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Assessing the population dynamics of *Escherichia coli* in a metropolitan river after an extreme flood event

Nicole M. Masters, Aaron Wiegand, Jasmin M. Thompson, Tara L. Vollmerhausen, Eva Hatje and Mohammad Katouli

ABSTRACT

We investigated *Escherichia coli* populations in a metropolitan river after an extreme flood event. Between nine and 15 of the 23 selected sites along the river were sampled fortnightly over three rounds. In all, 307 *E. coli* were typed using the PhP typing method and were grouped into common (C) or single (S) biochemical phenotypes (BPTs). A representative from each of the 31 identified C-BPTs was tested for 58 virulence genes (VGs) associated with intestinal and extra-intestinal *E. coli*, resistance to 22 antibiotics, production of biofilm and cytotoxicity to Vero cells. The number of *E. coli* in the first sampling round was significantly ($P < 0.01$) higher than subsequent rounds, whereas the number of VGs was significantly ($P < 0.05$) higher in isolates from the last sampling round when compared to previous rounds. Comparison of the C-BPTs with an existing database from wastewater treatment plants (WWTPs) in the same catchment showed that 40.6% of the river isolates were identical to the WWTP isolates. The relatively high number of VGs and antibiotic resistance among the C-BPTs suggests possessing and retaining these genes may provide niche advantages for those naturalised and/or persistent *E. coli* populations which may pose a health risk to the community.

Key words | *Escherichia coli*, faecal contamination, flood, health risk, wastewater

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INTRODUCTION

Transmission of waterborne pathogens predominantly occurs via the faecal–oral route when exposed to contaminated water. Faecal contamination of water bodies can result from various point and non-point sources, such as untreated discharge from wastewater treatment plants (WWTPs) (McCarthy *et al.* 2012), failing septic systems (Ahmed *et al.* 2005) agricultural and urban surface run-off, and local wildlife (Garcia Armisen & Servais 2007). Typically, the greatest deterioration in water quality occurs during intense precipitation events, during which, sewage and WWTP infrastructure can become overburdened, resulting in excessive wastewater being diverted to urban run-off drains and discharged into adjacent waterways (Ham *et al.* 2009; Passerat *et al.* 2011). In extreme circumstances, raw wastewater may be diverted without any treatment, although due to the large volume of water entering the waterway this might also have a potential dilution benefit. Such discharge entering the waterways not only introduces many faecal contaminants directly, but additional pollutants which provide ideal conditions for the replication of many microorganisms (Ham *et al.* 2009; Passerat *et al.* 2011).

Recreational water quality guidelines recommend enumerating thermotolerant faecal coliforms and enterococci, in addition to sanitary inspection and risk management (ANZECC/ARMCANZ 2000; NHMRC 2008). This water quality monitoring strategy was originally based on the assumption that faecal indicator bacteria (FIB) strongly correlated with enteric pathogens. However, in the past two decades, numerous studies have found limited correlation between FIB and various bacterial, viral and protozoan

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enteric pathogens or their virulence determinants (Anderson *et al.* 2005; Wohlsen *et al.* 2006; Ahmed *et al.* 2009; Masters *et al.* 2011; Wu *et al.* 2011). Yet, direct screening of all waterborne pathogens is neither economically nor technically viable for water management agencies, and so enumeration of FIB is still the preliminary method for evaluating surface water microbial loading.

E. coli are commonly isolated from the gut of humans and many other animals as well as diverse terrestrial and aquatic environments. While most *E. coli* are commensals, certain strains are pathogenic and are frequently implicated in waterborne disease outbreaks. Alarmingly, there has been an increase in the number of reported *E. coli* outbreaks and related deaths in recent years (Scheutz *et al.* 2011). Non-pathogenic, commensal strains of *E. coli* may also acquire a combination of virulence genes (VGs), which enable them to cause intestinal infections or extra-intestinal infections (Johnson & Stell 2000). This can be done by horizontal acquisition of VGs including antibiotic resistance (ABR) genes in the environment such as WWTPs (Johnning *et al.* 2013) or surface waters (Aminov 2011).

The health implications of these pathogenic *E. coli* strains are well known. However, to the best of our knowledge, the population dynamics of pathogenic *E. coli* in metropolitan surface waters after an extreme flood have not been investigated.

In this study, we investigated the population structure of pathogenic *E. coli* in a metropolitan river after an extreme wet weather event which resulted in overflow and discharge of several WWTPs, and inundation of residential and industrial areas. Our findings highlight a significant health risk associated with exposure to pathogenic *E. coli* strains by the general public who are either directly impacted by flood or use waterways for recreational activities long after the flood.

METHODS

Sampling sites and collection of water samples

The lower Brisbane River catchment is highly modified and urbanised and supports >1.8 million inhabitants. The upper reaches of this catchment are scattered with grazing land, forestry and natural bushland, however much of the downstream catchment area is highly urbanised and industrialised, dissecting Brisbane, the state capital of Queensland, Australia (ABS 2012).

On 11th January 2011, the Brisbane River broke its banks, following a prolonged and extreme precipitation event, and many adjacent metropolitan areas were inundated with flood water. The flood peak measured 4.46 metres Australian Height Datum at the Brisbane central business district gauge (Station Number 540198). Based on indirect information from the local council and various media transcripts, an estimated 20,000 houses and various other infrastructures were inundated, including damage to the sewerage infrastructure of nine WWTPs within the catchment boundary. A further 20,000 residential properties and many commercial and industrial buildings in the wider metropolitan area of Brisbane were also impacted by the flood. Due to the widespread damage and inundation of the Brisbane metropolitan WWTPs, untreated sewage was discharged directly into residential areas, public parks and adjacent waterways, which subsequently flowed into the Brisbane River for up to 10 weeks after the flood occurred.

The Brisbane River runs 309 km from the Great Dividing Range to Moreton Bay, a shallow sheltered bay that feeds into the Pacific Ocean. Wivenhoe Dam, situated 70 km upstream of the Brisbane River mouth, receives roughly 50% of the catchment overflow, and serves to supply water to the wider Brisbane community. During the height of the flood, the Wivenhoe Dam reached the capacity of 190% and excess water was released, to prevent dam collapse, into the lower Brisbane River catchment for over 5 weeks. This coincided with the first round of sampling.

