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Summary

Antibiotic resistance is one of the biggest threats to human health. Due to the misuse of Beta lactam (β-lactam) antibiotics, some Gram-negative *Enterobacteriaceae* have developed the genes to produce Extended Spectrum Beta Lactamase enzymes (ESBLs), which can render the antibiotic ineffective. In the last ten years, ESBL-producing *Enterobacteriaceae* such as $bla_{\text{CTX-M}}$ groups, bla_{TEM}, bla_{SHV} and bla_{OXA} have risen very rapidly in many countries. Societal changes (e.g. greater international travel) increase the likelihood of ESBLs transmission as individuals excrete the resistant bacterial strains, which can then enter the wider environment through wastewater treatment plants (WWTPs). When WWTP discharge ESBL-producing *Enterobacteriaceae* into the water environment, bacteria can associate with suspended sediment particles, in particular during storm events, then transport a wide range area such as estuaries, the coastline, offshore, leading to be a risk for human health, food and wildlife. This PhD studentship aimed to improve our understanding of the fate of ESBL-producing *Enterobacteriaceae* within a WWTP and following subsequent release. Chapter 3 assessed the presence of ESBL-producing *Enterobacteriaceae* within the WWTP (influent, primary sediment tank, aeration tank and effluent) in the city of Bangor. The treatment of wastewater significantly reduced 99% (*P*-value <0.05) of presumptive *E. coli* and other faecal coliforms (OFCs) between the influent and effluent. However, ESBL-producing *E. coli* and OFCs were detected in effluent site (post UV-disinfection) 2/26 and 5/28, respectively. We estimate that 300 billion each of both ESBL-producing *E. coli* and OFCs enter the water environment per day through the effluent released from this WWTP. Overall, across all sampling times and points, ESBL genes were found in 4/123 of *E. coli* and 18/136 of OFCs. The $bla_{\text{CTX-M}}$ group 1 was the most frequent ESBL gene among *E. coli*, while bla_{SHV} was most predominant in OFCs. In Chapter 4, we investigated how large changes to human population (before and after the students' arrival during "welcome week") might affect the presence of ESBL-producing *Enterobacteriaceae* in the WWTP. Of the *E. coli* isolated, a number of *E. coli* were ESBL-producers (7/208) before welcome week, and a similar number were found after students had arrived (6/238). However, there was an increase in the number of ESBL-producing OFCs recovered (18/155 before and 26/220 thereafter). Of note, the diversity of genes detected increased after the arrival of students (4 vs 6 for *E. coli* and 8 vs. 10 for OFC). The new *bla* genes detected after the arrival of students were $bla_{\text{CTX-M-14}}$, ($bla_{\text{CTX-M-15}}$ + $bla_{\text{OXA-1}}$) and ($bla_{\text{CTX-M-27}}$ + $bla_{\text{OXA-1}}$) in *E. coli*. In OFCs, these were ($bla_{\text{TEM-1}}$ + $bla_{\text{SHV-2}}$), ($bla_{\text{TEM-19}}$ + $bla_{\text{SHV-12}}$ + $bla_{\text{OXA-1}}$), ($bla_{\text{TEM-120}}$ + $bla_{\text{SHV-12}}$ + $bla_{\text{OXA-1}}$) and $bla_{\text{SHV-12}}$. $bla_{\text{CTX-M-15}}$ was the most frequent enzyme-producing *E. coli* in both periods, while the most frequently detected ESBL gene among OFCs was $bla_{\text{SHV-2}}$ and then $bla_{\text{TEM-19}}$ before welcome week, but $bla_{\text{SHV-12}}$ and then $bla_{\text{TEM-1}}$ predominated thereafter. Chapter 5 simulated, in microcosms, the impact of suspended sediment concentrations (low, medium and high) on the inactivation rate of $bla_{\text{CTX-M-15}}$-producing *E. coli* derived from human wastewater in seawater and freshwater under simulated duration of UV light in winter and summer of north Wales. Survival was greater under higher sediment concentrations, and was much better in freshwater than seawater. From all the experiments conducted, it shows that ESBL-producing *Enterbacteriaceae* is prevalent and persistent in WWTP, with an increase diversity of genes during population changes, and higher suspended sediment concentrations could be contribute to the survival of ESBL-producing *Enterobacteriaceae* in the water environment after discharging by WWTP. The presence of ESBL-producing *Enterobacteriaceae* in the water environment poses a risk for human health.

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Abbreviations

AMR	Antimicrobial resistance
ARB	Antibiotic resistant bacteria
ARGs	Antibiotic resistance genes
bla gene	beta-lactamase gene
β-lactam	Beta-lactam
BLAST	Basic Local Alignment Search Tool
BOD	Biochemical oxygen demand
CFU	Colony-forming unit
CPD	Cefpodoxime
CSO	Combined Sewer Overflow
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EC	Electrical conductivity
ESBL–producing *E. coli*	Extended Spectrum Beta-Lactamase -producing *E. coli*
ESBL–producing OFCs	Extended Spectrum Beta-Lactamase-producing other faecal coliform
ESBLs	Extended-Spectrum β lactamases
FTU	Formazin Turbidity Unit
G3CR	3rd generation cephalosporins
HGT	Horizontal Gene Transfer
ISs	Insertion Sequences
K. ascrbara	*Kluyvera ascrbara*
K. georgiana	*Kluyvera georgiana*
K. pneumonia	*Klebsiella pneumonia*
lacZ gene	Gene for β-galactosidase
MGEs	Mobile genetic elements
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
OFCs	Other faecal coliforms
P. aeruginosa	*Pseudomonas aeruginosa*

PBPs	Pencillin-binding proteins
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
rpm	Rounds per minute
S. aureus	*Staphylococcus aureus*
STP	Secondary treatment plant
TEM	Temoneira
TOC	Total organic carbon
TON	Total organic nitrogen
uidA gene	Gene for β-glucuronidase
UTIs	Urinary tract infections
UV	Ultraviolet
VBNC	Viable but non-culturable
WWTPs	Wastewater treatment plants

Chapter 1: Introduction

1 Overview of thesis

1.1 General introduction

This study was funded by a scholarship from the government of the Kingdom of Saudi Arabia. The aim of the work was to explore the role of wastewater treatment plants (WWTPs) in spreading antibiotic resistant bacteria (ARB) in the wider environment.

Resistance to antibiotics has become an increasing cause of mortality and morbidity to society (Ventola, 2015; WHO, 2015). Beta lactam (β-lactam) antibiotics are one of the most significant classes of antimicrobials used for the treatment of infectious diseases in both human and veterinary medicine (Madigan et al., 2012). In the last decade, antibiotic resistance in *Enterobacteriaceae* has risen very rapidly, particularly the spread of extended spectrum-beta-lactamase (ESBL) genes, the most common genes of which are $bla_{\text{CTX-M}}$ groups, bla_{SHV}, bla_{TEM} and bla_{OXA}. The presence of these genes enable these bacteria to disable a wide range of β-lactam antibiotics. ESBL-producing *Enterobacteriaceae* has become a serious issue in public health; leading to the death of around 1,700 people each year in the USA alone (Frieden, 2013).

The ESBL genes originated in several countries around the world, though recent work has shown that they can be mobilised and spread by travellers (Bengtsson-Palme et al., 2015; Tängdén et al., 2010; Hawkey, 2015). Upon becoming infected, individuals excrete ESBL-producing *Enterobacteriaceae* and it is purported that these strains will then enter the wider environment through ineffective treatment at wastewater treatment plants (WWTPs) (Everage et al., 2014). It is well known that WWTPs are considered as an important point for the spread of ESBL-producing *Enterobacteriaceae* in environmental waters (Bréchet et al., 2014). This is largely due to ineffective removal of ARB and antibiotic resistance genes, as reported by Berglund et al. (2015), Everage et al. (2014) and Rizzo et al. (2013).

During storm events, ARB derived from treated effluent can attach with suspended sediment particles that facilitate survival by protecting from UV sunlight radiation and providing substrates (Malham et al., 2014; Lawler et al., 2006). Once in the environment, these bacteria can enter a wide and complex cycle, aiding spread of resistance to areas from food, to wildlife (Wellington et al., 2013). However, Gao et al. (2012) noted a lack of clarity about the prevalence of antibiotic resistance genes and resistant bacteria via wastewater systems. Furthermore, it is unclear the extent to which turbidity levels affect the survival of ABR within

environmental waters post-release from a WWTP (Malham et al., 2014). Moreover, understanding the fate and transport of ARB in sediments is complicated by variable conditions such as waves and tides (Hassard et al., 2016).

There is therefore a real need to improve our understanding of the role of wastewater in the spread of antibiotic resistance. Notably, studies are needed that reveal the persistence of ESBL-producing *Enterobacteriaceae* from WWTP outputs to the water environment, which can lead to further cycling between different environmental compartments.

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Chapter 2: Literature review

2.1 *Enterobacteriaceae*

Enterobacteriaceae are the largest and the most heterogeneous family of bacteria, encompassing over 40 genera and hundreds of species and subclasses (Cullik et al., 2010), which include faecal coliform bacteria. Over the past decade, E*nterobacteriaceae* have become an increasing threat to public healthcare, and present a substantial challenge to infection control (Wellington et al., 2013).

2.1.1 Faecal coliform bacteria

Faecal coliform bacteria are Gram-negative, rod-shaped, oxidase negative bacteria, comprising of aerobes and facultative anaerobes (APHA, 1976). On fermentation, they produce acid and gas from lactose within 48 h at 44 ± 0.5°C (Doyle & Erickson, 2006). Coliforms mostly live in intestinal warm-blooded humans or animals as normal flora. Thus, their presence in the environment indicates faecal contamination source (Wutoret al., 2009). *Escherichia coli* (*E. coli*), *Kebsiella pneumonia* (*K. pneumonia*), *Citrobacter freundii* (*C. freundii*) and *Enterobacter areogenes* (*E. areogenes*) are classified as faecal coliforms, as illustrated in Figure 2.1.

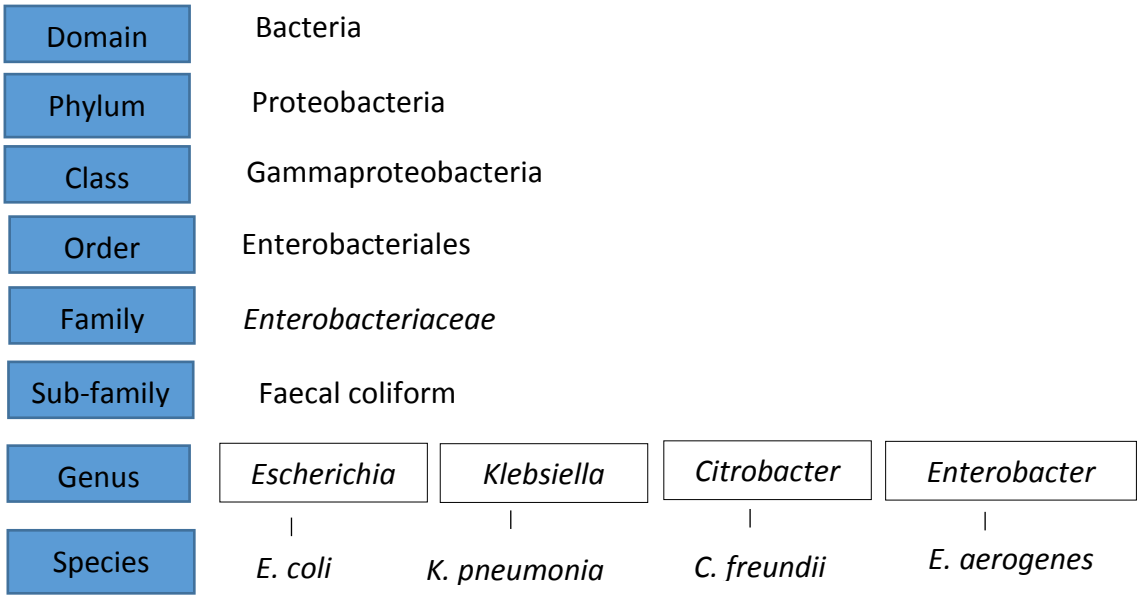

Figure 2.1 Taxonomic classification of *Enterobacteriaceae* bacteria (Faner et al., 2017)

2.1.1.1 *Escherichia coli*

E. coli is a sub-group of faecal coliforms (Doyle & Erickson, 2006). These bacteria exist in human faecal matter at a concentration of approximately 10^8 CFU g^{-1} (Drieux et al., 2016). Most *E. coli* are harmless in humans and animal intestines, but some strains have a variety of virulence factors that cause a significant disease burden, such as over 80% of community-acquired urinary tract infections (UTIs), and also a variety of hospital-acquired infections (Murray et al., 2009). Pathogenic *E. coli* strains can be divided into two types: (i) intestinal pathogenic *E. coli* (IPEC) and extraintestinal pathogenic *E. coli* (ExPEC). IPEC cause a variety of diarrhoeal diseases, and are further classified as enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), necrotoxic *E. coli*, and cell-detaching *E. coli* (CDEC). ExPEC cause urinary tract infections, including uropathogenic *E. coli* (UPEC) (Hamelin et al., 2007).

E. coli can be differentiated from other members of coliforms via biochemical reactions, as they are indole-positive bacteria. Therefore, this organism is used as an indicator of faecal contamination in drinking water and food (Park, 2013). Of course, these are commonly found in wastewater due to human faeces (Gündoğdu et al., 2013).

2.1.1.2 Other Faecal Coliforms (OFCs)

Other faecal coliforms are indole-negative bacteria. Although the U.S. Environmental Protection Agency (EPA) (2014) state that most of these bacteria are not harmful and are part of the normal digestive system, some are pathogenic to humans, such as *Citrobacter*, *Enterobacter* and *Klebsiella* (Cullik et al., 2010). In general, these bacteria live in the intestines of warm-blooded animals, therefore, are also associated with human and animal wastes.

