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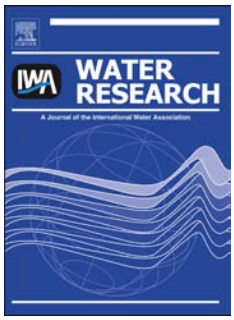

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Survival of *Escherichia coli* in two sewage treatment plants using UV irradiation and chlorination for disinfection

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Running title: Survival of *E. coli* post STP disinfection.

Key words: Sewage treatment plant, disinfection, *E. coli*, chlorination, UV irradiation

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Abstract

We investigated the survival of *E. coli* in two STPs utilising UV irradiation (STP-A) or chlorination (STP-B) for disinfection. In all, 370 *E. coli* strains isolated from raw influent sewage (IS), secondary treated effluent (STE) and effluent after the disinfection processes of both STPs were typed using a high resolution biochemical fingerprinting method and were grouped into common (C-) and single (S-) biochemical phenotypes (BPTs). In STP-A, 83 BPTs comprising 123 isolates were found in IS and STE, of which 7 BPTs survived UV irradiation. Isolates tested from the same sites of STP-B (n=220) comprised 122 BPTs, however, only two BPTs were found post chlorination. A representative isolate from each BPT from both STPs was tested for the presence of 11 virulence genes (VGs) associated with uropathogenic (UPEC) or intestinal pathogenic (IPEC) *E. coli* strains. Strains surviving UV irradiation were distributed among seven phylogenetic groups with five BPTs carrying VGs associated with either UPEC (4 BPTs) or IPEC (1 BPT). In contrast, *E. coli* strains found in STP-B carried no VGs. Strains from both STPs were resistant to up to 12 out of the 21 antibiotics tested but there was no significant difference between the numbers of antibiotics to which surviving strains were resistant to in these STPs. Our data suggests that some *E. coli* strains have a better ability to survive STPs utilising chlorination and UV irradiation for disinfection. However, strains that survive UV irradiation are more diverse and may carry more VGs than those surviving SPTs using chlorination.

1. Introduction

The treatment stages used in many sewage treatment plants (STPs) contain multiple barriers including physical, biological and chemical processes, during which numbers of undesirable microorganisms are reduced in wastewater (Spellman, 2008). STPs are not specifically designed to remove pathogenic microorganisms (Koivunen et al., 2003), but depending on the type of treatment process (i.e. activated sludge) and the disinfection regime, they may remove up to 99.96% of microorganisms (Guardabassi et al., 2002; Johnson & Stell, 2000; Okoh et al., 2007). Disinfection is usually accomplished either alone or as the final 'cleaning' of wastewater in an STP (NHMRC, 2004). Chlorination and other alternative disinfectants, such as ultraviolet (UV) irradiation and ozonation are three of the most common and currently used disinfection regimes worldwide (Hijnen et al., 2006; Koivunen et al., 2005). Factors involved in deciding whether a particular disinfection regime is effective can include the nature and concentration of the disinfection regime, the type of microorganism, suspended solids content, turbidity, temperature, pH and contact time (Ct) (NHMRC, 2004) i.e. the length of time the disinfecting agent and the wastewater remain in contact (Spellman, 2008).

Chlorine, the most widely used disinfectant in various countries (Hijnen et al., 2006), is an oxidant that acts by destroying nucleic acids and cell membranes of microorganisms. Chlorine may be added to effluent as a gas, but can be applied as a liquid or solid (Okoh et al., 2007). The second form of disinfection used is UV irradiation, which is emitted by mercury-amp vapour lamps, and is described as a physical treatment process that leaves no chemical residue (Locas et al., 2008; Okoh et al., 2007). Advantages of chlorination include its availability, cost, ease of dosing control and a moderate to average time for the effluent to pass through the disinfection stage or the hydraulic residence time (HRT). Furthermore, the chlorination process can be effective at inactivating bacterial cells due to its relatively short Ct. It generally requires 20-60min of Ct with chlorine doses of approximately 0.5 mg/L to

ensure that the remaining residual is enough for the continued inactivation of microorganisms downstream. The Ct and doses of chlorine can be dependent on the water quality of the effluent to be treated (NHMRC, 2004) and the amount of organic and particulate material present. In comparison, UV irradiation tends to have a shorter Ct with wastewater. UV irradiation is effective at a very short HRT i.e. 5 - 30sec at doses of 30-60mW.s/cm^2 (Darby et al., 1993; Kay et al., 2008). Despite these, pathogenic microorganisms have been shown to multiply in treated wastewater, during and after treatment (Anastasi et al., 2010). For instance, it has been shown that up to 10^2 CFU/mL survive treatment processes (Reinthaler et al., 2003), some of which may harbour virulence genes (VGs) and/or antibiotic resistance genes that may help them to survive and proliferate in receiving waters. Some strains of *E. coli* with uropathogenic characteristics (UPEC) have also been shown to survive all stages of treatment, including chlorination of effluent that has undergone the activated sludge process (Anastasi et al., 2010). These UPEC strains have also been demonstrated to persist in environmental waters (Anastasi et al., 2012). There have been an increasing number of reports describing the inadequacies of the disinfection processes used in STPs (Hijnen et al., 2006; Tree et al., 2003). Chlorine has been shown to be less effective against *Cryptosporidium* or protozoan oocysts at concentration levels routinely used (Okoh et al., 2007). UV irradiation has also been shown to be less effective against faecal coliforms, such as *E. coli* due to a process called photoreactivation (Hallmich & Gehr, 2010; Locas et al., 2008). *E. coli* strains can sometimes repair and reverse the destructive effects when UV is applied at low doses (Okoh et al., 2007). Nonetheless; high densities of bacteria can be found after any disinfection process (Darby et al., 1993; Okoh et al., 2007).