Water samples were collected from 23 selected sites along the lower Brisbane River catchment (1,195 km^2), however due to logistical problems samples could not be collected from all sites in each sample round. Samples were collected 4 (9 sites), 6 (15 sites) and 8 (12 sites) weeks after the major flooding events of January 2011 (Figure 1). Sampling commenced on the bottom of low tide at the mouth of the river. Grab water samples were collected in 1 L sterile bottles from 30 cm below the water surface and transported on ice to the laboratory where they were processed within 12 h of collection from the first sampling site.

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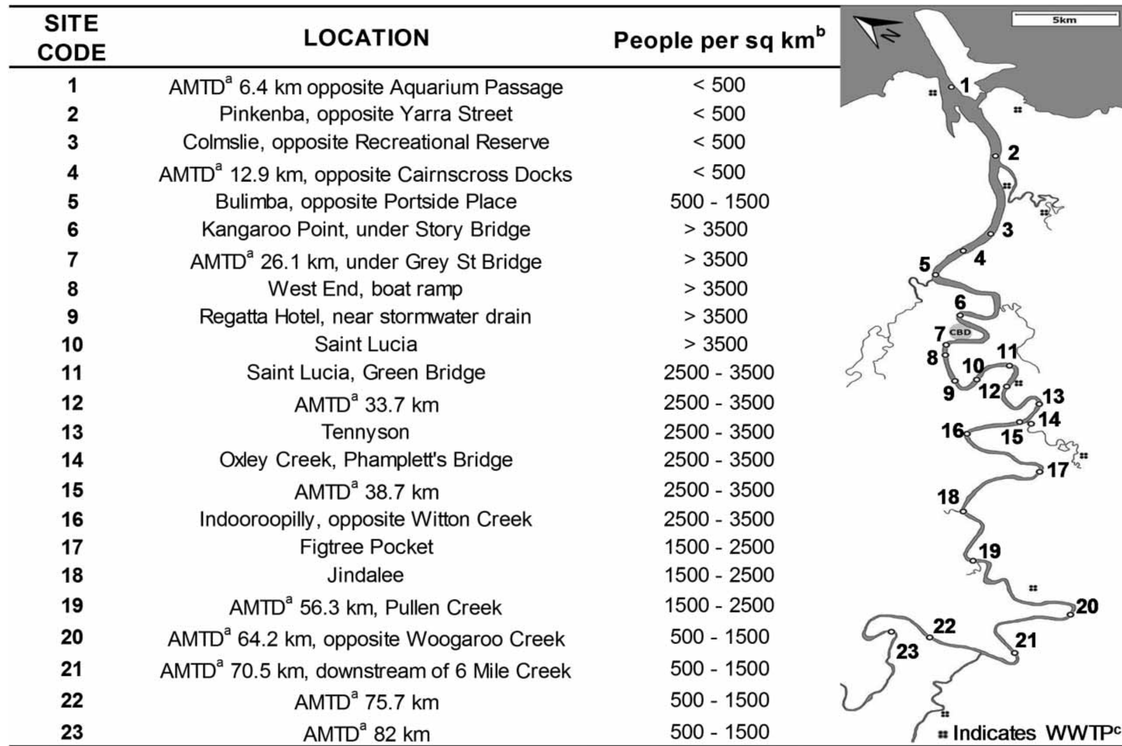

SITE CODE	LOCATION	People per sq km[b]
1	AMTD[a] 6.4 km opposite Aquarium Passage	< 500
2	Pinkenba, opposite Yarra Street	< 500
3	Colmslie, opposite Recreational Reserve	< 500
4	AMTD[a] 12.9 km, opposite Cairnscross Docks	< 500
5	Bulimba, opposite Portside Place	500 - 1500
6	Kangaroo Point, under Story Bridge	> 3500
7	AMTD[a] 26.1 km, under Grey St Bridge	> 3500
8	West End, boat ramp	> 3500
9	Regatta Hotel, near stormwater drain	> 3500
10	Saint Lucia	> 3500
11	Saint Lucia, Green Bridge	2500 - 3500
12	AMTD[a] 33.7 km	2500 - 3500
13	Tennyson	2500 - 3500
14	Oxley Creek, Phamplett's Bridge	2500 - 3500
15	AMTD[a] 38.7 km	2500 - 3500
16	Indooroopilly, opposite Witton Creek	2500 - 3500
17	Figtree Pocket	1500 - 2500
18	Jindalee	1500 - 2500
19	AMTD[a] 56.3 km, Pullen Creek	1500 - 2500
20	AMTD[a] 64.2 km, opposite Woogaroo Creek	500 - 1500
21	AMTD[a] 70.5 km, downstream of 6 Mile Creek	500 - 1500
22	AMTD[a] 75.7 km	500 - 1500
23	AMTD[a] 82 km	500 - 1500

Figure 1 | Map of the Brisbane River with the location of 23 sampling sites. [a]Adopted Middle Thread Distance [AMTD], [b]Australian Bureau of Statistics (2012), [c]Waste Water Treatment Plants located in lower Brisbane River catchment.

Enumeration, isolation and confirmation of *E. coli*

The membrane filtration method was used to enumerate *E. coli* from all water samples. Triplicate serial dilutions of samples were filtered through 0.45 μm pore size nitrocellulose membranes (Millipore, Australia), and placed on modified mTEC agar (Difco, Australia) plates and incubated at 35 °C for 2 h to revive cells and then 44.5 °C for 22 h. *E. coli* were enumerated on plates with 30–300 colonies.

Twelve presumptive *E. coli* were then isolated from each sample, purified twice on MacConkey agar no. 3 (Oxoid, Australia) and stored at −80 °C. Presumptive identification of the isolates was done on SIM and Simmons Citrate agars (Oxoid), and polymerase chain reaction (PCR) was used to confirm identification using a highly specific *E. coli* universal stress protein (*usp*A) gene (Chen & Griffiths 1998).