2.2 Antibiotics

Antibiotics are chemical substances that have antagonistic effects on the growth of specific microorganisms. It is calculated that 100,000–200,000 tonnes of antibiotics are produced globally per year (Laxminarayan et al., 2013). Whilst many are used for livestock farming and animal therapy, a significant proportion will be for human therapy (Martinez, 2009). Consequently, hospital and household wastes release many classes of antibiotics into wastewater treatment plants (WWTP) as a significant proportion are excreted in patients' stools or urine (Meena et al., 2015). Globally, the six groups of antibiotics most frequently isolated in WWTP are Beta lactams

(β-lactams), sulfonamides, fluoroquinolones, tetracyclines, macrolides, and other types of antibiotics (trimethoprim, thiamphenicol, chloramphenicol, lincomycin and clindamycin) (Zhang & Li, 2011). β-lactam antibiotics (Cefalexin), for example, have been detected at a concentration of 64,000 ng l^{-1} pre-treatment (raw sewage) and 250 ng l^{-1} post-treatment (effluent) in urban WWTP in Australia (Watkinson et al., 2009). Other antibiotics have been found at high concentrations in raw wastewater, including ofloxacin at 32000 ng l^{-1}, roxithromycin at 17000 ng l^{-1}, and ciprofloxacin at 14000 ng l^{-1} (Meena et al., 2015). Thereafter, antibiotics either degrade or settle in sludge or are released in the environment by treated effluent (Singer et al., 2016).

2.2.1 Antibiotic mode of action and classes

Antibiotics work by targeting particular regions or functions of bacterial cells by inhibiting the multiplication and growth of bacteria, and ultimately causing death. Mechanisms for this include, 1) Inhibition of cell wall synthesis by interference with the enzymes (e.g. β-lactams, vancomycin, cycloserine and bacitracin), 2) Disruption of cell-membrane function (e.g. polymyxins and daptomycin), 3) Inhibition of protein synthesis (e.g. tetracycline, aminoglycosides and macrolides), 4) Inhibition of nucleic acid synthesis (DNA and RNA), and 5) antimetabolite action to block the folic acid synthesis (e.g. trimethroprim and sulphonamides) (Sengupta et al., 2013; Manaia et al., 2012), as illustrated in Figure 2.2.

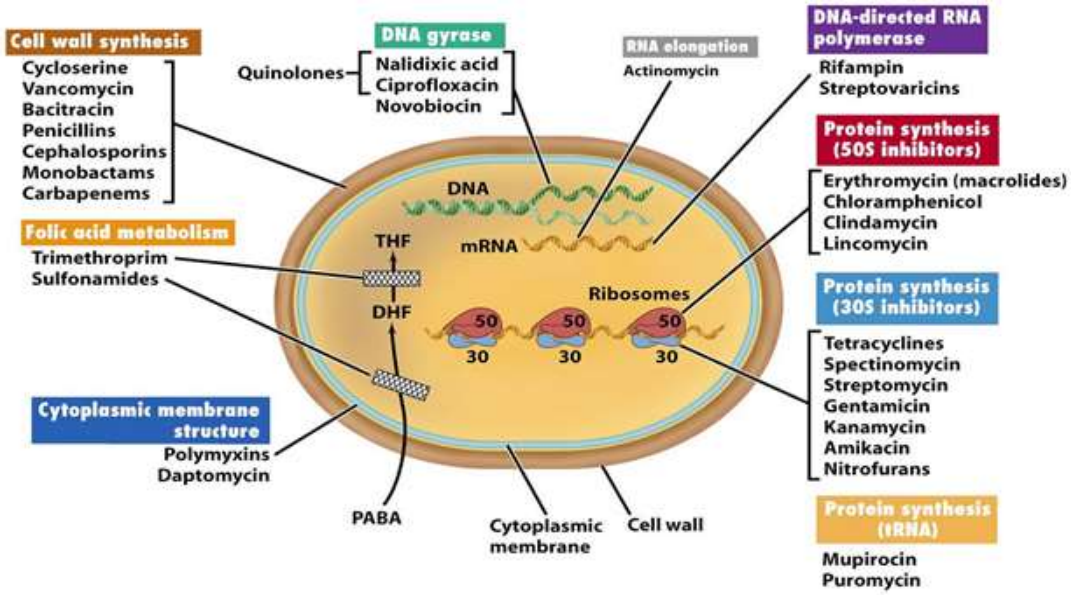

Figure 2.2 Classes of antibiotics or antibacterial agents and their modes of action on bacteria (Madigan et al., 2012)

2.2.2 Beta-lactam antibiotics

β-lactam antibiotics are one of the most significant classes of antimicrobials for the treatment of bacterial infections in both human and veterinary medicine (Li et al., 2007). By weight, these group of antibiotics account for approximately 60% of the total antibiotics used, because of their efficacy and safety (Madigan et al., 2012; Rubtsova et al., 2010; Livermore & Woodford, 2006). β-lactam antibiotic encompasses four major groups: penicillins, cephalosporins, carbapenems, and monobactams (Table 2.1) (Lakshmi et al., 2014).

Table 2.1 β-lactam classes of antibiotics (Ajao, 2011)

Class	Antibiotics	
Penicillins	Narrow Spectrum	Benzylpenicillin, Clometocillin, Benzathine Benzylpenicillin, Procaine benzylpenicillin, Azidocillin, Penamecillin, Phenoxymethylpenicillin, Propicillin, Benzathine phenoxymethylpenicillin, Pheneticillin, Oxacillin, Meticillin, Nafcillin
	Extended Spectrum	Aminopenicillins (e.g. Amoxicillin, Ampicillin, Epicillin), Carboxypenicillins (e.g. Carbenicillin, Ticarcillin, Temocillin) and Ureidopenicillins (e.g. Azlocillin, Piperacillin, Mezlocillin)
Cephalosporins	1st Generation	Cefazolin, Cefacetrile, Cefadroxil, Cefalexin, Cefaloglycin, Cefalonium, Cefaloridine, Cefalotin, Cefapirin, Cefatrizine, Cefazedone, Cefazaflur, Cefradine, Cefroxadine, Ceftezole
	2nd Generation	Cefaclor, Cefamandole, Cefminox, Cefonicid, Ceforanide, Cefotiam, Cefprozil, Cefbuperazone, Cefuroxime, Cefuzonam, Cephamycin Carbacephem
	3rd Generation	Cefixime, Ceftazidime, Ceftriaxone, Cefcapene, Cefdaloxime, Cefdinir, Cefditoren, Cefetamet, Cefmenoxime, Cefodizime, Cefoperazone, Cefotaxime, Cefpimizole, Cefpiramide, Cefpodoxime, Cefsulodin, Cefteram, Ceftibuten, Ceftiolene, Ceftizoxime, Oxacephem
	4th Generation	Cefepime, Cefluprenam,Cefozopran, Cefpirome, Cefquinome
	5th Generation	Ceftobiprole
Carbapenems		Biapenem, Doripenem, Ertapenem, Imipenem, Meropenem, Panipenem
Monobactams		Aztreonam, Tigemonam

β-lactams contain a β-lactam ring in their structure (Figure 2.3), that is considered crucial for antimicrobial interaction (Sherris et al., 2004). This consists of a heteroatomic ring structure of three carbon atoms and one nitrogen atom (Salyers and Whitt, 2005). Each group differs from the other by additional rings, e.g. a β-lactam ring is fused with thiazolidine ring for penicillins, β-lactam ring is fused with dihydrothiazine ring for cephalosporins (Finley et al., 2013), non-fused β-lactam ring for monobactams, and double ring structure for carbapenems (Samaha-Kfoury & Araj, 2003).

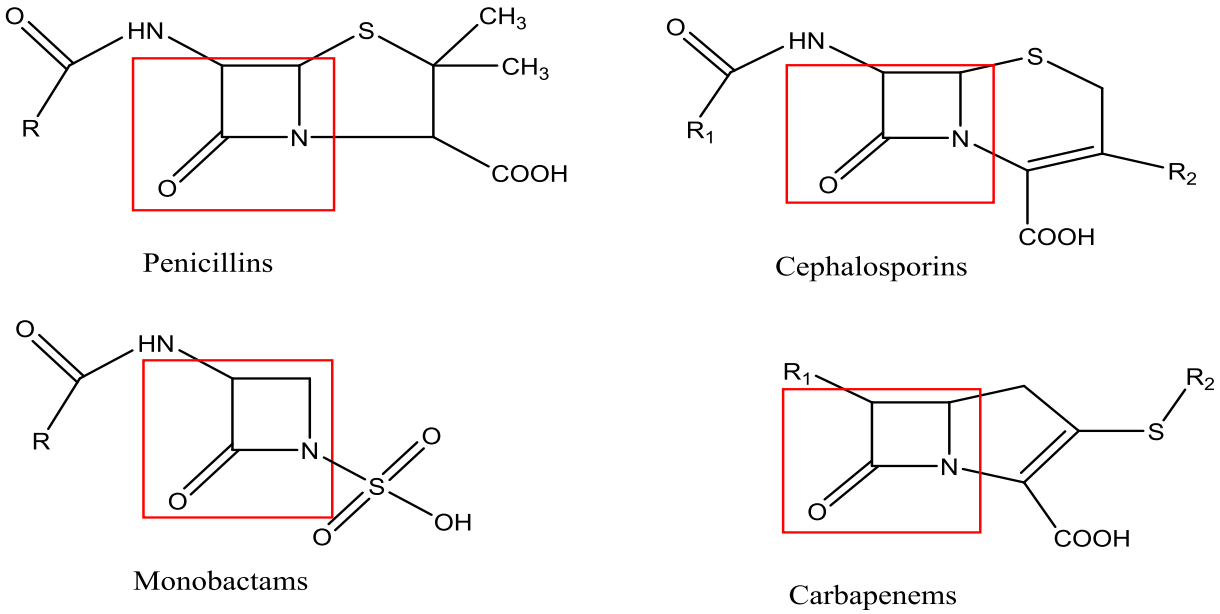

Figure 2.3 β-lactam ring structure (red square) of Penicillin, Cephalosporin, Monobactam and Carbapenem (Rubtsova et al., 2010)

2.2.2.1 History of β-lactam antibiotics

In 1928, penicillin was the first antibiotic discovered by Alexander Fleming in England (Li et al., 2004). He understood that a strain of the mould *Pencillium* produced a diffusible antibacterial agent that inhibited the growth of bacteria. Fleming did many studies on the filtrate from liquid cultures of *Pencillium*. He interpreted that the antibacterial activity of penicillin *in vitro* when injected into mice and rabbits was non-toxic. However, he failed to show its ability to overcome bacterial infection (Rolinson, 1998). Several years later, in 1940, Howard Florey and Ernst Chain illustrated that penicillin was highly effective against a streptococcal infection (Goldsworthy & McFarlane, 2002). In 1943, penicillin was used to treat those wounded in battle during the World War (Demain and Arnold, 1999). In 1944, streptomycin was discovered by Selman Abraham Waksman and the first antibiotic useful for infections caused by Gram-negative bacteria (Kingston, 2000). The period between the 1950s and 1960s led to the development of many new classes of antibiotic which were more efficient in treating infectious diseases (Aminov, 2010). For instance, beta-lactamase inhibitors (e.g. amoxicillin plus clavulanic acid) was a good treatment for infections caused by the *Enterobacteriaceae* family (Wellington et al., 2013). In the 1970s, there was a gradual decrease in the development of antibiotics and it appeared that some bacteria were developing resistance (Aminov, 2010). In the 2000s, bacterial production of Extended-Spectrum Beta-Lactamases (ESBLs), enzymes that can render β-lactams inactive, became increasingly prevalent, causing increasing morbidity and mortality (Wellington et al., 2013). Concurrently, the development of novel antibiotics rapidly reduced (Da Costa et al., 2013).

2.2.2.2 Mechanism of β-lactam antibiotics

The function of the cell wall is to maintain the characteristic shape of bacteria and prevent it from bursting due to osmotic pressures (Kohanski et al., 2010). The structure of cell wall of Gram-negative bacteria is more complex than those of Gram-positive bacteria. The former contain an extra layer of cells, called the outer membrane, which comprises one layer of phospholipids and one layer of lipopolysaccharide (Salyers and Whitt, 2005), conferring greater barrier to many antibiotics than Gram-positive strains (Greenwood et al., 2006). Peptidoglycan is a substantial part of the bacteria cell wall in both Gram-positive and Gram-negative bacteria (Figure 2.4).

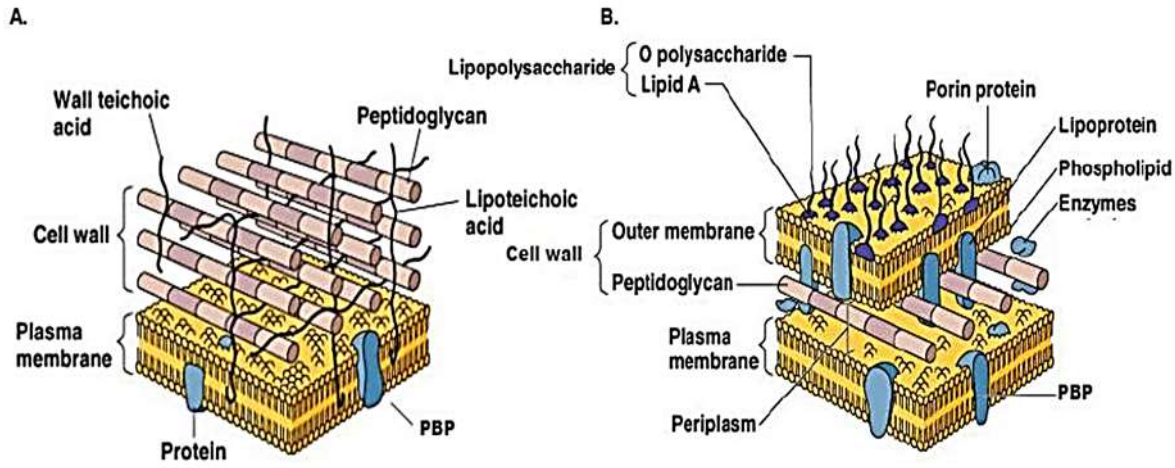

Figure 2.4 (A) Gram-positive and (B) Gram-negative cell wall structures (Tortora et al., 2010)

It is a complex meshwork of cross-linked glycan chains (Salyers and whitt, 2005), consisting of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid alternatively, (NAM) linked by a peptide cross-bridge (Hogg, 2013). During the cell wall synthesis, transpeptidase enzymes create peptide cross bridges that provide strength to peptidoglycan (Figure 2.5). Penicillin-binding proteins (PBPs) are set in the cytoplasmic membrane of bacterium (Pitout et al., 1997).