The aim of the present study was to investigate survival of *E. coli* strains in two STPs using UV irradiation or chlorination for disinfection and to identify whether surviving strains carried any virulence properties.

2. Materials and Methods

2.1. STPs and sample collection

Between September and November 2010, nine weekly samples were collected from two STPs located within a 20 km distance from each other in Southeast Queensland. The two STPs were distinguished by different disinfection processes, with one STP, designated STP-A, using UV irradiation and the second STP, designated STP-B, using chlorination. The characteristics of each STP included in this study are summarised in Table 1. The original sample design was to obtain samples during the dry season (i.e. from September to November); however, due to unexpected torrential rain events, during the nine weeks of sampling, all grab samples were collected on days where rainfall varied from 5 mm to 108 mm per week. Rainfall was recorded according to measurements from the Bureau of Meteorology (BOM) and is given in Table 3. Each week, grab samples were collected from three different sites of STPs, which included influent raw sewage (IS), secondary treated effluent prior to disinfection (STE), and post-disinfected effluent (PD). Samples were collected either in 600 mL sterile disposable sodium thiosulphate treated plastic bottles, or sterile 5 L plastic containers in accordance with Australian/New Zealand Standards (AS/NZS 4276.7:2007) (Standards Australia, 2007). From sites IS and STE, 600 mL grab samples were obtained from both STPs as well as 5 L grab samples from the PD sites. Samples were transferred to the laboratory on ice and processed within a maximum of 4-6 h.

2.2. Sample processing

IS and STE samples were initially serially diluted (10 fold) and filtered through 0.45 µM membrane filters (MSI Westboro, MA), whereas PD samples were filtered without any dilution. All membrane filters were then placed on Difco™ m-FC agar (Bacto Laboratories, NSW, Australia) plates and incubated at 44.5 ℃ for 24 h. From each of the samples, up to 28 *E. coli*-like colonies (where possible) were isolated and stored in nutrient broth (Oxoid, Adelaide, Australia) containing 20% v/v glycerol and stored at –80 °C for further analysis.

2.3. Typing of isolates

A rapid and highly discriminatory biochemical fingerprinting method which has been specifically developed for typing *E. coli* i.e. PhP-RE plates (PhPlate AB, Stockholm, Sweden) was used. In brief, each isolate was suspended in the first well of each row of each plate containing 325 µL of growth medium (0.011% w/v bromothymol blue and 0.1 % w/v proteose peptone) and 25 µL aliquots of the suspension in the first column were transferred into the other 11 wells containing 150 µL of growth medium (Anastasi et al., 2012). Plates were scanned after 7, 24 and 48 h using a HP Scanjet 4890 scanner and images were imported into the PhPlate software (PhPWin4.2) to create absorbance (A_{620}) data from all scanned images, generated from each individual time interval (Anastasi et al., 2010). Using the PhPlate software, the biochemical fingerprint of each isolate was calculated via the mean absorbance values of all readings and the similarity between isolates was calculated as a correlation coefficient and clustered according to the un-weighted pair-group method with arithmetic averages (UPGMA) to yield a dendrogram (Kühn et al., 1991; Saeedi et al., 2005; Sneath & Sokal, 1973). Non-inoculated PhP growth medium served as a negative control and all absorbance values were normalized against the negative control according to the manufacturers' instructions. Positive controls included ten previously typed *E. coli* strains with known biochemical fingerprints (Anastasi et al., 2012). The control strains were also tested in duplicate and the mean similarity between duplicate assays minus two standard deviations was used to calculate the identity level (ID) of the system (Anastasi et al., 2012). Isolates with similarity above the ID of >0.950 were termed common (C-) biochemical phenotypes (BPTs) and those with one isolate were referred to as single (S-) BPTs. Representative isolates belonging to each C- and S-BPT (as identified by the PhPlate software) from both STPs were selected and used for further comparison and/or tests.

2.4. Chromosomal DNA extraction and universal stress protein gene amplification

The universal stress protein A (*usp*A) gene is unique to *E. coli* and can be used to genetically confirm the identification of isolates (Chen & Griffiths, 1998). Chromosomal DNA extraction of all isolates was performed using a modified version of the boiling method as described previously (Anastasi et al., 2012). Extracted DNA (150 µL) from each isolate was transferred to a 1.5 mL Eppendorf tube (Eppendorf, Germany) and stored at –20 ℃. The primer pairs used for identification of the *usp*A gene were F5'-CCGATACGCTGCCAATCAGT-3' and R5'-ACGCAGACCGTAGGCCAGAT-3' (9) at final concentrations of 50 µM, which generate an 884 base pair (bp) fragment. Preparation of the PCR mastermix and amplification cycle used has been described before (Anastasi et al., 2010).