Sampling site *E. coli* DNA extraction

For whole sampling site *E. coli* DNA extractions a 1 L replicate of each sample was filtered through 0.45 μm pore size nitrocellulose membranes (Millipore), and placed on modified mTEC agar (Difco) plates and incubated at 35 °C for 2 h to revive cells and then at 44.5 °C for 22 h. Depending on the turbidity of water samples which caused clogging during filtration, multiple membranes were used to filter water samples. Filters were then transferred to sterile flasks containing 50 mL of tryptic soy broth (Oxoid) and incubated at 37 °C overnight. This enrichment step was included to ensure low numbers of *E. coli* containing VGs could be detected. DNA was extracted from 2 mL enriched broth using a DNeasy blood and tissue kit (Qiagen, Australia) according to the manufacturer's instructions, and DNA was stored at −20 °C.

Typing of the isolates

In all, 307 *E. coli* strains, from 36 samples across 23 sites, were typed using a high-resolution biochemical fingerprinting method (PhPlate system), specifically developed for typing of *E. coli* strains (PhP-RE; PhPlate AB, Stockholm, Sweden). The fingerprinting method was performed

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according to the manufacturer's instructions and described in more detail elsewhere (Vollmerhausen *et al.* 2011). Strains showing similarity above the established identity level of the system (97.5%) were regarded as identical and assigned to the same biochemical phenotype (BPT), BPTs containing more than one isolate were designated as common (C) BPTs, and those with one isolate as single (S) BPTs.

ABR

A representative of each C-BPT was tested for their resistance to 20 commonly used antimicrobial agents according to the Clinical Laboratory Standard Institute (CLSI) (2011) method. The antimicrobial impregnated discs (Oxoid) tested were cefoxitin (30 μg), tetracycline (30 μg), piperacillin-tazobactam (110 μg), sulphufurazole (300 μg), trimethoprim (5 μg), amikacin (30 μg), cephalothin (30 μg), ampicillin (10 μg), ceftazidime (30 μg), amoxycillin/clavulanic acid (30 μg), chloramphenicol (30 μg), cefepime (30 μg), nalidixic acid (30 μg), gentamicin (10 μg), nitrofurantoin (300 μg), cefpodoxine (10 μg), cefotaxime (30 μg), imipenem (10 μg), cefotetan (30 μg) and aztreonam (30 μg). Extended-spectrum beta-lactamase (ESBL) activity was also tested according to the calibrated dichotomous sensitivity method (Bell *et al.* 2012). The synergy between the beta-lactam antibiotic (AB) disc and clavulanate was monitored by placing a disc of beta-lactam inhibitor/beta-lactam AB combination drug and a disc of beta-lactam AB on an inoculated Mueller-Hinton agar plate 25 mm apart (centre to centre). These included amoxicillin-clavulanic acid (60 μg), ticarcillin-clavulanate (85 μg), cefotaxime (5 μg), ceftazidime (10 μg) cefepime (10 μg) and aztreonam (30 μg). The presence of a clear extension of the edge of the beta-lactam AB inhibition zone toward the disc containing beta-lactam inhibitor was regarded as the ESBL producer. The test was repeated placing the AB discs 20 mm and 15 mm apart (centre to centre) if resistance zones were small making it difficult to identify clear zone between discs. Two ESBL-producing *E. coli* strains K6 and EC10 and a susceptible *E. coli* strain ACM5185 (kindly provided by S. M. Bell, The Prince of Wales Hospital, Sydney, Australia) were included in all tests as the positive and negative controls, respectively.

PCR detection of *E. coli* VGs

From each C-BPT, previously screened for ABR, genomic DNA was extracted using a DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. The DNA of each C-BPT representative and the whole site DNA extracts were tested for the presence of 58 VGs associated with *E. coli* strains causing intestinal and extra-intestinal infections, using a combination of multiplex and uniplex PCR. Primers used for PCR detection of these VGs, the size of the PCR products for corresponding targets and PCR cycling conditions have been previously reported (Chapman *et al.* 2006). The PCR amplification was performed using the Eppendorf Master Cycler (Eppendorf, Germany). PCR products were size-separated on a 2% agarose gel in 0.6 × TBE buffer and visualised following ethidium bromide staining. Identification of the bands was established by comparison of the band sizes with molecular weight markers of 100-bp ladder (Gene-Works, Australia). Samples were considered to be positive for a specific VG when the visible band was the same size as that of the positive control DNA. To identify false positive results, filtered double-distilled water was used as template for the negative control in all experiments.

Cytotoxicity assay

To assess production of Shiga-like toxin (*stx*), Vero cells (ATCC CCL-81) derived from African green monkey kidney cells, were grown to confluence in Eagle's Minimal Essential Media (Lonza, Australia) substituted with 10% foetal bovine serum (Lonza) in a 96-well plate. Bacterial suspensions were prepared as previously described (Fiorentini *et al.* 1998). Cell-free supernatant (100 μL) was diluted (1:10 and 1:100) in sterile phosphate buffer saline (pH 7.4) and inoculated into wells containing confluent Vero cells in 200 μL of AB-free growth media. *E. coli* strains HMLN-1 and JM109 were used as positive and negative controls, respectively. Vero cells were observed at 4, 24 and 48 h after inoculation using an inverted microscope (×400). Production of toxin was concluded when a cytopathic effect was observed in >50% of Vero cells at a dilution of 1:10 or greater (Fiorentini *et al.* 1998). All tests were performed in triplicate.

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Biofilm formation

A representative isolate from each C-BPT was tested for biofilm formation by growing cultures overnight in Luria Bertani broth at 37 °C and diluting 1:100 in M9 minimal salt broth according to Schembri & Klemm (2000). Biofilm formation was tested at 25 °C and 37 °C. Biofilm formation was scored as follows: −, non-biofilm forming (A_{600} nm ≤ 1); +, weak ($1 < A_{600}$ nm $1 \leq 2$); ++, moderate ($2 < A_{600}$ nm ≤ 3); +++, strong (A_{600} nm >3) (Duprè *et al.* 2003). All tests were conducted in triplicate.

Comparison with *E. coli* library from WWTPs

A representative isolate from each C-BPT was also compared against an existing library of *E. coli* fingerprints previously developed in our laboratory from a bigger study that investigated the changes in population structures and survival of *E. coli* in WWTPs in the South-East Queensland region (Anastasi *et al.* 2012; Gündoğdu *et al.* 2013). The library contained *E. coli* ($n = 662$) isolates obtained from two WWTPs within the lower Brisbane River catchment and two WWTPs within the wider South-East Queensland region, collected within three months before and four months after the flood event.