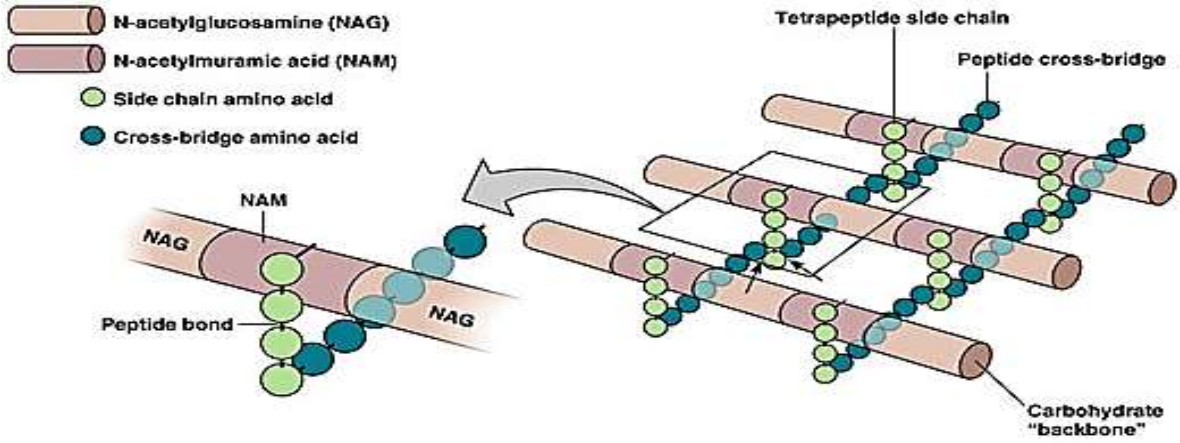

Figure 2.5 Peptidoglycan layer (AG Scientific, 2014)

In Gram-negative bacteria, β-lactam antibiotics pass through the outer membrane by the porin channels to PBPs that are in the periplasmic location, and then inhibit the growth or multiplication of bacterial cell wall synthesis (Pitout et al., 1997). As a result, the function of transpeptidase enzymes from the peptide cross bridge is blocked, thus killing the bacteria (Sosa et al., 2010).

2.3 Bacterial resistance to β-lactam

Bacterial resistance to β-lactam antibiotics includes a variety of mechanisms, particularly amongst Gram-negative bacteria, 1) changes to the antibiotic target site of PBPs, in order to reduce affinity for β-lactam antibiotics or the lack of antibiotic target, 2) low permeability of the bacterial cell wall leading to unreached target sites or moving the drug out of the cell by efflux pumps, contributing multi resistance to the bacteria against the antibiotic, 3) production of β-lactamase enzyme that degrade the β-lactams' activity or stop the reaction of the antibiotic (Galán et al., 2013; Saini, 2012; Van Hoek et al., 2011) (Figure 2.6).

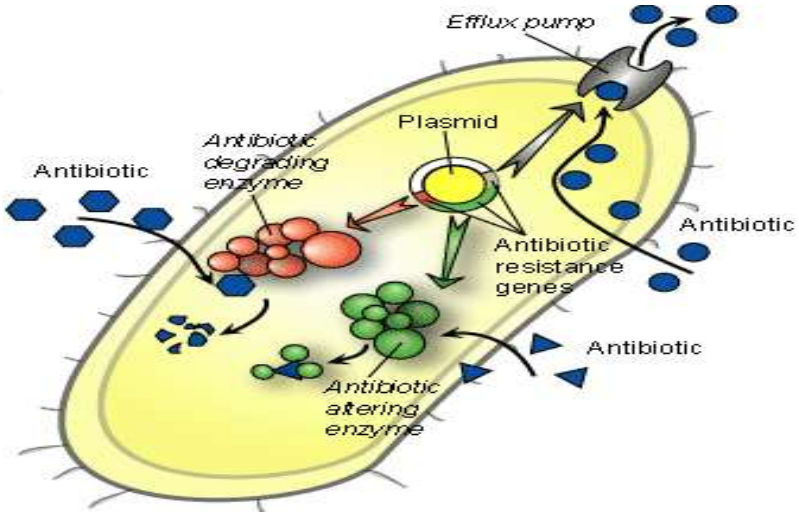

Figure 2.6 Mechanisms of β-lactam resistance (Sosa et al., 2010)

2.3.1 β-lactamase enzymes and their classification

β-lactamase enzymes are the most important resistance mechanisms to inhibit β-lactam antibiotics by hydrolysing the β-lactam ring (Kong et al., 2010). These enzymes cleave the peptide

bond in the β-lactam antibiotics, which leads to deactivation (Zhivich, 2017), as illustrated in Figure 2.7.

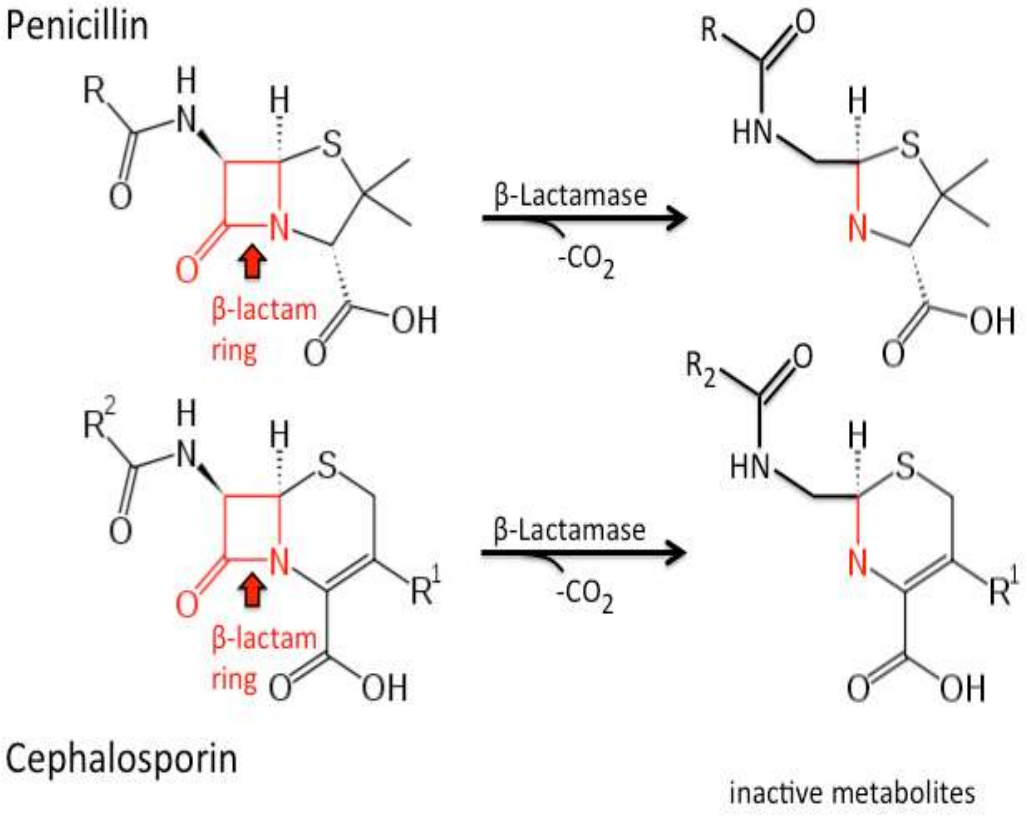

Figure 2. 7 Inactivation of β-lactam antibiotics by β-lactamase enzyme (http://tmedweb.tulane.edu/pharmwiki/doku.php/betalactam_pharm)

In the first set, as shown in Figure 2.8, the enzyme binds non-covalently with the β-lactam antibiotic to become the non-covalent complex by a water molecule attached to the amino acid. The free hydroxyl on the side chain of a serine residue at the active site of the enzyme attaches the carbonyl carbon atom of the antibiotic ring causing a covalent acylation. Ultimately, the protonation of the β-lactam nitrogen and cleavage of the C-N bond means the antibiotic is inactivated (Livermore, 1995).

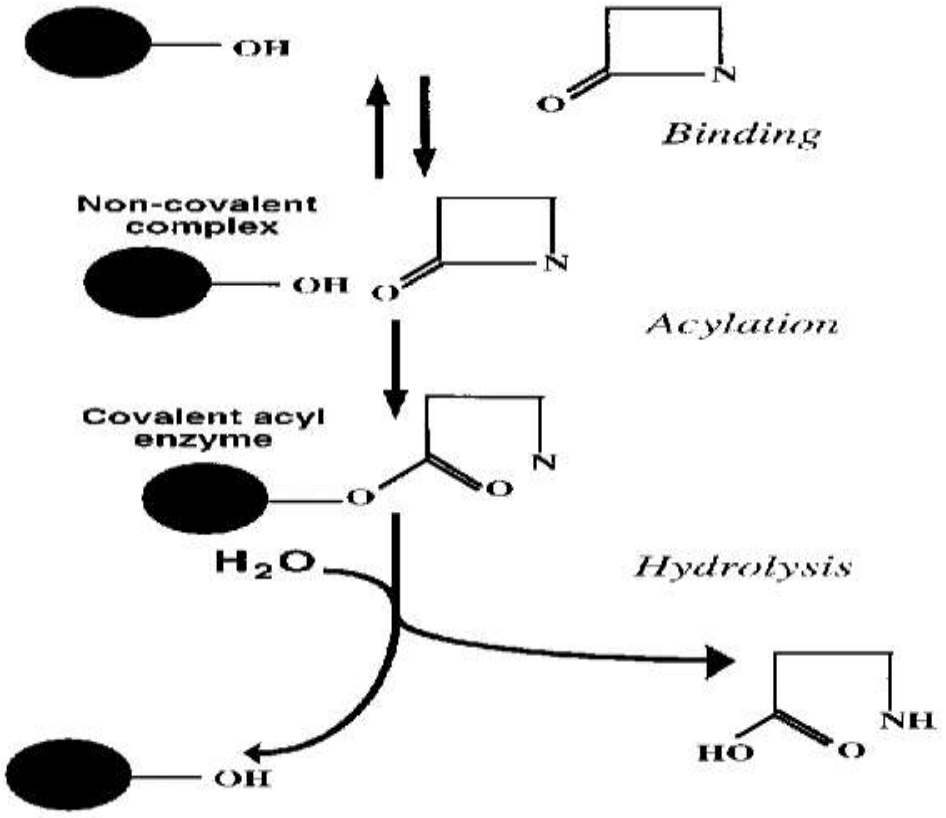

Figure 2. 8 β-Lactamases hydrolyse the β-lactam ring (Livermore, 1995)

These enzymes have been found more common in Gram-negative bacteria than positive bacteria (Cantónv et al., 2012). The first β-lactamase enzyme was discovered in *E. coli* before the use of penicillin in healthcare settings; more than 850 protein sequences for β-lactamases are known (Drawz and Bonomo, 2010). Another study discovered that about 1000 enzymes are associated with β-lactamase groups, and these enzymes have been rapidly increasing in number for more than two decades (Laxminarayan et al., 2013).

Classification schemes have been described for β-lactamase enzymes, as shown in Table 2.2. The first molecular classification based on amino acid sequence homology was identified by Amber and co-workers (Amber classification) and are divided into four classes (A, B, C and D) (Drawz and Bonomo, 2010; Bush and Jacoby, 2010). Classes A, C, and D are represented by serine β-lactamases mechanism, whereas class B act as metallo β-lactamases which need zinc for their action (Hall and Barlow, 2005).

Secondly, functional classification schemes of β-lactamases have been divided to up to three groups and subgroups (a-f), such as 2a, 2b, 2be, 2c, 2e/f, 1, 2d, 2df, and 3 based on activity and

inhibitor profile (Drawz and Bonomo, 2010). Activity group 1 cephalosporinases (molecular class C) are those which are not well inhibited by clavulanic acid. Group 2 β-lactamases (classes A and D) are those inhibited by clavulanic acid, and the group 3 metallo β-lactamases require a zinic ion at the active site (Bush and Jacoby, 2010).

Table 2.2 Classification scheme of β-lactamases (Pfeifer et al., 2010; Drawz and Bonomo, 2010)

Ambler class	Group	β-lactamases	Bacterial strains	Examples of enzymes	Agents inactivated
A **(Serine-β-lactamases)**	2a	Penicillinases	*Staphylococcus aureus*	PC1	Penicillin
	2b	Penicillinases Cephalosporinases	*Enterobacteriaceae*	bla_{TEM-1}, bla_{TEM-2}, bla_{SHV-1}, bla_{SHV-11}	Ampicillin
	2be	Extended spectrum β-lactamases (ESBLs)	*Enterobacteriaceae*	bla_{TEM-3} – $bla_{TEM-167}$, bla_{SHV-2} – $bla_{SHV-117}$, all bla_{CTX-M}, PER/VER/GES	Penicillins, 3rd generation Cephalosporins (G3CR)
	2br	Penicillinases	*Enterobacteriaceae*	bla_{TEM-30} – 41, 44, 45, 51 and 54; bla_{SHV-10}	Penicillins
	2c	Carbapenemases	*Enterobacteriaceae*	PSE/NMC/IMI/KPA-1/2/3, GES-2 and bla_{SHV-38}, SME/KPC	Penicillins, Carbapenems
	2e/f	Cephalosporinases	*Enterobacteriaceae*		Cephalosporins /Monobactams
C **(Serine-β-lactamases)**	1	Cephamycinases	*Enterobacter* spp. *Citrobacter* spp.	AmpC	Cefoxitin, G3CR
D **(Serine-β-lactamases)**	2d	AmpC Cephamycinases	*Enterobacteriaceae*	CMY, DHA, MOX, FOX, ACC	Cefoxitin G3CR
		Broad spectrum β-lactamases	*Enterobacteriaceae*, *A. baumannii*	bla_{OXA-1}, bla_{OXA-9}	Oxacillin, Ampicillin
		ESBL bla_{OXA}	*Enterobacteriaceae*, *A. baumannii*		Penicillin, G3CR
	2df	Carbapenemases	*Enterobacteriaceae*, *A. baumannii*	bla_{OXA-48}, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-25}	Carbapenems
B **(Metallo-β-lactamases)**	3	Carbapenemases	*Enterobacteriaceae*, *P. aeruginosa,* *A. baumannii*	VIM, IMP, NDM-1	All β-lactams except Monobactams
Not classified	4	Not determined			

2.4 Extended-Spectrum β-Lactamases (ESBLs)

Exposure to a multitude of β-lactam antibiotics have allowed bacteria to produce novel β-lactamase enzymes; so-called Extended-Spectrum β-lactamases (ESBLs) (Pitout et al., 2005; Samaha-Kfoury and Araj, 2003). ESBL enzymes attribute to the molecular class A and D β-lactamases, which confer resistance to penicillins, cephalosporins, monobactams, and are inhibited by clavulanic acid, but not the cephamycins (e.g. cefoxitin and cefotetan) and carbapenems (e.g. imipenem, meropenem, and ertapenem) (Pitout and Laupland, 2008; Bush and Jacoby, 2010).