2.5. Phylogenetic grouping

All phenotypically confirmed *E. coli* isolates were subjected to phylogenetic grouping analysis via multiplex PCR amplification using two genes, *chu*A, *yja*A and an anonymous DNA fragment TSPE4.C2 (Clermont et al., 2000). Primer pairs used for the multiplex PCR are included in Table 2. Each of these fragments when amplified produce 279, 211 and 152 bps respectively (Clermont et al., 2000). Preparation of the mastermix and amplification cycle for the multiplex PCR were performed according to Anastasi *et al*. (2010) and strains were assigned to phylogenetic groups A, B1, B2 and D (Clermont et al., 2000), with further classification into their respective genotypes A_0 and A_1 (A phylo-group), B1, (B1 phylo-group) $B2_2$ and $B2_3$ (B2 phylo-group) and D_1 and D_2 (D phylo-group) (Clermont et al., 2011). Positive controls confirmed by sequencing, were included in all reactions and have been previously described (Anastasi et al., 2010). For a negative control, filtered MilliQ water was used (Table 2).

2.6. Detection of *E. coli* virulence genes

Representative strains from each C- and S-BPT were tested for the presence of 11 VGs commonly associated with UPEC or IPEC strains. A series of four multiplex and two uniplex PCR sets were performed using an Axygene Maxygene PCR thermocycler, of which are described in Table 2. The PCR protocol for *pap*AH*, pap*EF, *pap*C*, hly*A, CNF1 and iroN$_{E.coli}$ was modified according to Anastasi *et al.* (2012). The multiplex PCR conditions for *elt*A and *est*II were; denaturation 95 ℃ for 4 min, 94 ℃ for 30 s, 58 ℃ for 30 s, 68 ℃ for 3 min for 25 cycles and a final extension step at 72 ℃ for 10 m in. Positive controls for all VGs are shown in Table 2. All PCR products were separated electrophoretically at 100 V for 90 min on a precast 2 % agarose gel (AMRESCO, Astral Scientific) suspended in 0.6 x TrisBase EDTA (TBE) buffer. Ethidium bromide (0.1 % w/v) was used to visualize each product at their respective size (720 bp (*pap*AH), 336 bp (*pap*EF), 200 bp (*pap*C), 1177 bp (*hly*A), 498 bp (CNF1), 665 bp (iroN$_{E.coli}$), 696 bp (*elt*A), 172 bp (estII), 384 bp (*eae*A), 255 bp (stx_1) and 180 bp (stx_2) under UV light produced by the Syngene camera.

2.7. Antibiotic susceptibility testing

E. coli strains were also tested for their antibiotic susceptibility against 21 antibiotics via the calibrated dichotomous sensitivity (CDS) method (Bell et al., 2006). Briefly, *E. coli* strains were grown on nutrient agar (Oxoid) plates at 37 ℃ for 24 h. One colony of each bacterial strain was inoculated into 2.5 mL of 0.9 % w/v saline solution (Biolab, Ltd, Victoria, Australia). Mueller Hinton agar (Oxoid) plates were then inoculated with 1 mL of the bacterial solution and loaded with selected antibiotics (all from Oxoid). These included ceftazidime (10 and 30 µg), augmentin (60 µg), ampicillin (25 µg), amoxicillin/clavulanic acid (30 µg), ciprofloxacin (5 µg), cefpirome (30 µg), amikacin (30 µg), cefepime (10 µg), cefoxitin (30 µg), cefotetan (30 µg), cephalexin (100 µg), cefotaxime (5 µg), cephazolin (30 µg), gentamycin (10 µg), imipenim (10 µg), nitrofurantoin (300 µg), sulphamethoxazole (100 µg), tazocin (55 µg), tetracycline (30 µg), trimethoprim (5 µg), and ticarcillin/clavulanic acid (85 µg). Plates

were incubated at 35-37 ℃ for 24 h. The CDS method reports drug susceptibilities as either "susceptible" or "resistant" and therefore results were not expressed as minimal inhibitory concentration (MIC) values and interpreted as either 'susceptible' or 'resistant' (Bell et al., 2006).

2.8. Statistical analysis

A Students' T-test was used to compare the significance of data between two STPs and a two-tailed p value of < 0.05 was considered as significant.