Statistical analysis

A one-way analysis of variance test was used to compare the significance of difference between compared groups and fortnightly sampling rounds.

Linear regression analysis was applied to investigate the degree of correlation between the number of *E. coli* and (1) the number of BPTs at each site, (2) the diversity index of isolates and (3) the number of VGs observed at each site. Linear regression analysis was also used to correlate the number of ABs to which each C-BPT was resistant to and the number of VGs it possessed.

The phenotypic diversity among the isolates was measured using Simpson's index of diversity (Di). Di is high (maximum 1) for a population consisting of different BPTs and is low (minimum 0) if the population consisted of few BPTs. The similarity between *E. coli* populations in two or more samples was calculated as a population similarity (Sp) coefficient. The Sp coefficient calculates the proportion of isolates that are identical in two or more compared bacterial populations (Möllby *et al.* 1993). For example, if two populations contain similar dominating BPTs, the Sp-value is high (maximum 1), but if they contain different BPTs, the Sp-value is low (minimum 0). Clustering of Sp coefficients was performed according to the UPGMA method to yield a dendrogram. In the dendrogram, each line represents the number of isolates in that population. All data analyses were performed using the PhPWIN v6.1 software (PhPlate AB).

A profile of 58 *E. coli* VGs was established from total DNA of each water sample by assigning a value of 1 for the presence of each VG and a value of 0 for its absence. Using the PhPWIN v6.1, similarities among VG profiles were measured after pairwise comparison of the VGs profile of each sample, and the obtained similarity matrix was clustered according to the UPGMA method to yield a dendrogram.

RESULTS

Enumeration of *E. coli*

The highest number of *E. coli* detected during the first round exceeded 50,000 CFU/100 mL at sampling site 9, which is downstream (approximately 4 km) of a WWTP and a major storm water outlet. Apart from this site, the mean number of *E. coli* found in the water samples during round 1 (1,123 ± 602 CFU/100 mL) was significantly higher than that of round 2 (268 ± 183 CFU/100 mL [$P < 0.01$]) and round 3 (121 ± 139 CFU/100 mL [$P < 0.01$]) (Table 1).

Typing of the isolates

A total of 307 isolates were confirmed as *E. coli* using a combination of culture-based techniques and PCR detection of the *usp*A gene. Typing of the isolates using the PhPlate system yielded 31 C-BPTs comprising 243 isolates (79.9%) and 62 S-BPTs. Among the C-BPTs, eight were found in more than one sampling site and on more than one occasion. One C-BPT (i.e., C31) was found in all (except two) sites and in all three sampling rounds (Figure 2).

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Table 1 | Number of *Escherichia coli* (CFU/100 mL) at each sampling site

Site code	Sampling round 1[a,b]	Sampling round 2[a]	Sampling round 3[b]
	Mean ± SD	Mean ± SD	Mean ± SD
1	NT	397 ± 58[c]	NT
2	NT	613 ± 172[c]	393 ± 72[c]
3	NT	NT	23 ± 6
4	NT	197 ± 17[c]	NT
5	NT	NT	20 ± 10
6	1,413 ± 111[c]	317 ± 58[c]	40 ± 10
7	NT	220 ± 49[c]	NT
8	1,680 ± 73[c]	NT	47 ± 6
9	50,867 ± 8,697[c]	NT	93 ± 32
10	1,260 ± 104[c]	NT	NT
11	440 ± 67[c]	NT	73 ± 32
12	NT	395 ± 65[c]	NT
13	1,413 ± 111[c]	NT	113 ± 15[c]
14	693 ± 114[c]	NT	457 ± 140[c]
15	NT	450 ± 22[c]	NT
16	110 ± 14[c]	597 ± 100[c]	70 ± 20
17	1,973 ± 308[c]	263 ± 34[c]	37 ± 31
18	NT	187 ± 71[c]	80 ± 15
19	NT	170 ± 43[c]	NT
20	NT	63 ± 5	NT
21	NT	47 ± 17	NT
22	NT	60 ± 36	NT
23	NT	43 ± 5	NT
Mean ± SD	1,123 ± 602*	268 ± 183*	121 ± 139V*

NT, not tested.
[a,b]Indicates significant difference ($P < 0.01$).
[c]Sites having *E. coli* numbers above the ANZECC/ARMCANZ Recreational Water Quality Guidelines (ANZECC/ARMCANZ 2000).
*$P < 0.01$ for sampling round 1 versus 2 and 3.

Strains of this BPT constituted 23.3% of the total *E. coli* strains tested.

Similarity among the populations of *E. coli* at the different sites was measured as a population similarity (Sp) value. It was found that six sites (cluster B) showed the highest similarity to each other (Figure S1, available with the online version of this paper). Furthermore, eight sampling sites (cluster E) contained BPTs that were totally unrelated to each other (see Figure S1). These sites also contained BPTs found in the main metropolitan area where the river was impacted by the overflow of three WWTPs (see Figure S1). However, there were no differences between the number of VGs and ABR carried by strains belonging to C-BPTs in these clusters (Figure S1).

Correlation between the C-BPT and their virulence properties

Linear regression analysis was applied to each C-BPT and its VGs and ABR to identify if there was any correlation between the number of times a C-BPT appeared across the samples and its pathogenic characteristics. No significant correlation was found to exist between the number of times a C-BPT appeared and the number of VGs it possessed ($P = 0.0686$) (Figure S2(a)) and the number of ABs it was resistant to ($P = 0.7455$) (Figure S2(b)). Furthermore, there was no correlation ($P = 0.2106$) between the number of VGs an individual C-BPT possessed and the number of ABs to which it was resistant (Figure S2(c)). (Figure S2 is available with the online version of this paper.)

Comparison with *E. coli* library from WWTPs

Comparison of C-BPTs identified in this study with those previously isolated from WWTPs showed ten C-BPTs representing 40.6% of the *E. coli* populations had identical fingerprints to *E. coli* isolates derived from WWTPs located within the lower Brisbane River catchment. An additional five C-BPTs representing 7.8% of the *E. coli* populations were also found to be identical to *E. coli* strains from WWTPs in the South-East Queensland region (Figure 2).