2.4.1 ESBLs Types

Many ESBLs genotypes have been isolated in clinical settings (Pitout et al., 2008), the majority of ESBLs (class A) identified as *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$ types (Cantón et al., 2012; Pitout et al., 2005), and ESBL (class D) such *bla*$_{OXA}$ (Bradford, 2001). These enzymes are most prevalent in *Enterobacteriaceae* members such as *E. coli* and *Klebsiella pneumonia* (Pitout et al., 2005).

2.4.1.1 *bla*$_{TEM}$

By the middle of the 1960s, the first *bla*$_{TEM}$ enzymes were identified from *E. coli,* which is so-called *bla*$_{TEM-1}$ in Greek (Paterson & Bonomo, 2005). *bla*$_{TEM-1}$ is able to degrade β-lactam antibiotics such as penicillins, cephalosporins (Shaikh et al., 2015). *bla*$_{TEM-2}$ is derived from *bla*$_{TEM-1}$ with a similar sequence of β-Lactamases, but distinguished by one amino acid substitution (Paterson and Bonomo, 2005). In the year 1984, the new plasmid encoded *bla*$_{TEM-1}$ enzyme was isolated from *K. pneumonia* in France, which conferred resistance to cefotaxime (Saladin et al., 2002). The enzyme, named *bla*$_{TEM-3}$, differed from *bla*$_{TEM-2}$ by two amino acid substitutions (Paterson and Bonomo, 2005).

Around 170 types of *bla*$_{TEM}$ enzymes exist, and the beta-lactamase database (http://www.lahey.org/studies) is steadily increasing (Salverda et al., 2010); with > 90 types of *bla*$_{TEM}$ known ESBL (sub-group 2be) and the rest are non ESBL (sub-group 2b, 2br and 2ber) (Rubtsova et al., 2010; Bradford, 2001). The most predominant ESBL- *bla*$_{TEM}$ isolated globally are *bla*$_{TEM-3}$, *bla*$_{TEM-5}$, *bla*$_{TEM-10}$, *bla*$_{TEM-12}$, *bla*$_{TEM-26}$ and *bla*$_{TEM-52}$ (Bradford, 2001). The ESBL- *bla*$_{TEM}$ types are usually carried on the cell plasmid and result from mutations in *bla*$_{TEM}$ genes (*bla*$_{TEM-1}$ and *bla*$_{TEM-2}$) by one or many amino acid substitution (Ur Rahman et al., 2018).

2.4.1.2 *bla*$_{SHV}$

bla$_{SHV}$ enzymes (*s*ulf*h*ydryl *v*ariable) are predominantly isolated in *Klebsiella* spp. (Samaha-Kfoury and Araj, 2003) and are the second most prevalent after *bla*$_{TEM}$ enzymes (Rubtsova et al., 2010). Furthermore, 68% of *bla*$_{TEM-1}$ and *bla*$_{SHV-1}$ are of similar sequence in β-Lactamases (Reynolds et al., 2006).

The *bla*$_{SHV}$ enzyme was recognised in *K. ozaenae* in the early 1980s in Germany and they are capable of breaking down cefotaxime, and to lesser extent ceftazidime (Paterson and Bonomo, 2005). The first SHV-1 was found in *K. pneumonia* and has the capability to hydrolyse penicillin antibiotics (Shaikh et al., 2015). Approximately 200 *bla*$_{SHV}$ types of β-lactamase have been isolated in healthcare facilities, most commonly from the *Enterobacteriaceae* family, and *Pseudomonas aeruginosa* (*P. aeruginosa*) (Bin Li et al., 2009; Paterson & Bonomo, 2005). *bla*$_{SHV}$ types have divided into three categories, 1) ESBL- *bla*$_{SHV}$ (belong to subgroup 2be (*n* = 46) degrade oxyimino-cephalosporins such as cefotaxime, ceftazidime and aztreonam), 2) non-ESBL- *bla*$_{SHV}$ (belong to subgroup 2b (*n* = 37) degrade penicillins and cephalosporins and well inhibited by clavulanic acid and tazobactam), 3) others types of *bla*$_{SHV}$ (*n* = 106) such subgroup 2br (*n* = 7) broad-spectrum β-lactamases pose resistance to clavulanic acid and the rest (*n* = 99) are not classified because of the unclear of biochemical detection (Liakopoulos et al., 2016). The most dominant of ESBL- *bla*$_{SHV}$ are *bla*$_{SHV-2}$, *bla*$_{SHV-5}$, *bla*$_{SHV-12}$, *bla*$_{SHV-29}$, *bla*$_{SHV-152}$, *bla*$_{SHV-153}$, *bla*$_{SHV-160}$, and *bla*$_{SHV-165}$ (Liakopoulos et al., 2016). The first *bla*$_{SHV-2}$ plasmid encoded of ESBL isolated in 1983 was resistant to G3CR (Liakopoulos et al., 2016). These enzymes result from mutations and these genes could move to other bacteria by plasmids that acquire resistance to β-lactams antibiotics (Bourouis et al., 2015).

2.4.1.3 *bla*$_{OXA}$

bla$_{OXA}$ enzymes were refered to ''oxacillinases'' due to their enzymes' capability to degrate at least 50% of oxacillin antibiotics (Drawz and Bonomo, 2010). In addition, these enzymes confer resistance to penicillins, cephalosporins, carbapenems and extended-spectrum cephalosporins (*bla*$_{OXA}$-type ESBLs) (Drawz and Bonomo, 2010). *bla*$_{OXA}$ enzymes are often detected in *Pseudomonas aeruginosa* and others Gram-negative bacteria like *bla*$_{OXA-1}$ found most frequently in *E. coli* (Paterson and Bonomo, 2005). Four hundred and ninety eight types of *bla*$_{OXA}$ enzyme have been isolated, including ESBL-*bla*$_{OXA}$ (class D; subgroup 2d) and non ESBL-*bla*$_{OXA}$, based on

molecular similarities and reported in the database (http://www.lahey.org/ Studies/other.asp#table1) (Ur Rahman et al., 2018).

These ESBL- *bla*$_{OXA}$ enzymes extracted from *bla*$_{OXA-10}$ such *bla*$_{OXA-11}$, *bla*$_{OXA-14}$, *bla*$_{OXA-16}$, and *bla*$_{OXA-17}$. For instance, *bla*$_{OXA-14}$ differs from *bla*$_{OXA-10}$ by only one amino acid substitution, *bla*$_{OXA-11}$ and *bla*$_{OXA-16}$ differ by two, and *bla*$_{OXA-13}$ and *bla*$_{OXA-19}$ differ by nine (Bradford, 2001). *bla*$_{OXA-10}$ enzyme confer weak resistance to cefotaxime, ceftriaxone and aztreonam, while *bla*$_{OXA-11, -14, -16, -17, -19, -15, -18, -28, -31, -32, -35}$ and $_{-45}$ confer high resistance to cefotaxime and sometimes ceftazidime and aztreonam (Rawat & Nair, 2010). The first ESBL-OXA type enzyme was detected in *Pseudomonas aeruginosa* from a patient in Turkey in 1991 (Evans & Amyes, 2014). Recently, many newer types of *bla*$_{OXA}$ have also been found in *Pseudomonas aeruginosa* (in France and Turkey). Paterson and Bonomo (2005) recommended that further studies are required to elucidate the spread *bla*$_{OXA}$ enzymes on worldwide distribution.

2.4.1.4 *bla*$_{CTX-M}$

bla$_{CTX-M}$ types are described as novel enzymes of plasmid-mediated ESBLs class A, which are not very closely (about 40% sharing amino acid) referred to *bla*$_{SHV}$ or *bla*$_{TEM}$ β-lactamases (Monstein et al., 2007). The *bla*$_{CTX-M}$ enzymes confer a higher level of resistance to cefotaxime than ceftazidime, but some of them like *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-19}$ can be resistant to ceftazidime (Pitout et al., 2005). The *bla*$_{CTX-M}$ family is large, and have different group of enzymes. To date, over 40 types of *bla*$_{CTX-M}$ have been found (Pitout et al., 2005), mostly in *Enterobacteriaceae* such *E. coli* and *K. pneumoniae* (Eckert et al., 2004; Pitout et al., 2005). As a result of alignment of amino acid sequence similarities, the *bla*$_{CTX-M}$ types have been divided into five groups (*bla*$_{CTX-M}$ group 1, *bla*$_{CTX-M}$ group 2, *bla*$_{CTX-M}$ group 8, *bla*$_{CTX-M}$ group 25 and *bla*$_{CTX-M}$ group 9), each of which have been further divided into sub-groups (Stuart et al., 2010), as shown in Table 2.3.

Table 2.3 *bla*$_{CTX-M}$ types and origin (Cantón and Coque, 2006; Bonnet, 2004; Pitout et al., 2005)

bla$_{CTX-M}$ group					
	1	2	8	9	25
Year	1989	1986	1996	1994	2000
Enzyme	*bla*$_{CTX-M-1}$	FEC-1	*bla*$_{CTX-M-8}$	*bla*$_{CTX-M-9}$	*bla*$_{CTX-M-25}$
Source	Baby	Dogs	Patients	Spain	Patients
Country	Germany	Japan	Brazil	*K. georgiana*	Canada
Bacteria	*K. ascorbata*	*K. ascorbata*	*K. georgiana*		Unknown
Sub-groups	*bla*$_{CTX-M-1}$	*bla*$_{CTX-M-2}$	*bla*$_{CTX-M-8}$	*bla*$_{CTX-M-9}$	*bla*$_{CTX-M-25}$
	bla$_{CTX-M-3}$	*bla*$_{CTX-M-4}$		*bla*$_{CTX-M-13}$	*bla*$_{CTX-M-26}$
	bla$_{CTX-M-10}$	*bla*$_{CTX-M-4L}$		*bla*$_{CTX-M-14}$	
	bla$_{CTX-M-12}$	*bla*$_{CTX-M-5}$		*bla*$_{CTX-M-18}$	
	bla$_{CTX-M-15}$	*bla*$_{CTX-M-6}$		*bla*$_{CTX-M-16}$	
	bla$_{CTX-M-28}$	*bla*$_{CTX-M-7}$		*bla*$_{CTX-M-17}$	
	bla$_{CTX-M-30}$	*bla*$_{CTX-M-20}$		*bla*$_{CTX-M-19}$	
	FEC-1	Toho-1		*bla*$_{CTX-M-21}$	
				bla$_{CTX-M-24}$	
				bla$_{CTX-M-27}$	
				Toho-2	

This illustrates each of the first subgroup is originated by a chromosomal gene of different *Kluyvera* species, such as *K. cryocrescens* in *bla*$_{CTX-M}$ group 1, *K. ascorbata* in *bla*$_{CTX-M}$ group 2, *K. ascorbate* in an enzyme *bla*$_{CTX-M-3}$, *K. georgiana* in *bla*$_{CTX-M-8}$ and *Kluyvera* spp. in *bla*$_{CTX-M}$ group 9 (Pfeifer et al., 2010; Pitout et al., 2005). The relationship between members of a single group share >94% amino acid identity but ≤90% amino acid identity between groups (Bonnet, 2004). Each type of *bla*$_{CTX-M}$ group has sub-types and differ from the original gene by one or many mutations in amino acid positions (Rubtsova et al., 2010), as illustrated in Figure 2.9. The most frequent mutations are found in positions 77, 114 and 288 in *bla*$_{CTX-M}$ group 1 and 231 in *bla*$_{CTX-M}$ group 9.

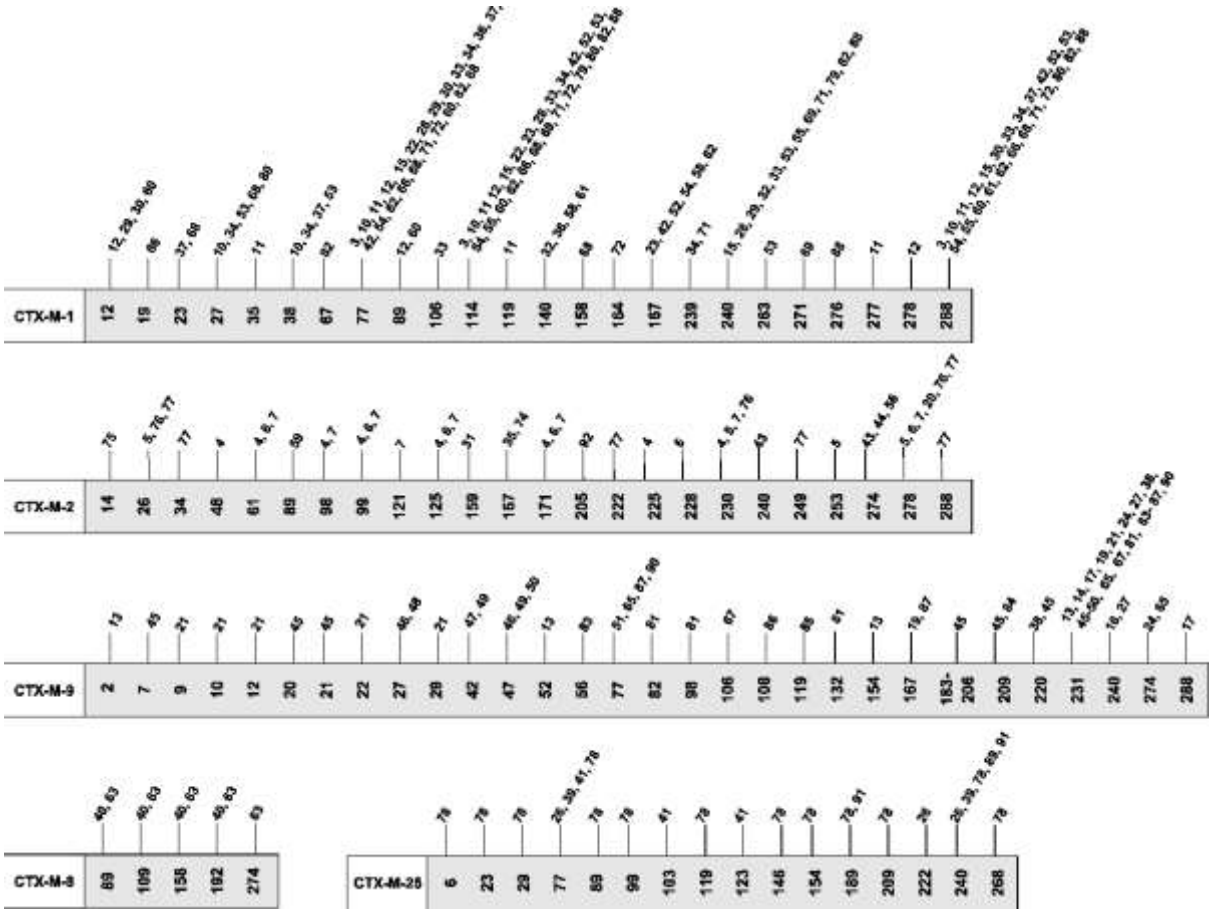

Figure 2.9 Five types of *bla*$_{CTX-M}$ (1, 2, 9, 8 and 25) classified into sub-types based on similarities of amino acid sequence (Rubtsova et al., 2010). The numbers above indicate positions of mutations in amino acids