3. Results

In all, 370 *E. coli* strains were isolated from the three sampling sites of STP-A (n=146) and STP-B (n=224). The mean number of *E. coli* strains initially detected in samples collected from IS sites of STP-A and STP-B (i.e. 12778 ± 4143 CFU/mL and 11100 ± 4306 CFU/mL) and at the STE sites (i.e. 48 ± 14 CFU/mL and 60 ± 36 CFU/mL for STP-A and B respectively) did not differ significantly over nine weeks of sampling. However, the number of *E. coli* strains isolated from the PD site of STP-A compared to STP-B during the first (8±3 and 0 CFU/mL) and second (14±2 and 0 CFU/mL) weeks of sampling was much higher (Table 3). In general, there was an approximate 2.5 log-reduction (99.5 %) in the *E. coli* population from the IS site to the STE sites in both STPs followed by a further 1.5 log-reduction (95.0 %) in samples collected from PD sites (Table 3). The mean number of *E. coli* strains in collected from the IS of STP-A (12288 ± 4395 CFU/mL) and the same site of STP-B (11000 ± 4567 CFU/mL) (p=0.4387) also did not differ significantly. Similar results were found when the mean number of surviving *E. coli* strains at the STE sites of both STPs (i.e. 48 ± 15 CFU/mL for STP-A versus 60 ± 39 CFU/mL STP-B; p=0.3926) were compared. In weeks 1, 4 and 5, there was a high level of rainfall, which was associated with an increase in the *E. coli* population, particularly in STP-A, which used UV irradiation (Table 3). There was also a higher UV flow rate and a higher chlorine dose reported in STP-A and B respectively.

247 This resulted in the isolation of *E. coli* strains from the PD sites of both STPs only in weeks 1
248 and 2 in STP-A (Table 3).
249
250 Of the 370 *E. coli* isolates among each sampling site from both STPs, a high resolution
251 typing method showed the presence of 22 common (C-) and 78 single (S-) BPTs in STP-A
252 and 37 C- and 87 S-BPTs in STP-B. The number of isolates found in each site as well as the
253 number of C-and S-BPTs they represent is given in Table 4. There was a decrease in the
254 number of both C-and S-BPTs found amongst all sampling sites, and therefore, not all
255 isolates belonging to such C- or S-BPTs, were found in the subsequent sampling sites of
256 both STPs. This indicates that some of the strains belonging to either C- or S-BPTs, did not
257 survive the treatment process. However, the number of S-BPTs was always higher than C-
258 BPTs demonstrating the prevalence of phenotypically different isolates surviving in samples
259 of each site (Table 4). Within each STP, several *E. coli* strains with identical BPTs were
260 found in two or all of the sampling sites in this study. Some of these BPTs contained more
261 than one isolate (i.e. C-BPTs) or represented one isolate (i.e. S-BPTs). In STP-A using UV
262 irradiation, 31 isolates belonging to seven BPTs (i.e. BPTs 1-7) were found in the IS and
263 STE sites and were all detected post-disinfection. In contrast, only 8 strains belonging to two
264 BPTs (i.e. STP-B1 and B2) were found pre- and post-disinfection. BPTs with one isolate
265 were also found in IS, STE as well as PD, demonstrating the survival of strains belonging to
266 the same BPT throughout the subsequent sampling sites (Table 5).
267
268 Five out of the seven surviving BPTs in STP-A (i.e. STP-A1, A2, A3, A5 and A6), harboured
269 one or more VGs mainly associated with UPEC and they belonged to phylogenetic groups
270 B1, $B2_2$, D_1 and D_2. In contrast, strains found in STP-B did not carry any VGs and belonged
271 to phylogenetic group B1 which are normally found in commensal strains of *E. coli* (Table 6).
272 One of the two isolates belonging to BPT STP-A1, although belonging to phylogenetic group
273 D_2, it harboured the *elt*A gene. This gene is responsible for production of heat labile (LT)
274 toxin among *E. coli* causing diarrhoea and is associated with IPEC strains (Table 6).

Within strains of each BPT, different antibiotic resistance profiles were found. The resistance ranged between 1 to 12 antibiotics although the mean number of antibiotics to which surviving strains were resistant, did not differ between the strains of the two STPs (5.5 ± 3.7 antibiotics for STP-A versus 7.8 ± 4.3 antibiotics; p=0.3040) (Table 6). The resistance to sulphamethoxazole and gentamycin was present in all BPTs in both STPs with resistance to amoxicillin and cefoxitin being presented in six BPTs (Table 6).

4. Discussion

Many factors involved in sewage treatment may limit the ability to effectively reduce pathogen numbers in STPs. These are known to include, capacity and the type of treatment process (Kistemann et al., 2008; Koivunen et al., 2003), retention time, other biological flora present in activated sludge, oxygen concentration, pH, temperature and the efficiency of removing suspended solids (NHMRC, 2004; Okoh et al., 2007). Despite these, an optimally functional biological system in an STP should be efficient at reducing pathogen and indicator bacterial numbers. In this study, we investigated the survival of *E. coli* strains during different stages of treatment processes in two STPs using UV irradiation or chlorination. To eliminate the possibility of a coincidence that might happen in a one-off sampling regime, we extended our sampling from all sample sites of both STPs for nine weeks.

Whilst the number of *E. coli* strains in the IS and STE sites did not differ significantly between the two STPs, the number of *E. coli* isolates belonging to different BPTs (C- and S-BPTs) that survived post-UV irradiation was higher than those found in STP-B. Furthermore, the surviving strains in STP-A, harboured VGs as opposed to STP-B, which carried no VGs. Despite this, considering the low number of *E. coli* surviving both STPs, it might be difficult to conclude whether *E. coli* strains carrying VGs have a better survivability against UV treatment (STP-A) than chlorination (STP-B).