Correlation between the numbers of *E. coli* and the presence of VGs

A linear regression analysis was applied to calculate any possible correlation between the number of *E. coli* and the presence of VGs in total DNA extract from each sample. It was found that the number of *E. coli* in the water samples did not correlate ($P = 0.2106$) to the number of VGs (data not shown).

Prevalence of *E. coli* pathogenic determinants

In total, 40 (69%) of the 58 VGs tested were detected in at least one C-BPT and the total DNA extract of water samples.

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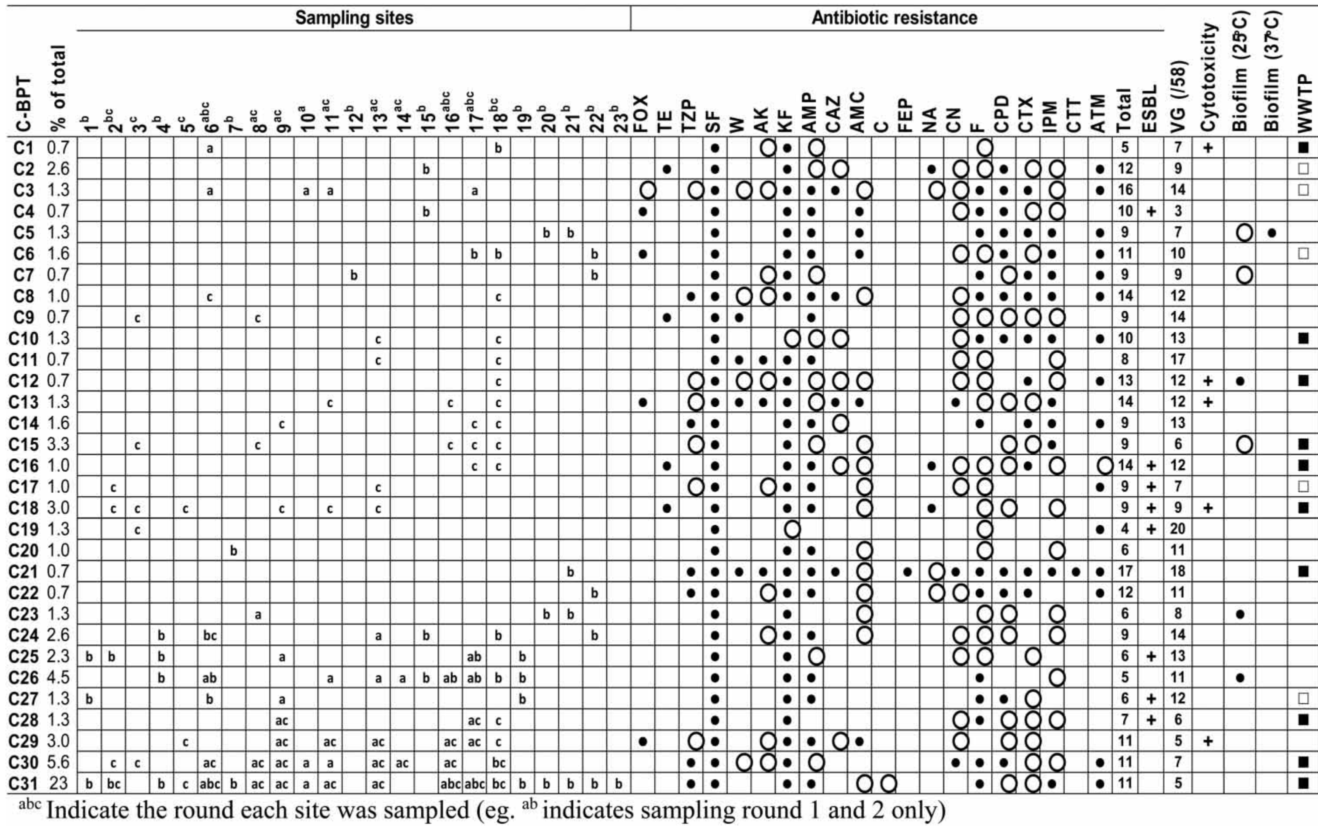

Figure 2 | Common (C) BPTs of *E. coli* found at sampling sites along the Brisbane River across all rounds. A representative isolate from each C-BPT was screened for resistance to different antimicrobial agents, 58 *E. coli* VGs, cytotoxic activity against Vero-cell line and biofilm formation. + indicates production of cytotoxin; ○ indicates weak biofilm production, ● indicates moderate biofilm production. Strains with BPTs identical to those found in WWTP within the catchment ■ or within the region □. For AB resistance, ● indicates resistance and ○ indicates intermediate resistance to ABs tested. FOX, cefoxitin; TE, tetracycline; TZP, piperacillin-tazobactam; SF, sulphurazole; W, trimethoprim; AK, amikacin; KF, cephalothin; AMP, ampicilin; CAZ, ceftazidime; AMC, amoxycillin/clavulanic acid; C, chloramphenicol; FEP, cefepime; NA, naladixic acid; CN, gentamicin; F, nitrofurantoin; CPD, cefpodoxine; CTX, cefotaxime; IPM, Imipenem; CTT, cefotetan; ATM, aztreonam; ESBL, extended spectrum beta-lactamase producing strains.

The most common VGs found in both the C-BPTs (Figure 3) and the total DNA extracts (data not shown) included the extra-intestinal pathogenic *E. coli* (ExPEC) adhesion gene *fim*H (100% for C-BPTs and total DNA extracts), the Shiga-toxin producing *E. coli* (STEC) toxin gene stx_2 (94% and 83%, respectively), the ExPEC non-categorised VG *yja*A and DNA fragment TSPE4C2 (71% and 75% for C-BPTs and 68% and 81% for the total DNA extract, respectively) and the STEC siderophore VG *chu*A (74% and 92%, respectively). The STEC/enteropathogenic *E. coli* (EPEC) toxin gene *exh*A, together with the ExPEC toxin gene *hyl*A and the enteroaggregative *E. coli* (EaggEC) toxin gene *east*1 were only found in one, two and five samples of the total DNA extracts, respectively. Moreover, all C-BPTs were found to possess VGs belonging to two or more *E. coli* pathotypes, including toxin genes (Figure 3).

