	+
	CBGP3 (2)	1.5×10^3	7.0×10^3	3.4×10^1	-	-	-	+
	CBGP1 (3)	5.4×10^3	3.5×10^3	-	-	-	-	-
	CBGP2 (3)	3.2×10^3	9.2×10^3	-	-	-	-	-
	CBGP3 (3)	9.6×10^2	1.3×10^3	-	-	-	-	-
	CBGP1 (4)	2.8×10^3	4.0×10^3	-	-	-	-	-
	CBGP2 (4)	1.9×10^3	1.7×10^3	3.0×10^1	-	-	-	-
	CBGP3 (4)	3.7×10^2	8.0×10^2	-	-	-	-	-
Oxley Creek	OC1 (1)	7.2×10^2	1.2×10^3	-	-	-	-	-
	OC2 (1)	8.0×10^1	6.8×10^2	-	-	-	+	+
	OC3 (1)	9.0×10^1	3.2×10^2	-	-	-	+	+
	OC4 (1)	5.8×10^1	3.0×10^2	-	-	-	+	-
	OC1 (2)	1.3×10^3	6.2×10^2	-	-	-	-	-
	OC2 (2)	6.7×10^2	1.5×10^3	-	-	-	+	+
	OC3 (2)	1.4×10^2	2.0×10^3	-	-	-	+	+
	OC4 (2)	8.0×10^1	9.8×10^1	-	-	+	+	+
	OC1 (3)	4.2×10^2	2.3×10^2	+ [a]	-	+	+	+
	OC2 (3)	4.8×10^2	9.0×10^1	-	-	-	-	-
	OC3 (3)	4.7×10^3	1.5×10^2	-	-	-	-	-
	OC4 (3)	7.1×10^2	2.2×10^2	-	-	-	-	-
	OC1 (4)	1.9×10^2	2.8×10^2	-	-	+	+	+
	OC2 (4)	5.6×10^1	1.6×10^2	+ [a]	-	-	+	+
	OC3 (4)	5.0×10^1	1.3×10^2	-	-	-	-	-
	OC4 (4)	5.3×10^1	1.2×10^2	-	-	-	-	-
Blunder Creek	BC1 (1)	1.0×10^0	1.0×10^3	+ [a]	-	-	-	-
	BC1 (2)	1.1×10^2	2.5×10^3	-	-	-	-	-
	BC1 (3)	1.0×10^2	5.5×10^1	-	-	+	+	+
	BC1 (4)	1.1×10^2	2.1×10^2	-	-	-	+	+
Total	$n = 32$	-	-	8/32	1/32	9/32	14/32	15/32

[a] Below detection limit; (+) : Pathogen present, (-) Pathogen absent; [b] The number of gene copies found in 500 ml of water samples was converted to 100 ml