The biochemical fingerprinting method (i.e. PhP-RE plates) was used in this study to give a high level of discrimination among the strains found in treatment stages of the two STPs. This method has been shown to have a high discriminatory power (Ansaruzzaman et al., 2000; Saeedi et al., 2005) to type environmental strains of *E. coli* and is a highly useful typing method for an ecological study such as this one. The importance of detecting different BPTs among *E. coli* strains in this study is that strains can be differentiated based on their ability to metabolise different substrates. The system is based on evaluation of the kinetics of 11 highly discriminatory substrates, and the normal evolution will induce changes in the *E. coli* strains making them diverge into different similarity groups (i.e. BPTs) (Kühn et al., 1991). Using this typing system we found that certain BPTs of *E. coli* irrespective of belonging to a C- or an S-BPT were found in two or all sampling sites in both STPs. Some of these BPTs were dominant in the primary stage (i.e. IS sampling site) of sewage treatment and appeared in the subsequent treatment stages, including after UV irradiation in this study. One possible explanation for this is that the higher prevalence of strains belonging to these BPTs (C-BPTs) will give them a better chance to survive treatment to the extent that they can be detected in samples from the subsequent stage(s). Alternatively, it is possible that these strains had a better survivability than other BPTs, or a combination of both. The fact that many S-BPTs were also found to survive different treatment stages supports the latter conclusion. Considering that the surviving strains, particularly in STP-A, carried certain VGs and/or antibiotic resistance genes, we postulate that the presence of VGs and/or antibiotic resistance genes in these strains could have contributed to their survivability during the treatment process as suggested by others (Al-Ahmad et al., 1999; Backhaus & Grimme, 1999).

In our study we found that strains belonging to some of the dominant BPTs from STP-A (i.e. A2 (n = 12) and A3 (n = 9) respectively) and some of the isolates in STP-B (i.e. B2 (n = 6)) were resistant to 11 or 12 antibiotics. Interestingly most of these isolates, although they shared the same BPTs with other strains, they did not carry any VGs. On the contrary, most strains with exhibiting a lower antibiotic resistance profile carried VGs although this was not

329 consistent. One reason for this inconsistency could be due to the small number of isolates
330 tested. In this study, we selected representative strains from each C- and S-BPT from both
331 STPs as they represented a large number of strains or a specific clone in the population and
332 tested them for their antibiotic resistance pattern. Therefore, it is not possible to make a
333 conclusion of whether the antibiotic resistant strains had a better survivability in STP-A
334 opposed to STP-B. Besides, identical strains belonging to the same BPT may independently
335 acquire antibiotic resistance genes STPs. Among the antibiotics tested, the highest
336 resistance among the strains belonged to quinolones such as sulphamethoxazole. The
337 presence of *E. coli* strains in STPs with resistance to penicillin and cephalosporin groups, as
338 well as quinolones have been reported before (Reinthaler et al., 2003). The high presence of
339 strains with resistance to sulphamethoxazole and cephalosporins such cefoxitin, is in
340 agreement with previously published work (Reinthaler, et al. 2003). Some of the surviving
341 strains were resistant to aminoglycosides such as gentamycin. The aminoglycoside resistant
342 phenotype has been reported in *E. coli* and other coliform bacteria in wastewaters from
343 STPs (Stelzer et al., 1988). It is not clear however, that the presence of antibiotic resistance
344 genes has any impact on survival of these bacteria during disinfection process of the STPs.
345 Despite this, Rizzo and co-workers (2013) have shown that the presence of multiple
346 antibiotic resistance genes may have an effect on the survival of *E. coli* after UV irradiation,
347 although this depended on the type of antibiotics. These workers also showed that the
348 disinfection achieved through chlorination would result in a lower inactivation rate of
349 antibiotic resistant *E. coli* strains after 1 h of Ct compared to UV irradiation.
350
351 One the *E. coli* strains that survived post-UV irradiation, harboured the *elt*A gene which is
352 commonly found among IPEC strains causing diarrhoea. *E. coli* strains that survive the
353 chlorination process during STP disinfection have been shown to carry VGs associated with
354 UPEC, but the presence of *elt*A among the surviving strains was not surprising, mainly
355 because STPs receive *E. coli* strains from diverse sources including domestic animals which
356 may carry IPEC strains with toxin genes such as *elt*A or *est*II (Hamilton et al., 2010).