Strains belonging to different C-BPTs at each sampling round carried a number of VGs ranging from five to 20 (Figure 3). However, the numbers of VGs carried by C-BPTs isolated during each sampling round were not significantly different (Table S1, available with the online version of this paper). When the numbers of VGs in the total DNA extracts were compared across sampling rounds, it was found that samples collected in round 3 carried the highest number of VG, and it was significantly ($P < 0.05$) higher than sampling round 2 (Table S1).

In general, C-BPTs possessing a greater number of VGs and/or were more resistant to the tested ABs, were detected occasionally at few sites and never in more than one sample round. On the contrary, those C-BPTs carrying fewer VGs and/or were less resistant to ABs, were observed on more occasions (Figure 2). These isolates although they were

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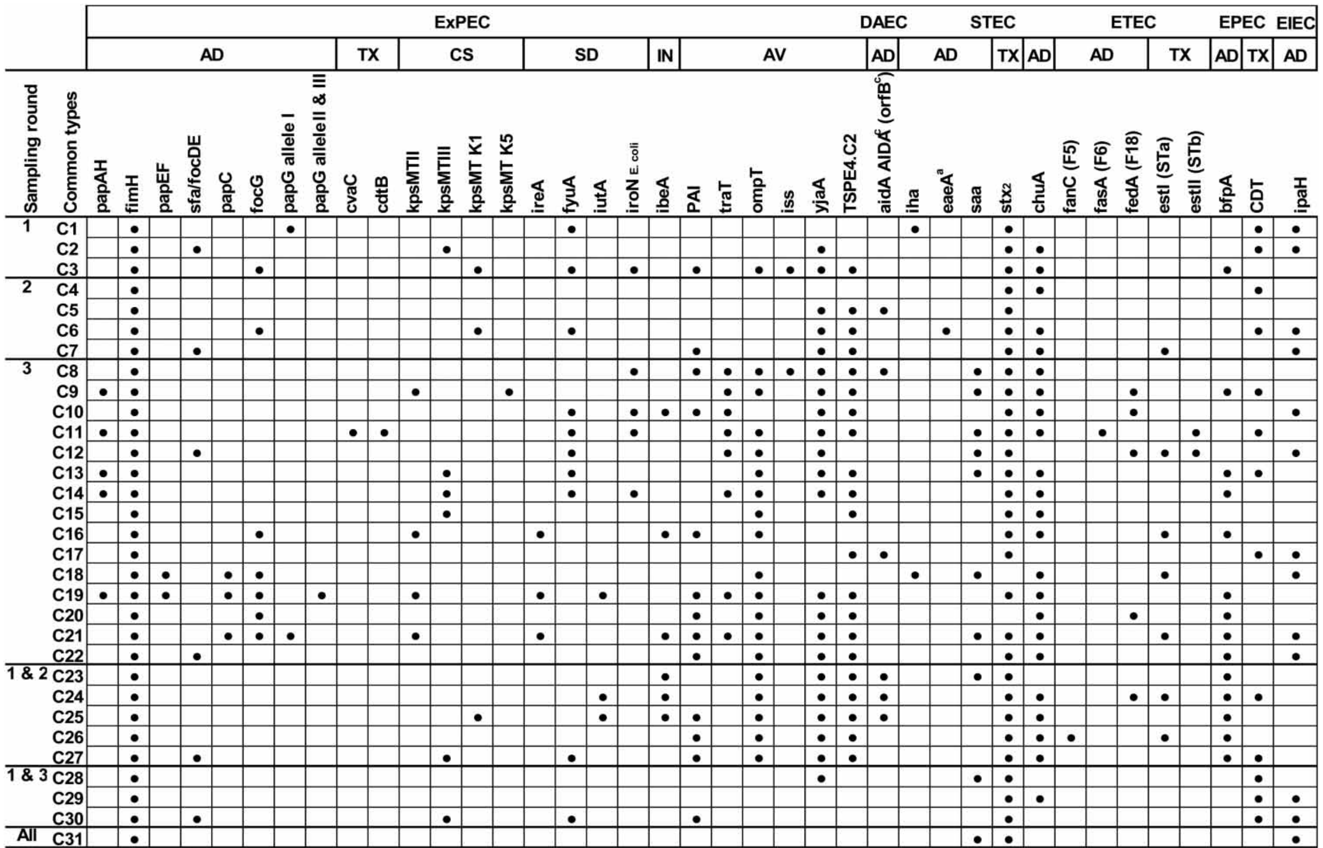

		ExPEC																									DAEC	STEC					ETEC					EPEC		EIEC
		AD								TX		CS				SD				IN	AV						AD	AD			TX	AD	AD			TX		AD	TX	AD
Sampling round	Common types	papAH	fimH	papEF	sfa/focDE	papC	focG	papG allele I	papG alleleII & III	cvaC	cdtB	kpsMTII	kpsMTIII	kpsMT K1	kpsMT K5	ireA	fyuA	iutA	iroN E. coli	ibeA	PAI	traT	ompT	iss	yjaA	TSPE4.C2	aidA AIDA[c] (orfB[c])	iha	eaeA[a]	saa	stx_2	chuA	fanC (F5)	fasA (F6)	fedA (F18)	estI (STa)	estII (STb)	bfpA	CDT	ipaH
1	C1		•					•									•												•			•								•	•
	C2		•		•							•												•						•	•							•	•
	C3		•				•						•			•		•		•		•	•	•	•					•	•						•		
2	C4		•																												•	•							•	
	C5		•																						•	•	•				•									
	C6		•				•						•			•								•	•			•		•	•							•	•
	C7		•		•																•				•	•					•	•				•				•
3	C8		•															•		•	•	•	•	•	•	•			•	•	•								
	C9	•	•									•			•							•	•		•	•				•	•	•			•			•	•	
	C10		•														•		•	•	•	•			•	•					•	•			•					•
	C11	•	•							•	•						•		•			•	•		•	•				•	•	•		•			•		•	
	C12		•		•												•					•	•		•					•	•				•	•	•			•
	C13	•	•										•				•						•		•	•				•	•	•						•	•	
	C14	•	•										•				•		•			•	•		•	•					•	•						•		
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	C21		•			•	•	•				•				•				•	•	•	•		•	•				•	•	•				•		•		•
	C22		•		•																•		•		•	•					•	•						•		•
1 & 2	C23		•																	•			•		•	•	•			•	•							•		
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1 & 3	C28		•																						•					•	•								•	
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All	C31		•																											•	•									•