2.4.2 Vehicle of dissemination of ESBL resistance genes

Acquired resistance could develop either vertically as a mutation on the chromosome, or horizontally as horizontal gene transfer (HGT) via plasmids, transposons, integrons, changes to sequences of insertion, etc. (Coculescu, 2009). Such a mutation is a change in sequence of DNA or exchange of chromosomes (perhaps as a result of selective antibiotic pressure) that reduces the susceptibility of the bacteria to the antibiotic (Soares et al., 2012; Tenover, 2006). For example, spontaneous mutation happens randomly when there are replication errors or under incorrect repair of a damaged DNA in cell proliferation (Dzidic et al., 2008). HGT is a process where genetic material contained in small packets of DNA can be transferred between individual bacteria of the same or different species (Lawrence, 2005). Mechanisms of HGT are divided into three types, namely A) Transformation where the bacteria take up DNA from another dead

bacterium from the environment, B) Transduction or transfection is when bacteria-specific viruses (bacteriophages) transfer DNA between two closely related bacteria. C) Conjugation, where two bacterial cells contact and transfer plasmids or chromosome fragments from bacteria to another by pilus. For example, the plasmids have a broad host range and can cross genus lines during the gene transfer (Dale and Park, 2004), as shown in Figure 2.10.

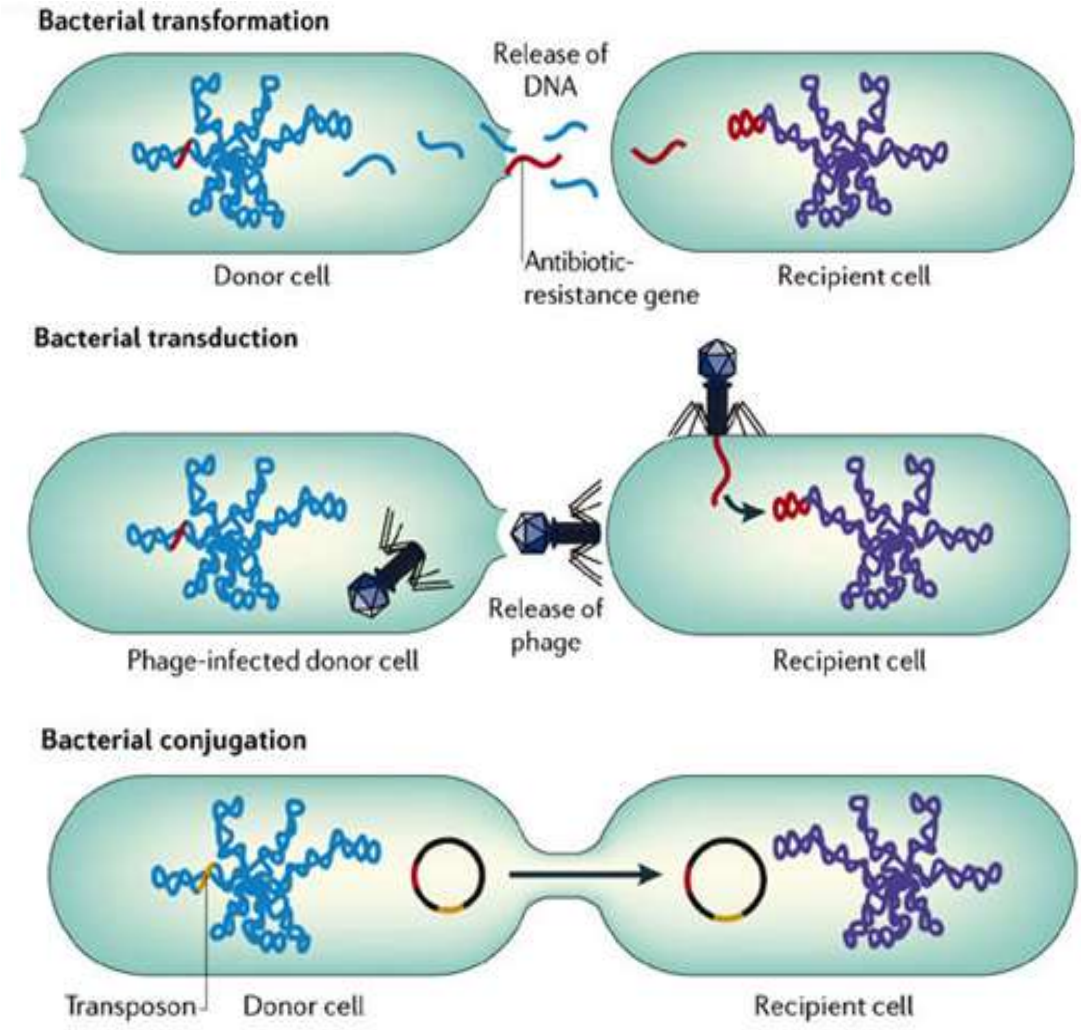

Figure 2.10 Ways of horizontal gene transfer antibiotic resistance (Nature Reviews-Microbiology, 2006)https://images.nature.com/full/natureassets/nrmicro/journal/v4/n1/images/nrmicro1325-f2.jpg

β-lactamase enzymes display either chromosomal (inherent to the organism) or plasmid-mediated synthesis (move between bacterial populations) of β-lactamase in class A and C (Cantónv et al., 2012), but class D β-lactamases were detectable only as plasmid-mediated (Evans and Amyes, 2014).

2.4.2.1 Plasmids

Plasmids play an important role in evolution and disseminating resistant genes among bacterial pathogens (San Millan, 2018). They are described as circular DNA, extrachromosomal and self-replication double-stranded DNA (Yamashita etal., 2014; Kelly et al., 2009). These plasmids carry genes for multi-resistance to other antimicrobial agents such as aminoglycosides, trimethoprim, sulphonamides, tetracyclines and chloramphenicol (Pitout et al., 2005). For example, ESBLs genes are more commonly attributed to conjugation by plasmids (Titelman et al., 2014). These plasmids can be harbouring many resistant genes including *bla*$_{\text{SHV-1}}$, *bla*$_{\text{SHV-2}}$, *bla*$_{\text{TEM-1}}$ and *bla*$_{\text{TEM-2}}$ (Shaikh et al., 2015). Phylogenetic analysis showed that *bla*$_{\text{CTX-M}}$ family are resulted by mobilization of chromosomal *bla* genes from *Kluyvera* spp (Cantón et al., 2012). These genes are found on mobile genetic elements which transfer among bacteria either the same or different species that confer resistance to β-lactam antibiotics (Chroma & Kolar, 2010). There are varieties of genetic elements that can participate *bla*$_{\text{CTX-M}}$ enzymes in the spread of antibiotic resistance genes through plasmids, transposons, integrons and insertion sequences (Coculescu, 2009)as shown in Figure 2.11.

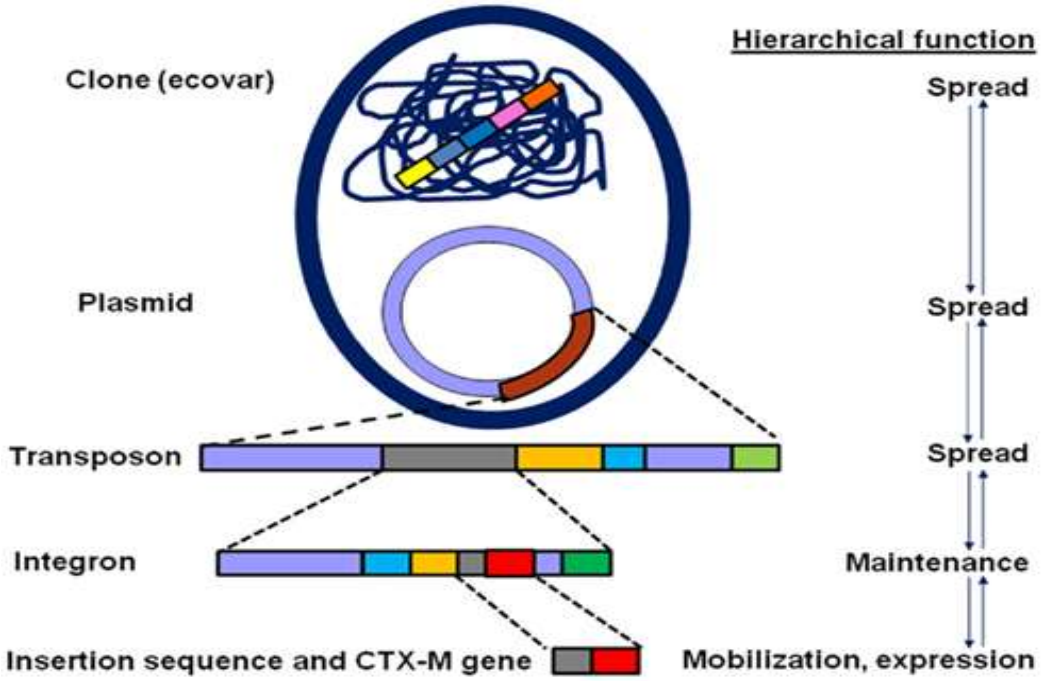

Figure 2.11 Structures of *bla*$_{CTX-M}$ genes in mobile genetic elements within bacterial clones (Cantón et al., 2012)

bla$_{CTX-M}$ encoding plasmids have been found to be transmissible between cells at a frequency of transfer of between 10^{-7} and 10^{-2} per donor cell (Bonnet, 2004), as found in *E. coli* and *K. pneumonia* (Vaidya, 2011).

Plasmids have been classified into at least 29 groups based on incompatibility (Inc) (Zhao & Hu, 2012). Plasmids of *bla*$_{CTX-M}$ genes are referred to as IncF, IncI, IncN, IncHI2, IncL/M and IncK groups; the most common in *bla*$_{CTX-M-15}$ genes is IncF (FIA, FIB and FII), whilst IncF, the *bla*$_{CTX-M-14}$ genes nearly included IncK and IncI1. Additionally, the *bla*$_{CTX-M-9}$ genes contained IncHI2, *bla*$_{CTX-M-1}$ genes are dominantly harboured by IncN and IncI1 and *bla*$_{CTX-M-3}$ genes by IncL/M and IncI1 (Zhao & Hu, 2012). The *bla*$_{CTX-M}$ genes are located on large plasmids ranging between 40 and >200 kb (Cullik et al., 2010).

2.4.2.2 Insertion sequences (ISs)

IS elements are defined as small transposable molecular vehicles (700-2500 bp long) that are distributed widely amongst bacteria strains by chromosomes or plasmids (Schneider & Lenski, 2004). ISs have three main functions among bacteria, (i) to activate non-vital genes or enhance expression of adjacent genes, (ii) to move of IS elements from one location to another via integrons, transposons, plasmids and chromosomes, and (iii) to encode transposase enzyme during transposable elements (Zhao & Hu, 2012).

Insertion sequences have been divided into nearly 20 groups, which depend on sequences of transposase enzymes and terminal inverted repeat (Ooka et al., 2009). IS*Ecp*1, IS*CR*1 and IS26 elements are usually isolated from *Enterobacteriaceae* in $bla_{\text{CTX-M}}$ genes that confer resistance to cefotaxime (Cantón et al., 2012).

IS*Ecp1* is considered as one of the most important transposable element, and was discovered at the end of 1990s, from the plasmid named pST01 in *E. coli* to the $bla_{\text{CTX-M-15}}$ (Zhao & Hu, 2012). The structure of this element is made up of one inverted repeat of IS*Ecp1*, DNA sequence and a second inverted repeat (Lartigue et al., 2004). The range $bla_{\text{CTX-M-1}}$ gene is 48 and 127 bp, from 34 to 42 bp in $bla_{\text{CTX-M-9}}$ gene cluster and around 40–52 bp in $bla_{\text{CTX-M-25}}$ and $bla_{\text{CTX-M-8}}$ gene clusters, respectively (Cantón et al., 2012). Insertion sequence IS*Ecp1* emerged on upstream of $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-10}}$, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-3}}$ genes and downstream of $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-10}}$, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-3}}$ genes that the same sequence to the right inverted repeat might mobilize via transposition roles (Lartigue et al., 2004). $bla_{\text{CTX-M}}$ genes could be transferred from clinical to community settings and the wider environment through mobile genetic element like IS*Ecpl*, which means that $bla_{\text{CTX-M}}$ types might lead to dissemination between animals and human (Pitout et al., 2005).

2.5 The impact and mode of antibiotic resistance

It is known that some bacteria have abilities to create strategic ways to survive in the presence of antibiotics, thereby halting the effectiveness of a number of drugs (Marti et al., 2014) and meaning that many infectious diseases have become difficult to cure (WHO, 2014). As a result, antibiotic resistance has become a global health problem (Marti et al., 2014). Indeed, Rahube and Yost (2010) have detected around 13,200 resistance genes and 630 antibiotic resistant genomes.