357 It has been generally accepted that the requirement for chlorination of wastewater varies
358 considerably, depending on the quality of the effluent (Lazarova et al., 1999). Higher doses
359 of chlorine are required for low quality wastewater. In this study, a higher dose of chlorine
360 and UV irradiation was applied during weeks where there was a higher level of rainfall (i.e.
361 weeks 1, 4 and 5). During weeks 1 and 2 of sampling, the UV irradiation chamber was
362 functioning at 95% of its capacity. To compensate for this, the UV flow rate was increased (it
363 exceeded the required UV dose of 35mW.s/cm^2). The increased UV flow rate was also
364 introduced during weeks 4 and 5 which resulted in removal of all *E. coli* strains post-UV
365 irradiation. An increase in the chlorination dose of STP-B was also observed in weeks 1, 4
366 and 5 which resulted in total removal of *E. coli* post-chlorination. This possibly occurred, as
367 higher volumes of wastewater are more easily disinfected by manually increasing the
368 chlorine dose rather than increasing the Ct (i.e. decrease the UV flow rate). Nonetheless at
369 no time did either of the STPs exceed their regulatory discharge licence requirements for *E.*
370 *coli*.
371
372 During the design of the study we tried to select STPs that were located within a short
373 distance to avoid the impact of rainfall events and climate conditions on the performance of
374 disinfection process. This also allowed us to make a reasonable comparison between the
375 types of *E. coli* surviving the two disinfection processes. The surviving *E. coli* strains in STP-
376 A belonged to 7 BPTs, five of which carried VGs associated with either UPEC or IPEC.
377 Frigon et al., (2013) demonstrated that between 26 and 51% of the *E. coli* populations in the
378 influent of four STPs using activated sludge were potentially pathogenic. They also reported
379 that extraintestinal pathogenic *E. coli* (ExPEC) formed a majority of the pathogenic strains
380 represented by 24% of all isolates. In contrast, IPEC strains constituted only 10% of the
381 isolates (Frigon et al., 2013). These workers however, did not investigate whether any
382 specific types of ExPEC or IPEC have a better survivability during the STP treatment
383 process. In our study, only one of the seven *E. coli* BPTs (14%) that survived UV irradiation
384 contained VGs associated with IPEC strains. Whether this is merely the result of their

relatively lesser prevalence in STP samples or their decreased survivability compared to UPEC strains, remains to be investigated. Contrary to UV irradiation, only two BPTs, in STP-B, were found post-chlorination and none carried VGs. Strains belonging to these two BPTs were also low in number (only four isolates) and all belonged to phylogenetic group B1 which is considered non-pathogenic (Duriez et al., 2001; Johnson & Stell, 2000). This may indicate that non-pathogenic strains, although small in number, may also survive chlorination which to our judgement is not a common phenomenon (Anastasi et al., 2010). Based on these data we postulate that UV-irradiation might not always be ideal to remove all types of *E. coli* strains, especially if they carry potential pathogenic attributes.

To the best of our knowledge, this is the only study that compares the ability of *E. coli* strains to survive post-disinfection in STPs. The impact of this finding on the presence of potentially pathogenic strains on surface waters and their implication in public health has yet to be identified. The fact that two of the 17 incidences of Shiga-toxin-producing *E. coli* (STEC) infections in the state of Queensland in 2010 have been reported to occur in the same region of this study (Communicable Disease Intelligence, 2012), a source of infection was not identified in most of the cases due to multiple potential sources of infection (e.g. person-to-person contact, food borne, hospital acquired, contact with animals, etc.), and there were no outbreaks associated with any of the cases reported. While this disease may be waterborne, the rates of disease and lack of outbreaks suggest that waterborne transmission was not a primary source of infection for these particular cases observed during this time period (Communicable Disease Intelligence, 2012).

4. Conclusions

The importance of our findings on the survival and release of *E. coli* with potential pathogenic attributes into surface waters indicates higher numbers of isolates may be a potential source of infection in the future. In view of the above, we postulate that a combined UV-irradiation and chlorination process might be a better and more effective system for

412 preventing pathogenic *E. coli* strains to be released into the environment from STPs. Further
413 investigation into this phenomenon is needed.
414
415

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Table 1. Characteristics of the two STPs used in this study. BNR: biological nutrient removal; AS: activated sludge; HRT: hydraulic residence time. IS: Influent raw sewage. STE: Secondary treated effluent.

STP characteristics	**STP-A** (UV irradiation)	**STP-B** (Chlorination)
Equivalent Population (EP) Served	100,000	25,000
Type of sewage	>95% domestic sewage	>95% domestic sewage
Pre-treatment	Band screens Vortex grit tanks	Step screen Aerated grit tanks
Primary treatment (IS)	Primary sedimentation tanks	Nil
Secondary treatment (STE)	BNR AS with 5-stage Bardenpho[a] configuration and secondary clarifiers	BNR AS with anaerobic zone + oxidation ditch configuration and secondary clarifiers
Bioreactor sludge age	16 d	25 d
Tertiary treatment	Cloth filters UV disinfection	Chlorination
[b]**Overall HRT** (at average flows)	42.5h	54.6h
Disinfection HRT (at average flows)	8s	55min
Disinfection dose	35 mW.s/cm^2 (min)	7 mg/L Cl_2 (aver.)
DEHP Licence Disinfection Requirements	1.5 CFU/mL (50 percentile) 6.0 CFU/mL (80 percentile)	1.5 CFU/mL (50 percentile) 6.0 CFU/mL (80 percentile)

[a]5-stage Bardenpho is a configuration used for biological nutrient removal (BNR) following 5 types of tanks in series i.e. anaerobic-anoxic-aerobic-anoxic-aerobic; [b] overall HRT is for all tanks in the entire sewage treatment process

Table 2. Summary of multiplex (sets 1-3, 6-7) and uniplex (sets 4-5) primer sets, amplified product size (bp) and the primer concentration (pmol) used in the PCR amplification of phylogenetic grouping (PG) and 11 *E. coli* virulence genes (VGs) analysed in this study. For each primer set, a specific positive control, previously tested and sequenced was used.