[a] Indicates shared by STEC and EPEC

Figure 3 | The VG profile of the 31 C-BPTs detected in the water samples. VG are grouped by *Escherichia coli* pathotypes: ExPEC, extra-intestinal pathogenic *E. coli*; DAEC, diffuse adherent *E. coli;* STEC, shiga toxigenic *E. coli*; ETEC, enterotoxigenic *E. coli*; EPEC, enteropathogenic *E. coli*; EIEC, enteroinvasive *E. coli*; AD, adhesin; TX, toxin; CS, capsule synthesis; SD, siderophore; IN, invasin; AV, non-categorised VG.

commonly found in water samples did not form biofilm or exhibit any cytotoxic effects (Figure 2). Of the five C-BPTs that produced biofilm at 25 °C, only one expressed biofilm formation at 37 °C. Cytotoxicity activity was found to be present in C-BPTs isolated in sampling rounds 1 and 3 only (Figure 2).

Diversity of *E. coli* at each sampling site

There was no significant difference between the total numbers of BPTs (both common and S-BPTs) of *E. coli* at each site across the three sampling rounds (Table S1). However, there was a significant ($P < 0.05$) difference in the overall Di between rounds 1 and 2 only, indicating a decrease in overall *E. coli* diversity between rounds 1 and 2 (Table S1). Interestingly, there was no decrease in the total number of VGs detected in each sample between rounds 1 and 2, even though the diversity of the *E. coli* populations decreased. There was a significant ($P < 0.05$) increase in the number of VGs detected in samples collected during round 1 when compared to round 2. This supports the increase in the overall diversity of the *E. coli* populations in round 3 when compared to round 2 (Table S1).

Comparison of *E. coli* VG profiles in water samples

A profile of *E. coli* VGs was created in total DNA extract of water samples in each site and compared with each other. The results indicated many sites had a high similarity coefficient (>80%) with each other (Figure 4). Sites with high similarity in their VGs profile were found to be in close proximity of each other and most of which were from the same sampling round. These sites were located upstream

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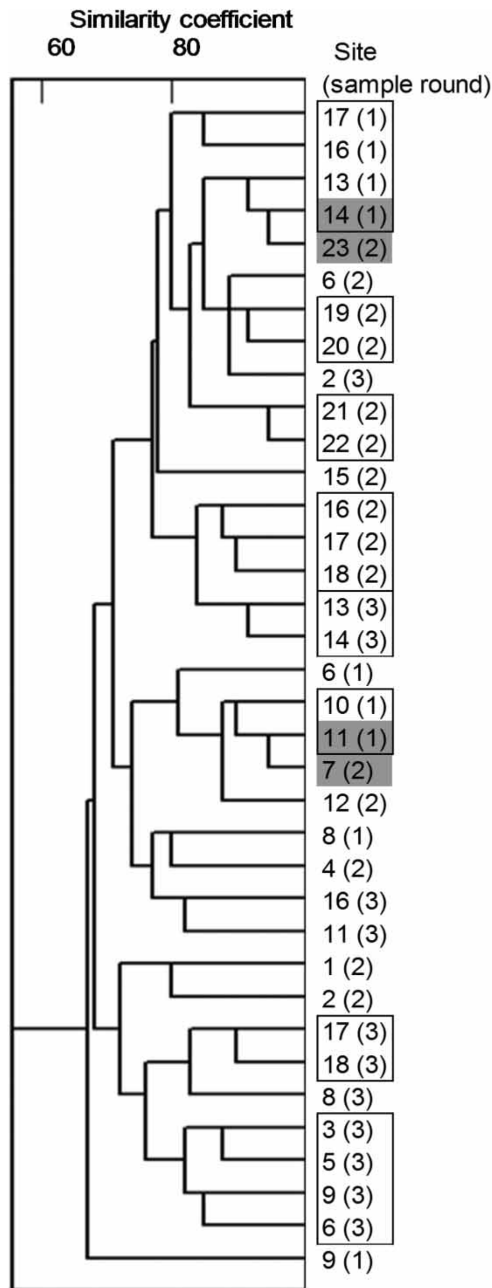

Figure 4 | Similarity among the *E. coli* VG profiles of water samples. The VG profile of each sample was constructed using values of 1 for the presence and 0 for the absence of each gene in the whole DNA extract and clustered using UPGMA. Sites with high similarity during the same sampling round and within direct proximity are indicated in a box. Sites with high similarity irrelevant of sampling round or location are highlighted with grey.

of the river and away from tidal mixing of the river mouth (refer to boxes in Figure 4). Interestingly, the sites with the highest similarity coefficients were sampled at remote locations over different sampling rounds (indicated by shaded boxes in Figure 4).

DISCUSSION

The numbers of *E. coli* in the water samples collected in the first sampling round were above recreational water guidelines and much higher than those collected in subsequent rounds. The first round of sampling coincided with the flood mitigating reservoir gates continuing to release water into the lower Brisbane River catchment and many of the metropolitan WWTPs still redirecting raw effluent into the Brisbane River. The significant decrease in *E. coli* numbers between the first and subsequent sampling rounds could have been attributed to the closing of the upstream Wivenhoe Dam flood gates 1 week prior to the second round of sampling. The numbers of *E. coli* in water samples collected during the third sampling round were mostly below the Australian and New Zealand water guideline for the environmental resources (ANZECC/ARMCANZ 2000). We acknowledge though there are many point and non-point source contributors typical of any large metropolitan river catchment such as large industrial, commercial, residential and semi-rural, rural and agricultural areas that would have influenced the *E. coli* population dynamics of the flood after such an event.

Interestingly, the decrease in *E. coli* numbers across sampling rounds was associated with a significant increase in the diversity of these bacteria and the number of VGs observed in the water DNA extracts. In addition to flood gates closing, these results may also have been the consequence of reduced WWTP overflow entering the waterway, allowing *E. coli* originating from less dominant pollution sources to enter the river. Under these conditions, it is likely that these sources introduced strains with a higher number of VGs. Our observation on the higher number of VGs in water samples collected during the second sampling round supports this possibility.