According to the CDC (Centers for Disease Control and Prevention) (2014), ''more than 70% of the bacteria that cause hospital-associated infections are resistant to at least one antibiotic''; causing over 2 million illnesses and 23,000 deaths per year in the USA. More specifically for ESBLs. in 2013, the CDC reported that about 26,000 (19%) of healthcare setting acquired *Enterobacteriaceae* infections and 1,700 patients die per year because of ESBLs in the USA alone (Frieden, 2013). Another study, conducted by the World Health Organization, found that the worldwide resistance to G3CR had increased significantly for *E. coli* (50–80%) (Kuenzli et al., 2014).

To create a novel antibiotic, it costed at least $1.87 billion in 2006, which is more expensive than the annual cost involved in treatment of influenza in the USA. In the UK, the NHS spends about £1 billion per year for treatment of patients infected with ARB (Westwood et al., 2014). In 2014, Jim O'Neill estimated that if the issue of antibiotic resistance cannot be resolved, it will cost $100 trillion for treatment and can cause an annual death of around 10 million people by 2050 (O'Neill, 2014). Given the growing spread of resistance, coupled with the costs and time involved with developing new antibiotics, it is important that scientists, health authorities and politicians implement measures to reduce antibiotic use in order to slow the spread of antibiotic resistant bacteria (ARB) (Galán et al., 2013).

2.6 Antibiotic resistance in the environment

Although ARB and their genes were first detected in a clinical setting, they are becoming increasingly prevalent in the wider environment (Da Costa et al., 2013). Many studies have confirmed that bacterial antibiotic resistance is found in environments such as wastewater, water, sediments, soils, food, fish and plants (Gao et al., 2012; Kümmerer, 2004; Wellington et al., 2013). This is due to the extra selection pressure of antibiotics or the misuse of antibiotics for human and animal treatment over the past decades (Allen et al., 2010; Cantón et al., 2012).

Metabolites or antibiotic resistant genes may circulate in the environment via a number of pathways (Figure 2.12) (Wellington et al., 2013). The cycle of ARB entering the environment starts when the antibiotics used for human and animal medicine are excreted via urine or faeces as compounds and metabolites. Human waste may, or may not be subsequently treated (e.g. at a WWTP) (Tao et al., 2014; Cha et al., 2006). A WWTP could ultimately discharge resistant bacteria and genes into the wider aquatic environment (e.g. river, surface water, drinking and

groundwater (Dolejska et al., 2011)), or by application of sewage sludge onto agricultural soils (Rahube & Yost, 2010).

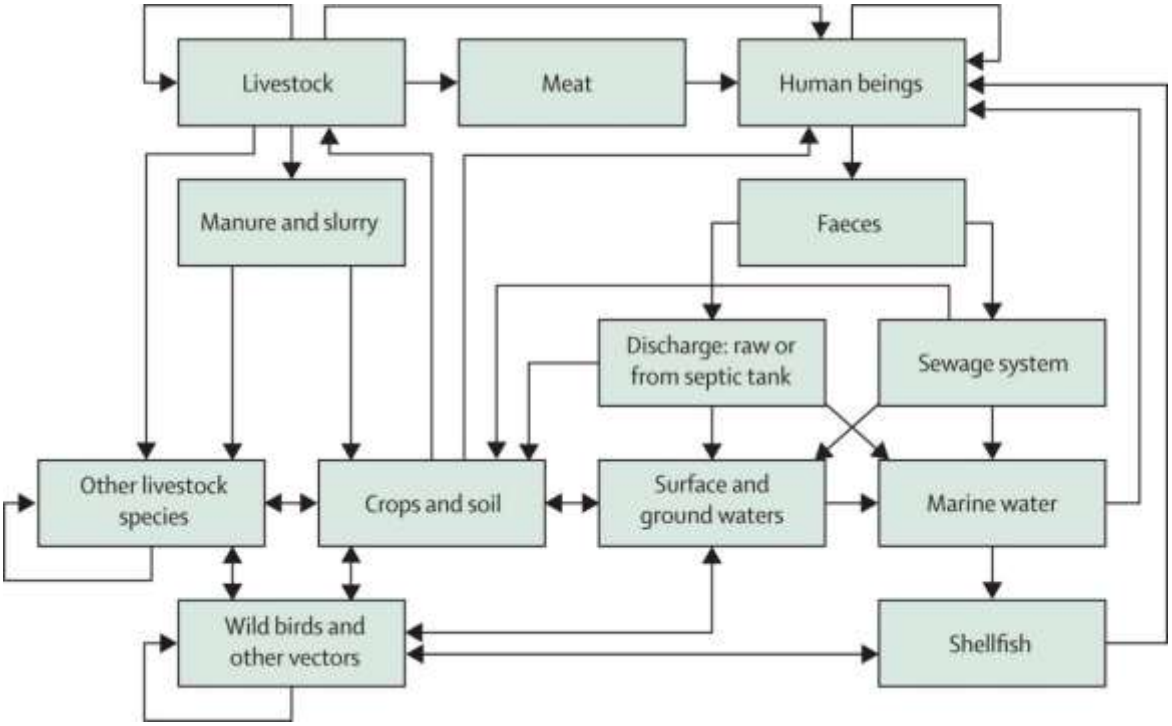

Figure 2.12 The scheme of transfer of ARB between humans, animals in the environment (Wellington et al., 2013)

The release and circulation of ARB into the environment is a serious problem to public health (Lazarus et al., 2015). Wellington et al. (2013) interpreted how antibiotic resistance spread through (i) consumption of crops contaminated by sludge, manure and slurry, (ii) consumption of livestock produce from animals treated with veterinary drugs, (iii) consumption of fish contaminated by aquaculture treatments, (iv) drinking contaminated groundwater and surface water, and (v) consumption of seafoods from contaminated coastal waters. Others have shown or suggested that the ARB could be spread by routes such as international medical travellers, the global trade in food, and pet animals (Cantas et al., 2013; Finley et al., 2013).

Once released into the environment, ARB can enter the food chain; where, together with the misuse of antibiotics in livestock production, it can augment the foodborne spread of resistance bacteria and their genes (CDC, 2018). Fahrenfeld et al., (2016) demonstrated that many studies have detected ARB with livestock and food. A study in China found that, between 2004 and 2012,

ARB-producing *E. coli* were isolated from chickens, ducks, pigs, and cows (Yassin et al., 2017). In Bangladesh, multi-antibiotic resistance in *E. coli* were detected from chicken (cloacal swab, intentional fluid, egg surface, faecal material of chicken and hand wash of chicken handlers) (Akond et al., 2009). Ma and co-workers (2012) found that domestic ducks helped spread $bla_{CTX\text{-}M}$ -producing *E. coli* genes by direct excretion to surface water or agricultural land, and which could enter humans via the food chain. Processing of livestock (e.g. slaughterhouses) can also lead to release of ARB into the environment (Diallo et al., 2013; Fahrenfeld et al., 2016).

Several studies showed that domestic animals such as cats and dogs also carry ARB (Allen et al., 2010); however, a recent report found that wild animals may have become a more important source (Saif et al., 2014). Wild birds and mammals can uptake ARB from the natural environment or those that are released into the environment (Radhouani et al., 2014). Birds can acquire ARB from the environment by direct contact with human or animal faeces, from the transfer of genes to native wild birds (Costa et al., 2013), and by drinking polluted waters (Radhouani et al., 2014). Birds, and migratory waterfowl in particular, move considerable distances (Allen et al., 2010), and Wellington et al. (2013) clearly illustrated the increased rates of resistant strains in wild birds in the last five years. Over the last decade, the most common ESBL-producing genes detected in *E. coli* in wild birds and mammals were $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}14}$, $bla_{TEM\text{-}15}$ and $bla_{SHV\text{-}12}$ (Wellington et al., 2013). Since then, several species of birds pose as a reservoir of ESBL genes (Costa et al., 2013). Another study, conducted in Bangladesh, found that birds acquired a high level of multi-resistance genes ($bla_{CTX\text{-}M\text{-}15}$ -producing *E. coli*) following feeding on healthcare waste (Hasan, 2013).

Although several studies report the occurrence of ESBL in wildlife, it is not clear whether these wild birds influence prevalence ESBL in wastewater during scavenging for food (Costa et al., 2013). Guenther et al. (2011) emphasised that gulls exchanged resistant strains within wastewater. In 1975, pigeons were identified as the first wildlife to carry multi-antibiotic resistance in *E. coli* (Bonnedahl & Järhult, 2014). Over the last decade, ESBL-producing *E. coli* in wild birds and mammals were first observed for $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}14}$, $bla_{TEM\text{-}15}$ and $bla_{SHV\text{-}12}$ (Wellington et al., 2013). Since then, several species of birds pose as a reservoir of ESBL genes (Costa et al., 2013). For instance, a study conducted in Portugal found about 34% of seagulls carried $bla_{CTX\text{-}M\text{-}15}$ -producing *E. coli* in their faeces (Costa et al., 2013).

Collectively, it is clear that there are many potential sources of ARB in the environment, and that these bacteria and their genes can enter a large ecological cycle, ultimately augmenting further spread and dissemination. A key component of ARB cycling in the environment is wastewater.

2.7 Wastewater as a reservoir for antibiotic resistance

WWTPs play an important role in reducing chemical compounds and eliminate pathogen strains (Rahube and Yost, 2010). However, they could also act as a fundamental source of ARB (Wellington et al., 2013; Gao et al., 2012; Novo et al., 2013; Rizzo et al., 2013). Given that they also encompass high bacteria densities and diversity, it is postulated that they could act as hotspots for the spread of antibiotic resistance to other bacteria (Schwartz et al., 2003) via mobile genetic elements, by HGT or a mutation on the chromosome (Korzeniewska et al., 2013). Plasmids play a crucial role in carrying resistance genes between bacterial diversity in WWTP for transferring and evolving of antibiotic resistance genes (Rahube & Yost, 2010).

As discussed by Kümmerer (2004), unused drugs are sometimes disposed of in wastewater; antibiotics and then the metabolites enter sewage treatment systems. Raw wastewater can therefore contain a relatively high concentration of antibiotics, high bacterial diversity and detergent compounds (also known to drive antibiotic resistance) (Novo and Manaia, 2010; Wellington et al., 2013; Rahube et al., 2014). Preliminary treatment (screening and grit removal) passes raw influent through screening to remove larger materials such as plastic, paper, grit, solids such as sand, silt and stones, which could affect the treatment process. Primary treatment uses a sedimentation tank to settle floating materials such as oils, grease, fats, sand and grit, and heavier organic matter along the bottom of the sedimentation tank to reduce suspended solids. The raw sludge is then removed by pump or gravity feed to a sludge treatment process. The supernatant still consists of suspended matter in sedimentation tank that goes to a secondary treatment process. Approximately 60% of suspended solids can be removed at this stage, with a 35% reduction in biological oxygen demand. The secondary treatment uses aerated biological digestion by pumping air into a tank, which promotes the microbial growth in the wastewater and mixing wastewater with remaining suspended and dissolved material to degrade. Thereafter, the wastewater passes to a secondary sediment tank for solids to settle out. This process can reduce 90% of organic matter. Finally, the tertiary treatment consists of three phases: physical treatment removes components materials as suspended solids by filtration, chemical treatment

removes nitrates, phosphates, toxic or non-biodegradable organic matters that are not eradicated by the secondary treatment, and disinfection treatment using UV light or ozone to destroy microbes, nucleic acids, protein, lipids components. Thereafter, treated effluent is discharged into the environment (Hendricks and Pool, 2012; Naidoo and Olaniran, 2013) as illustrated in Figure 2.13.

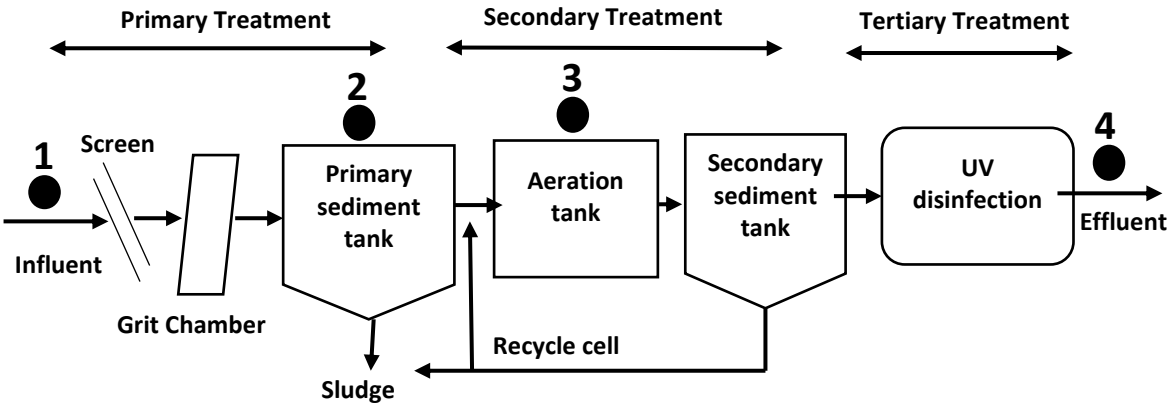

Figure 2.13 Flow diagram of a tertiary wastewater treatment process (Naidoo & Olaniran, 2013)

Theoretically, the tertiary stage of wastewater treatment kills 99% of microorganisms (Baquero et al., 2008). However, studies suggest that it may not always be sufficiently effective to remove bacteria strains and genes (Dolejska et al., 2011). Indeed, several reports have called for the redesign of wastewater treatment in order to avoid the dissemination of ARB and antibiotic resistance genes (Biswal et al., 2014). A study in the Netherlands by Blaak and co-workers (2014) indicated that the effluent (discharge point) contained a high percentage (50%) of ESBL-producing *E. coli*, with 71% of those being multi-antibiotics resistant. A recent study by Bréchet and colleagues (2014) in France calculated that treated wastewater containing more than 600 billion ESBL -producing *E. coli* were released daily in the receiving river. Amine (2013), asserted that the proportion of ESBL-producing Gram-negative bacteria was higher in effluent sewage than raw wastewater.

2.7.1 Factors that influence the prevalence of ESBLs in WWTP

2.7.1.1 Population demographics

Research conducted by Laupland et al. (2008) and Drieux et al. (2016) showed that elderly people, females and travellers are more likely to be introduced to ESBL producing *E. coli* in the community. Okeke and Edelman (2001) observed that people living in a largely populated city are more likely to be exposed to ARB, which then have the opportunity to evolve and spread. However, there is no available data in the scientific literature that elucidate the relationship between rates of antibiotic resistance in human populations and in raw wastewater (Fahrenfeld et al., 2016).