Primer Set	VG	Primer Name	DNA Sequence 5' → 3'	Product size (bp)	Primer Concentration	Positive control
1 (PG)	*chuA*	ChuA.1	GACGAACCAACGGTCAGGAT	279	20 pmol	RBH 2
		ChuA.2	TGCCGCCAGTACCAAAGACA			
	yjaA	YjaA.1	TGAAGTGTCAGGAGACGCTG	211	20 pmol	
		YjaA.2	ATGGAGAATGCGTTCCTCAAC			
	TSPE4.C2	TspE4C2.1	GAGTAATGTCGGGGCATTCA	152	20 pmol	
		TspE4C2.1	CGCGCCAACAAAGTATTACG			
2	*papAH*	PapA-F	ATGGCAGTGGTGTCTTTTGGTG	720	50 pmol	RBH 130
		PapA-R	CGTCCCACCATACGTGCTCTTC			
	papEF	PapEF-F	GCAACAGCAACGCTGGTTGCATCAT	336	50 pmol	
		PapEF-R	AGAGAGAGCCACTCTTATACGGACA			
3	*hlyA*	hly-F	AACAAGGATAAGCACTGTTCTGGCT	1177	50 pmol	RBH 130
		hly-R	ACCATATAAGCGGTCATTCCCGTCA			
	papC	PapC-F	GTGGCAGTATGAGTAATGACCGTTA	200	50 pmol	
		PapC-R	ATATCCTTTCTGCAGGGATGCAATA			
4	CNF*1*	cnf1-F	AAGATGGAGTTTCCTATGCAGGAG	498	50 pmol	RBH 136
		cnf2-R	CATTCAGAGTCCTGCCCTCATTATT			
5	$iroN_{E.coli}$	IRONEC-F	AAGTCAAAGCAGGGGTTGCCCG	665	50 pmol	RBH 136
		IRONEC-R	GACGCCGACATTAAGACGCAG			
6	*eaeA*	eaeA-F	GACCCGGCACAAGCATAAGC	384	50 pmol	*E. coli* 0157:H7
		eaeA-R	CCACCTGCAGCAACAAGAGG			
	stx_2	stx2-F	GGCACTGTCTGAAACTGCTCC	255	50 pmol	
		stx2-R	TCGCCAGTTATCTGACATTCTG			
	stx_1	stx1-F	ATAAATCGCCTATCGTTGACTAC	180	50 pmol	
		stx1-R	AGAACGCCCACTGAGATCATC			
7	*eltA*	LTA-1	GGCGACAGATTATACCGTGC	696	50 pmol	*E. coli* 0149
		LTA-2	CCGAATTCTGTTATATATGTC			
	estII	STb-1	ATCGCATTTCTTCTTGCATC	172	50 pmol	
		STb-2	GGGCGCCAAAGCATGCTCC			

Table 3. *E. coli* population found in sampling sites; influent raw sewage IS, secondary treated effluent (STE), and post-disinfection (PD) sites of STP-A (UV irradiation) and STP-B (chlorination) over nine weeks of sampling. Amount of rainfall (mm) per week (W), UV Flow and Dose and the amount of free and total chlorine are also presented over nine weeks. cfu: colony forming units, L/s: litres per second, and mW.s/cm^2: milliwatts per centimetre squared, mg/L: milligrams per litre. NA: data not available.

STP sites and disinfection regime	**Sampling weeks (level of rainfall)**								
	W1 (86mm)	**W2** (34mm)	**W3** (14mm)	**W4** (108mm)	**W5** (91mm)	**W6** (3mm)	**W7** (16mm)	**W8** (20mm)	**W9** (5mm)
STP-A (UV irradiation)									
UV flow (UV dose)	200L/s (50mW.s/cm^2)	105L/s (96mW.s/cm^2)	140L/s (56mW.s/cm^2)	225L/s (40mW.s/cm^2)	215L/s (41mW.s/cm^2)	140L/s (62mW.s/cm^2)	110L/s (93mW.s/cm^2)	105L/s (77mW.s/cm^2)	110L/s (80mW.s/cm^2)
	Number of *E .coli* isolates (CFU/mL)								
IS	17200±1400	13300±1500	14200±3000	15400±500	16100±1500	3700±200	8800±1700	16000±4900	10300±600
STE	37±12	54±9	38±6	82±16	52±4	45±6	45±2	31±6	48±3
PD	8±3	14±2	0	0	0	1	0	0	0
STP-B (chlorination)									
Free chlorine dose (total chlorine dose)	0.3mg/L (3.5mg/L)	NA (NA)	1.0mg/L (3.2mg/L)	0.1mg/L (2.9mg/L)	0.2mg/L (3.5mg/L)	0.1mg/L (1.4mg/L)	0.1mg/L (0.5mg/L)	0.1mg/L (0.6mg/L)	0.7mg/L (1.5mg/L)
	Number of *E .coli* isolates (CFU/mL)								
IS	7200± 900	16600±2000	14700±900	18900±1700	9000±1100	9600±300	9400±1000	9300±2000	17200±400
STE	13 ± 4	9±1	24±3	109±12	57±9	68±3	74±3	72±1	115±18
PD	0	0	0	0	1	1	0	2	0

Table 4. The number of *E. coli* isolates tested from each sampling site of STP-A and -B. Identical strains were grouped into common (C-) biochemical phenotypes (BPTs) and those with unique BPTs were named as single (S-) BPTs. Within each site several C-and S-BPTs were found.