In all, the number of observed *E. coli* VGs did not decrease during the entire study, and even though the number of *E. coli* decreased significantly, there was no correlation. This is consistent with our previous findings that the number of *E. coli* in a water sample does not necessarily

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reflect the number of VGs carried by these bacteria in the water (Masters *et al.* 2011).

The presence of toxin genes, an indicator of virulence potential, in the C-BPTs and the total DNA extract of water in some sites, suggests that those sites continued to pose a potential public health risk, in spite of many sites returning to an acceptable FIB concentration of *E. coli* by the third sampling round. In our study, all C-BPTs were positive for VGs belonging to more than one *E. coli* pathotype. The most prevalent of the toxin genes, found in all but three C-BPTs, was the Shiga-toxin (*stx*2) gene which is normally associated with STEC *E. coli*, and has been implicated in many cases of swimmers' gastroenteritis (Keene *et al.* 1994; Ackman *et al.* 1997). Wastewaters among others have been shown to be a major source of microorganisms that harbour and express the *E. coli stx* genes (Muniesa & Jofre 2000; Imamovic *et al.* 2010). These genes can withstand thermal stress and chlorination, suggesting the likelihood of their chronic release into adjacent waterways via treated wastewater (Muniesa *et al.* 1999; Imamovic *et al.* 2010). If this was the case, it would be expected that a high percentage of *E. coli* isolated from receiving water bodies harbour the *stx2* gene, as found in this study. The human health risk these strains present however is unknown and the presence of *stx2* and other toxin genes alone does not necessarily indicate bacterial pathogenesis. As shown in our study, only five of the 29 *stx2* positive C-BPTs exhibited cytotoxicity against Vero cells. However, *in vivo* tests would be necessary to confirm their pathogenicity.

The PhP system used in this study to type *E. coli* strains has been used in many epidemiological and ecological studies (Ansaruzzaman *et al.* 2000; Ahmed *et al.* 2006; Anastasi *et al.* 2012; Cassanovas-Massana & Blanch 2013) and was shown to have the same discriminatory power as genotypic methods (Blanch *et al.* 2006; Hamilton *et al.* 2010; Vollmerhausen *et al.* 2011). Using this method, we identified isolates belonging to both C-BPTs and S-BPTs had relatively low diversity among the samples, suggesting that many of the isolates were from similar sources. The fact that almost half of the *E. coli* isolates were found to have identical fingerprints to *E. coli* previously obtained from two WWTPs within the catchment area suggests that these sources may be the dominant contributors of *E. coli* in the river after the flood. However, it is also possible that some of those strains originated from agricultural run-off and/or wild animals. Ideally, concurrent collection and analysis of samples collected at the wastewater outlets would have provided more conclusive evidence, however this was not possible due to the extensive damage to many of the WWTPs facilities within the catchment at the time of sampling.

Population similarity of the C-BPTs indicated high similarity between adjacent sites, however this was not consistent for all sites. Interestingly, one cluster of sites had a significantly higher number of populations than the other population clusters, indicating that these sites contained similar bacterial population possibly introduced from similar sources. Interestingly, we found a similar number of VGs and AB resistance genes across the population clusters.

The largest C-BPT population, consisting of nearly one-quarter of the *E. coli* isolated, was found across all sampling rounds in all but three samples. This C-BPT could possibly represent a naturalised population of *E. coli* existing in the Brisbane River. This is in line with previous studies that found certain *E. coli* populations were persisting within various tropical, sub-tropical and temperate fresh and estuarine waters (Riehle *et al.* 2001; Walk *et al.* 2007; Perchec-Merien & Lewis 2013). *E. coli* populations persistent in environmental waters have shown lineages both distinct to those of host origin and others that are closely related to strains of human and animal origin (Riehle *et al.* 2001; Perchec-Merien & Lewis 2013). Natural selection and the high rate of recombination among closely related lineages enable their persistence in this particular ecological niche (Riehle *et al.* 2001; Gow *et al.* 2008; Perchec-Merien & Lewis 2013), and possibly those long-term surviving *E. coli* populations have since adapted to their aquatic environment. The strains of the dominant C-BPT in our study was found not to harbour many VGs, did not exhibit cytotoxic activity nor produce biofilm. However, the number of ABs to which they showed resistance was similar to other more virulent C-BPTs. The relatively high AB resistance profile of these strains may be the result of prolonged interactions with many *E. coli* of diverse origin. AB resistant genes are typically located on mobile DNA elements such as transposons and plasmids, which facilitate their transfer to other bacteria (Ibekwe *et al.* 2011) and also their distribution within the environment (Smith *et al.* 2002; Ahmed *et al.* 2008). The acquisition of such AB resistant genes could be

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anticipated if the population was indeed naturalised within the catchment, and if this is the case it suggests possessing and retaining these genes may provide niche advantages for those naturalised and/or persistent *E. coli* populations. If such close interactions are occurring, the gross influx of wastewater into receiving waters, during extreme wet weather events, could result in spread of AB resistance at an alarming rate.

The long-term implications of such gross faecal contamination in a metropolitan waterway have yet to be reported. Given the increased likelihood of extreme weather events due to climate change (Kjellstrom & Weaver 2009), the possible health risks associated with an increasing urbanised population need to be studied. A post-analysis survey of the *E. coli* populations to identify the persistence and characteristics of dominant BPTs would provide a robust picture of the dynamics of *E. coli* in this waterway.

CONCLUSION

We suggest that although extensive point and non-point contaminants entered the Brisbane River during the flood event, the *E. coli* population could be largely attributed to a few sources. Although other point and non-point sources may have contributed to the population structure of *E. coli*, the high-volume input from the inundated and damaged WWTPs entering the catchment for up to 10 weeks after the event, suggest that these sources are the primary factor influencing the *E. coli* population dynamics of the river observed during this study. With the increase in urbanised catchments, the close proximity of WWTPs to major waterways and the likelihood of increased incidences of extreme weather events, such as the one seen in this study, it is crucial to adapt better strategies for management of WWTPs in preparation for such events, thus reducing the potential risk posed to public health from untreated wastewater entering the catchment.

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