2.7.1.2 Worldwide movement of people

Changes in population demography is a driving factor influencing new antibiotic resistance in various regions of countries. In particular, global travel for holidays, by medical 'tourists', migrant employees and overseas students between countries could theoretically be a risk factor for dissemination of ARB and antibiotic resistant genes (Barlam & Gupta, 2015). In the UK, the movement of travellers increased from 39.5 to 50 million between 2002 and 2012 (Hawkey, 2015); such movement of people inevitably raises the level of risk. In 2004, the first travellers colonised by ESBLs-producing *E. coli* were found in Canada and New Zealand (Van der Bij & Pitout, 2012). In 2012, Wickramasinghe et al. describe how 732 faecal samples were collected from three groups of people living in Birmingham but of different global origin (Europe: *n*=571, Middle East/South Asia: *n*=145 and Africa *n*=7), UK. They found those of Middle Eastern/South Asian origin had the highest percentage of carriage of ESBLs-producing *E. coli* (22.8%), and concluded that changes in population might spread antibiotic resistant bacteria.

Several studies carried out on the movement of people around the world confirmed that traveller's faeces could carry ESBL, as illustrated in Table 2.4. Hawkey (2015) observed that travellers returning from India could harbour bacteria with genes such as $bla_{\text{CTX-M-15}}$. However, Laupland et al. (2008) concluded that the endemic areas with high colonisation of ESBL-producing *E. coli* are India, the Middle East and Africa.

Table 2.4 A summary table of studies reporting incidences of ESBL-producing *Enterobacteriaceae* colonisation acquired through travelling

International travel		Percentage positive for ESBLs post-travelling	Reference
Area of origin	Destination		
Scandinavia	Tropical and subtropical areas	Twenty one percent (90/430) of individuals were colonised with ESBL-producing *Klebsiella* post-travel. Colonisation was most frequent was in those that had visited South Asia	Kantele et al., 2015
Sweden	India or Central Africa	Sixty seven percent (12/18) of students acquired ESBL-producing *E. coli* whilst visiting India	Bengtsson-Palme et al., 2015
Netherlands	Southeast Asia, Indian subcontinent, Northern & Southern Africa, Southern Europe, Central America and South America	Prevalence of ESBL encoding $bla_{\text{CTX-M}}$ genes in 122 individuals increased from 9.0% to 33.6% post-travel. The greatest increase was in those that had visited south east Asia and the Indian sub-continent	von Wintersdorff et al., 2014
Switzerland	South Asia (India, Bhutan, Nepal and Sri Lanka)	Sixty nine percent of travellers to South Asia were colonised with ESBL-producing *E. coli* post-travel; with the highest percentage of colonisation (86.8%) in those who returned from India	Kuenzli et al., 2014
New York City, USA	Central and South America, Africa, Middle East and South, Southeast and East Asia	Twenty five percent (7/28) acquired ESBL- producing *Enterobacteriaceae* during travelling; with $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ being the most common genes found	Weisenberg et al., 2012
Canada	Asia, North America, Central America, Africa, India, Europe, South America, Middle East, Australia and New Zealand	Twenty three percent (26/113) of travellers harboured ESBL-producing *E. coli*; with travelling to the Indian sub-continent and Africa representing a major risk for rectal colonisation with $bla_{\text{CTX-M}}$ producing *E. coli*	Peirano et al., 2011
Sweden	Africa, Asia, Central America, Middle East, North & South America and Southern Europe	Twenty four percent (24/100) of travellers acquired ESBL-producing *E. coli*	Tängdén et al., 2010

There are many ways travellers can be colonised by ESBLs-producing *E. coli*. Collignon (2009) highlighted that one important route was consuming contaminated foods or drinking water. Hawkey (2015) added that tourists could get ESBL-producing *E. coli* infections from swimming in rivers to which WWTPs release effluent. Other reasons visitors can be infected with ESBL *Enterobacteriaceae* include poor hygiene, and direct contact with sick people, as mentioned by Kantele et al. (2015).

2.7.1.3 Healthcare and community settings

The sources of wastewater in sewers to the influent of treatment plants can be seen in Figure 2.14.

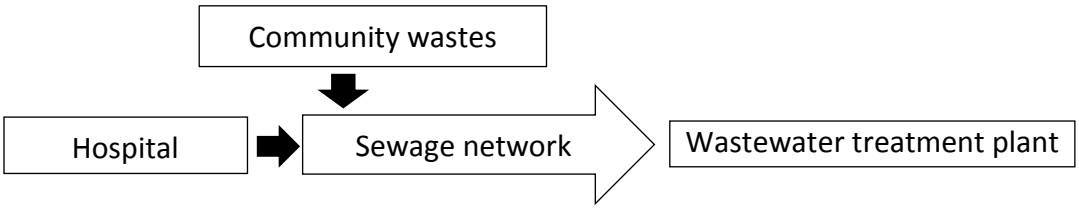

Figure 2.14 Schematic pathway of wastewater to the treatment plant through sewage systems

Hospital liquid effluent can include skin, sputum, saliva and faeces from sick populations (Fahrenfeld et al., 2016). It might contain resistant bacteria and antibiotic residues and then move to sewage networks without any treatment (Kümmerer, 2004). Thereafter, faecal community bacteria come into contact with non-faecal bacteria (Fahrenfeld et al., 2016). In sewer networks, ARB can survive, propagate and transfer their genes by HGT, or degrade. This sewage mix, with community wastes from household and industry wastes reach the influent point of wastewater treatment plants (Kümmerer, 2004; Harris et al., 2013; Katouli et al., 2009). The survival and persistence of bacterial populations will be affected by conditions within the wastewater environment, such as chemical and biological oxygen demand (BOD/COD), pH, temperature, nutrient content and competition from residual microbes (e.g. in biofilms) as well as the degree and mode of treatment.

In general, hospital effluent has the highest loads of ARB, because of the elevated levels of antibiotics used; this might also support non-resistant strains to convert to ARB (Jury et al., 2010). At the beginning of 1970s, the first report announced hospital discharge contained a

multitude of antibiotic resistant bacteria (Drieux et al., 2016). A study by Bréchet et al., (2014) found that ESBL-producing *E. coli* in hospital effluent was of a greater concentration than in community influent, they interpreted this was most probably due to developing HGT among ESBL-producing *E. coli* to non-producers. Other studies raised concern about ARB and their genes spreading more broadly to wider microbial populations where there is contact between hospital sewage and local sewage (Moges et al., 2014). Drieux et al. (2016) also found that the emergence of ESBL-producing *E. coli* in community wastewater setting was most probably due to pollution from hospital waste.

Others found that the concentration of ARB in hospital effluent waste might be reduced when mixed with community waste, due to dilution. Kwak et al. (2015) in Sweden showed that the percentage of ESBL-producing *E. coli* was decreased between hospital effluent and influent of WWTP from 13.6 to 2.3%. Kümmerer (2004) found that there was up to a 100-fold dilution in ARB derived from hospital effluent when it entered into the wider sewage system.

On the other hand, Kümmerer (2004) reported differences suggesting that the total consumption of antibiotics in the community was twice greater than in the hospital, implying that community-derived waste is the main source of ABR in influent sewage. Fahrenfeld et al., (2016) pointed out that several studies mentioned raw influent has higher numbers of ARB than hospital effluent.

As shown above, it is unclear how much hospital-derived ARB survive and mix with community waste and into the influent, and subsequent effluent, of wastewater treatment plants (Bréchet et al., 2014). Thus, the World Health Organization (WHO) suggests that wastewater from healthcare settings must be treated and disposed before discharge to environment (Harris et al., 2013). Unfortunately, many countries do not follow the standard of WHO limits (Harris et al., 2013). Kümmerer (2004) recommended that hospital effluent should be treated in private treatment plant to discard all ARB and its compounds before discharge to sewage pipes.

2.7.1.4 Storm events

2.7.1.4.1 Dilution

WWTPs are designed to decrease organic matter and to eliminate pathogens bacteria, viruses, and protozoa (Lucas et al., 2014). However, heavy rainfall events can fill the capacity of wastewater plants, triggering the release of raw wastewater via a combined sewer overflow, and/or the extra volume of water affects the quality control of the treatment plant (Passerat et al., 2011). Although several studies have focussed on the microbial quality of raw and treated sewage, the effectiveness of disposal methods and output to environment, only few papers have focused on how storm events influence microbial quality in influent water (Lucas et al., 2014).

2.7.1.4.2 Sediment particles

Eroding soils, rocks and land following a heavy rain event can cause an increase in suspended sediments in water bodies (Lucas et al., 2014). Pathogens can attach to such sediments, and this can play an important role in the survival and dissemination of bacteria within the water body (Lawler et al., 2006a). Sediments can provide bacteria with nutrients (e.g., carbon, phosphate and nitrogen), and can also increase exposure to heavy metals and raise salinity, which will have an impact on the bacteria directly, and on those microbes and protozoa that predate on them (Malham et al., 2014). The high levels of turbidity in water can cause a barrier to UV radiation and sustain the growth of bacteria (Lawler et al., 2006b). Walters et al. (2014) found that increased sediment concentration reduced UV inactivation of bacteria.

2.8 ESBL in the water environment

Contamination of different water sources (river, marine, lake and surface water) with ARB is a risk for human health. There are three main sources that can release ARB into the water environment: a WWTP, animal wastes, and aquaculture (Barcelos et al., 2018). WWTP effluent is one of the most important source of inputs to the aquatic environment.

A study from the United States reported the presence of ARB in marine animals such as fish, mammals and seabirds (Gaw et al., 2014). Recently, many studies, as shown in Table 2.5, isolated ESBL-producing *Enterobacteriaceae* from different places in the aquatic environment and sea life, they suggested that WWTP might cause contaminated water environment.

Table 2.5 Studies where ESBL-producing *Enterobacteriaceae* have been detected in the aquatic environment and marine animals

Ref.	Source	Findings
Alouache et al. (2012)	Algeria beaches	The most common gene detected in seawater was *bla*$_{\text{CTX-M-15}}$-producing *E. coli*.
Maravić et al. (2013)	Mussels in Adriatic Sea	Nineteen out of 21 strains of bacteria isolated from 28 mussels produced *bla*$_{\text{CTX-M-1}}$
Brahmi et al. (2015)	Fish from Mediterranean Sea near Algeria	ESBL were isolated from 22/300 (7.3%) fish samples, with *blaCTX*$_{\text{-M-15}}$ *E. coli* most frequently found (94%)
Manageiro et al. (2015)	Dolphin in Portugal	*bla*$_{\text{CTX-M-15}}$-producing *E. coli* was isolated from a marine dolphin
Maravić et al. (2015)	Croatian beaches	In beach seawater, 4.2% of *Enterobacteriaceae* were ESBL-producers
Chen et al. (2016)	River in Taiwan	Thirty percent of *E. coli* recovered from river water were ESBL-producers. The most frequently found ESBL was *bla*$_{\text{CTX-M}}$
Diab et al. (2018)	Estuary in Lebanon	ESBL-producing *Enterobacteriaceae* detected from 15 out of 22 (68.2%) different estuaries. *bla*$_{\text{CTX-M-15}}$ gene producing *E. coli* was predominantly isolated
Leonard et al. (2018)	UK beach	Only 0.1% of recovered *E. coli* were *bla*$_{\text{CTX-M-15}}$-producing
Singh et al. (2018)	River in India	*bla*$_{\text{CTX-M-15}}$ most frequently gene of ESBL family isolated from river water in India

2.9 Conclusions

It is obvious that the spread of ARB in the environment has become a big issue in worldwide. ESBL-producing *Enterobacteriaceae* are increasing in prevalence in many countries. WWTPs are hotspots for the spread of ESBL-producing *Enterobacteriaceae* into wider environmental compartments, especially recreational waters. However, post release to the environment, it is not clear how these strains survive and transport in water body, particularly during storm events.

Improved understanding the fate and transport ESBL-producing *Enterobacteriaceae* via WWTP and their survival in the water environment is needed to tackle some of these knowledge gaps and reduce contamination and risk for public health.

2.10 Aims, objectives, and hypotheses

The review of the literature above has identified knowledge gaps and the need for further work; particularly on how the fate of ABR during and after entering a WWTP is affected by different factors. The work within this PhD project aims to address some of the need for further study. Specifically, the aims and objectives of the work, and accompanying hypotheses were:

1. To assess ESBL-producing *Enterobacteriaceae* are present at Bangor WWTP sites and whether they release to water environment
   - It was hypothesised that ESBL-producing *Enterobacteriaceae* would be present in Bangor's WWTP.
2. To investigate how large changes to the local population affect the presence and genetic diversity of ESBL-producing *Enterobacteriaceae* in Bangor's WWTP.
   - It was hypothesised that changing demographics would affect the microbial populations of Bangor's WWTP.
3. To investigate how concentrations of suspended sediment in fresh and seawater affect the survival of $bla_{\text{CTX-M-15}}$-producing *E. coli* released from WWTP.
   - It was hypothesised that ESBLs would survive typical UV exposure rates in water of a high concentration of suspended sediment.

2.11 Plan of thesis

From this point, the thesis is divided into several chapters, as shown in Figure 2.15, starting with a review of existing research performed on ESBL-producing *Enterobacteriaceae*, above.

The first two experimental Chapters (3 and 4) detail the findings of studies that assessed the presence of ESBL-producing *Enterobacteriaceae* in Bangor's WWTP (Aims 1 and 2, above). Chapter 3 describes an assessment of Bangor's wastewater where the persistence of ESBL-producing *Enterobacteriaceae* was presented on four sites of the WWTP: the influent, primary sediment tank, aeration tank and effluent. Chapter 4 reports on an investigation of how the arrival of students to Bangor affect the presence of ESBL-producing *Enterobacteriaceae* in the WWTP.

The last experimental chapter (Chapter 5) examined how sediment concentrations in the water environment affects the survival and distribution ESBL-producing *E. coli* following release from a WWTP (Aim 3). Using laboratory microcosms, this Chapter details the findings of a study that investigated how three different concentrations of suspended sediment in seawater (Menai Strait) and freshwater (river Conwy) affect the survival of $bla_{\text{CTX-M-15}}$-producing *E. coli* released from WWTP under UV exposure to simulate outside exposures in north Wales.