Sampling site	**STP-A (UV irradiation)**			**STP-B (Chlorination)**		
	No. of *E. coli* isolates tested	**No. of C-BPTs** (no. of isolates found in C-BPTs)	**No. of S-BPTs**	No. of *E. coli* isolates tested	**No. of C-BPTs** (no. of isolates found in C-BPTs)	**No. of S-BPTs**
Influent raw sewage (IS)	71	12 (36)	35	107	20 (69)	38
Secondary treated effluent (STE)	52	7 (23)	29	113	16 (66)	47
Post-disinfection (PD)	23	3 (9)	14	4	1 (2)	2
Total	146	22 (68)	78	224	37 (137)	87

Table 5. *E. coli* strains with identical biochemical phenotypes (BPTs) found in each sampling site of STPs using UV irradiation (STP-A) or chlorination (STP-B) for disinfection. + / – signs indicate the presence and absence of the same isolate in each sampling site. ND: strains belonging to these BPTs were not detected in that sampling site

BPTs found in two or all sampling sites	**Presence of the strains in**		
	Influent raw sewage (IS) (no. of isolates)	**Secondary treated effluent (STE)** (no. of isolates)	**Post- disinfection (PD)** (no. of isolates)
STP-A1	+ (1)	ND	+ (1)
STP-A2	+ (5)	+ (6)	+ (1)
STP-A3	+ (4)	ND	+ (5)
STP-A4	+ (2)	+ (1)	+ (1)
STP-A5	ND	+ (1)	+ (1)
STP-A6	ND	+ (1)	+ (1)
STP-A7	ND	+ (1)	+ (1)
STP-B1	+ (1)	ND	+ (1)
STP-B2	+ (3)	+ (2)	+ (1)

Table 6. Distribution of virulence genes, phylogenetic groups (PG) and antibiotic resistance among *E. coli* isolates found in all sample sites of both STPs.

BPTs (no. isolates)	PG	Virulence genes profile of the isolates	Antibiotic resistance profile
STP-A1 (2)	D_2	-	FOX, CAZ, KZ, AMC (30), AMC (60), AMP, CPO, CL, CTX, TIM, W, FEP
	D_2	*elt*A	CL, CN, KZ, RL, FOX, AMC (30), AMC (60)
STP-A2 (12)	B1	*pap*AH, *pap*EF, *papC*, *hly*A, iroN$_{E.coli}$	RL
	D_2	-	IPM, CTX, CN, RL, FOX, KZ, F, AMC (30), AMC (60), AMP, CTT
STP-A3 (9)	D_2	*pap*AH, *pap*EF, *pap*C, *hly*A, iroN$_{E.coli}$	CL, CN, FOX, KZ, F, RL, CAZ, AMC (30), AMC (60), AMP, TZP, CTT
	B1	-	CN, RL, F
STP-A4 (4)	D_2	-	CAZ, CTX, CN, RL, F
	D_2	-	RL, CL, CN
STP-A5 (2)	$B2_2$	*pap*AH, *pap*EF, *pap*C, *hly*A	AMP, CN, RL, KZ
	A_0	-	RL, AMP, W
STP-A6 (2)	D_1	-	RL, F, KZ, IPM, AK, CN
	D_1	*pap*C, *hly*A	CTX, CL, W, FOX, F, RL, AMP, AMC (30), CTT, KZ
STP-A7 (2)	A_1	-	RL, F, CN
	A_1	-	CL, AK, RL
STP-B1 (2)	B1	-	CL, CAZ, CTX, AK, CN, FOX, KZ, F, RL, AMC (30), AMC (60), AMP
	B1	-	AK, RL, F, FOX
STP-B2 (6)	B1	-	CTX, CAZ, CL, CN, W, RL, KZ, FOX, F, AMC (30), AMC (60), AMP
	B1	-	CN, AK, RL

CAZ: ceftazidime, AMC (60): augmentin, AMP: ampicillin, AMC (30): amoxicillin/clavulanic acid, CIP: ciprofloxacin, CPO: cefpirome, AK: amikacin, FEP: cefepime, FOX: cefoxitin, CTT: cefotetan, CN: gentamycin, IPM: imipenim, F: nitrofurantoin, RL: sulphamethoxazole, TZP: tazocin, TE: tetracycline, W: trimethoprim, CL: cephalexin, CTX: cefotaxime, KZ: cephazolin, TIM: ticarcillin/clavulanic acid.

Highlights

- Certain strains of E. coli have a better ability to survive different treatment stages of sewage treatment plants (STPs) to be released into the environmental waters
- These strains belonged to certain types and most of them carried virulence properties commonly found in E. coli causing intestinal or extraintestinal infections
- Survival of these pathogenic E. coli strains was independent of the type of disinfection process used by STPs, although a higher number of pathogenic strains survive post UV irradiation than post chlorination