Chapter 6 is a general discussion of results from all previous experimental chapters. Conclusions are drawn and areas of further work identified. Lastly, an Appendix includes brief Figures and Tables, illustrations of all experimental work performed and physiochemical procedures of Chapter 5.

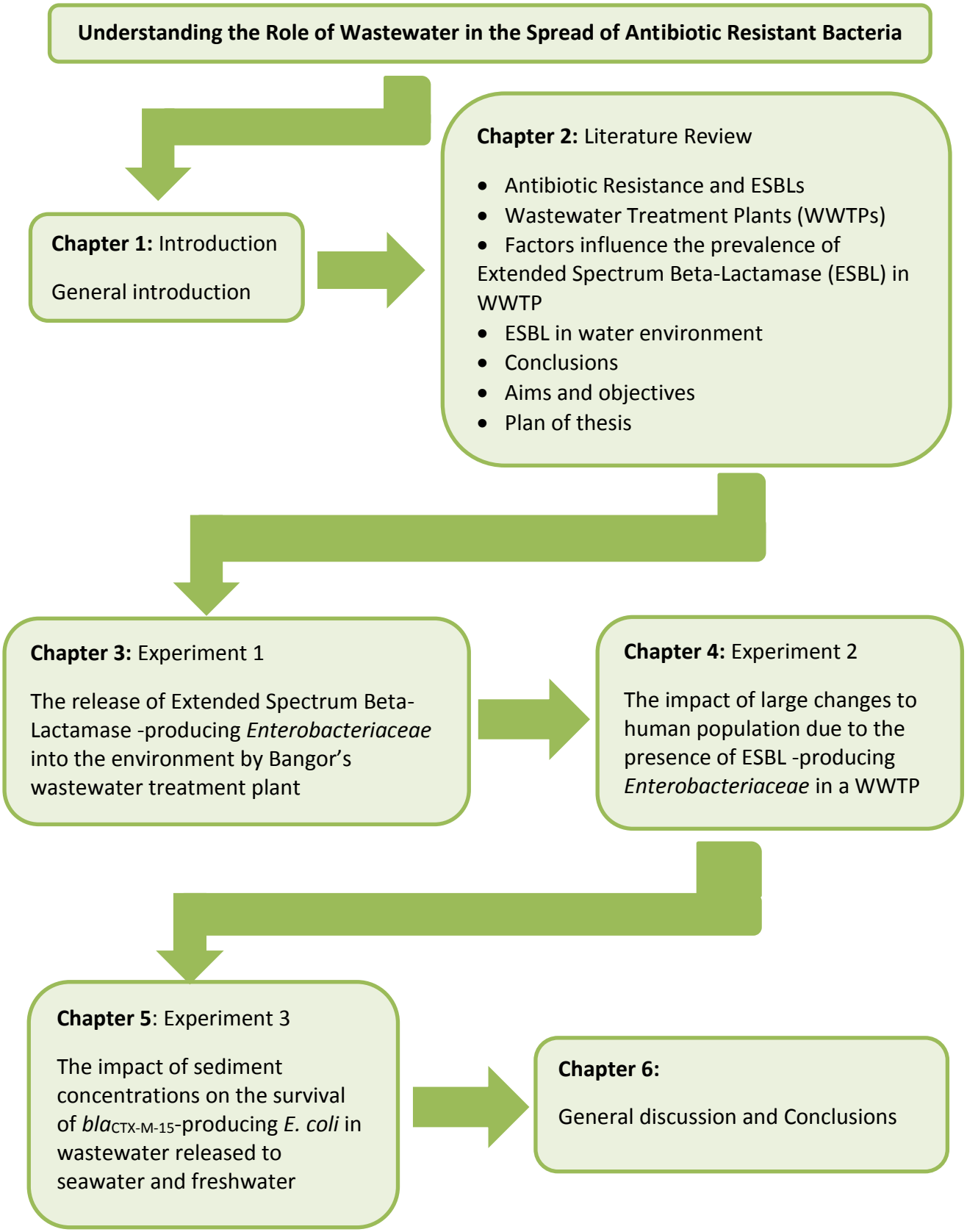

Figure 2. 15 The outline of the thesis

2.12 References

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Chapter 3: Experiment 1

The release of Extended Spectrum Beta-Lactamase -producing *Enterobacteriaceae* into the environment by Bangor's wastewater treatment plant

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3.1 Abstract

As a result of the misuse of antibiotics, bacterial resistance is an increasing threat to public health. Some Gram-negative *Enterobacteriaceae* possess genes that produce Extended Spectrum Beta Lactamase (ESBL) enzymes, enabling them to break down multiple groups of antibiotics. Wastewater treatment plants (WWTP) could play an important role in the development and dissemination of antibiotic resistance in ecological compartments. The aim of this study was to assess whether ESBL in *Escherichia coli* and other faecal coliforms (OFCs) were released in the environment through Bangor's WWTP. Samples were collected weekly over eleven sampling times from the influent (raw wastewater), primary sediment tank, aeration tank and effluent (post UV-disinfection) at the WWTP that serves Bangor and nearby areas. Counts and isolates of presumptive *E. coli* and OFCs were performed on selective agar medium. Isolates from seven sampling times were confirmed by oxidase and Polymerase Chain Reaction (PCR) tests. ESBL - producers were screened using combination disc method. Genotypic identification of $bla_{\text{CTX-M}}$ groups (1, 2, 8/25, 9), bla_{TEM}, bla_{SHV} and bla_{OXA} coding genes were performed by PCR. The results showed that there was a significant decrease of about 99% in presumptive *E. coli* and OFCs between the influent and effluent counts. In total, four out of 123 isolates were screened as ESBL-producing *E. coli* and eighteen out of 136 isolates as ESBL-producing OFCs. Genotypic identification showed that the $bla_{\text{CTX-M}}$ group 1 gene was detected in all four ESBL-producing *E. coli*, and that thirteen out of eighteen ESBL-producing OFCs harboured bla_{SHV}. From our results, we estimate that 300 billion each of both ESBL-producing *E. coli* and OFCs enter the water environment per day through the effluent released from this WWTP. The findings highlight that wastewater treatment process significantly reduced the counts of indicator bacteria from the influent to effluent stages, but does not completely eradicate such bacteria.

3.2 Introduction

Antibiotic resistant bacteria (ARB) have become a significant problem worldwide. Such bacteria have the capability to survive, and even grow, within high concentrations of antibiotics (Madhavan and Sowmiya, 2011). The problem has largely arisen because of misuse of antibiotics in healthcare and community settings, and is now linked to many diseases and deaths. Unless a resolution is found, O'Neill (2014) suggested that the issue will cause the death of around 10 million people per year by 2050, and cost incurred will be $100 trillion per annum. According to a World Health Organisation (WHO) report in 2015, ''this is the single greatest challenge in infectious diseases today''. Centers for Disease Control and Prevention (CDC) (2013) reported that two million people become infected with ARB per year in the USA, and as a result, at least 23,000 people die (Frieden, 2013).

Beta-lactam (β-lactam) antibiotics are one of the most important groups of antibiotics, accounting for approximately 60% of total antibiotics used (Madigan et al., 2012). They work via inhibition of cell wall synthesis, and consist of four groups: penicillins, cephalosporins, carbapenems, and monobactams (Lakshmi et al., 2014). β-lactams are facing the greatest challenges due to resistant bacteria (Van Hoek et al., 2011). Different mechanisms that enable bacteria to survive through β-lactam antibiotic includes: 1) changes to the antibiotic target site of penicillin binding proteins (PBPs), in order to decrease affinity for β-lactam, 2) prevention of the access of the antibiotic by altered permeability or the removal of the antibiotics by efflux pumps, 3) the creating of enzymes such as β-lactamases (Saini and Bansal, 2012). The production of β-lactamase enzymes in Gram-negative bacteria is considered the most important resistance mechanism to inactivate antibiotic function by the hydrolysis of β-lactam. Exposure to high rates of β-lactam antibiotics such as penicillins and cephalosporins may cause bacteria to develop systems to produce novel β-lactamase enzymes, so-called Extended Spectrum β-Lactamases (ESBLs) (Pitout et al., 2005; Samaha-kfoury & Araj, 2003). ESBLs are enzymes that are able to hydrolyse penicillins and cephalosporins but not cephamycins and carbapenems, and are inhibited by clavulanic acid, sulbatam and tazobactam (Pitout and Laupland, 2008; Bush and Jacoby, 2010). Resistance to 3rd generation cephalosporins has become most common in *Enterobacteriaceae*, including *E coli*, *Klebsiella pneumonia*, *Enterobacter* spp., *Serratia* spp., *Proteus* spp., *Providencia* spp., and *Morganella* spp. (Kahlmeter and Singh, 2017). ESBLs are a serious global issue (De Boeck et al., 2012). Recently, up to 300 ESBL subgroups have been found,

with *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{OXA}$ being the most common types (Korzeniewska and Harnisz, 2013).

The dissemination of antibiotic resistance genes (ARGs) from pathogenic bacteria to natural bacteria occurs via horizontal gene transfer (HGT) mechanisms, including: i) Conjugation by plasmid or transposon from one bacterium to another, ii) Transformation via integration of free-DNA from dead bacteria into a new chromosome, and iii) the transfer of resistance gene from one bacterium to another via bacteriophages, a process known as transduction (Wellington et al., 2013). There is evidence that the ESBL genes probably move via HGT throughout the bacterial chromosome by mobile genetic elements (MGEs) such as plasmids and integrons, transposons and chromosomes (Dzidic et al., 2008; Vaidya, 2011; Dhillon and Clark, 2012). Many of the known ARB and ARGs are found not just in a clinical setting but also in the wider environment; including wastewater, water, sediments, soils, and even food animals, fish, plants and vegetables (Lin et al., 2015). Szewzyk and Feuerpfeil (2013) have shown that the ARB or ARGs could enter the environment by the faeces of humans and animals.

In recent years, several studies have proven that wastewater treatment plants (WWTPs) can play a role in the dissemination of ARB and ARGs to natural environments (Yuan et al., 2014). Others have reported that WWTPs act as reservoirs for evolvement of ARB and their genes, as plasmid genes can transfer between different bacteria via HGT (Wellington et al., 2013). Bréchet et al. (2014) confirmed that WWTP spread a great number of ESBL producing *E. coli* to the environment. However, Gao et al. (2012) observed that the relationship between increasing levels of antibiotics and antibacterial resistance are ambiguous; and there is still no clarity about the prevalence of ARB and ARGs within WWTP systems.

The objective of this study was to assess whether ESBL-producing *Enterobacteriaceae* were released in the environment through Bangor's WWTP.

3.3 Materials and Methods

3.3.1 Study setting

The location of the study was Treborth WWTP, Bangor, north Wales, UK. The WWTP studied serves approximately 23,000 people from Bangor, Menai Bridge, Bethel and Felinheli areas (Rana, 2015) and processes more than 24.5 million litres of raw wastewater per day (approximately 300 l s^{-1}) (Welsh Water, personal communication).

The treatment process involves three phases: physical treatment removes components materials such as suspended solids by filtration, chemical treatment removes nitrates, phosphates, toxic or non-biodegradable organic matters which are not eradicated by the secondary treatment, and disinfection treatment by UV light to destroy microbes, nucleic acids, protein, lipids components (Bouki et al., 2013; Mounaouer and Abdennaceur, 2015). Thereafter, treated wastewater is released into the Menai Strait, which goes to the Irish Sea.

3.3.2. Wastewater sampling

In 2014, during the months of September, October and November, one sample per week was collected over a period of eleven weeks. Triplicate wastewater samples were collected into sterile 50 ml containers at each stage, from raw influent (before screens and grit removal), going into primary sediment tank (primary clarifier), aeration tank, to final effluent (post UV-disinfection) (Figure. 3.1; Figure I in the Appendix). Samples were analysed in the laboratory within 1-2 hours (h) of collection.

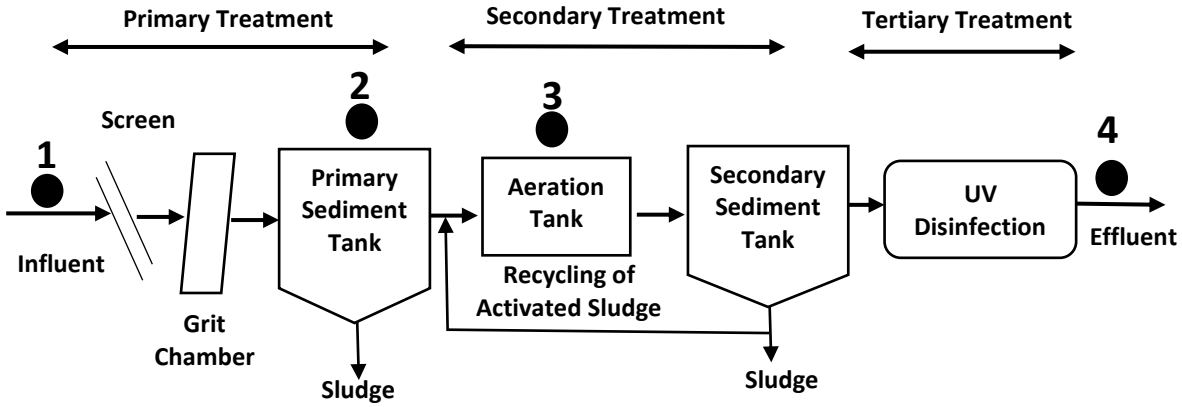


Figure 3.1 Flow illustration of the WWTP, with samples collected from points 1, 2, 3, and 4 (Naidoo and Olaniran, 2013)

3.3.3. Enumeration and isolation of presumptive *E. coli* and other faecal coliforms

To optimise the bacterial count, a series of decimal dilutions (10^{-1} - 10^{-2} and 10^{-3}) were prepared, where 1 ml of each sample was diluted individually in 9 ml of 1/4 strength Ringer's solution (Oxoid Ltd., Basingstoke, UK) and were then homogenized by vortexing. Subsequently, 100 µl volume of suspension from each sample was spread using L-Shaped Spreader (Thomas Scientific) over the entire surface of Chromogenic Primary UTI agar medium (PP3005; E&O Laboratories Ltd.). Plates

were incubated at 37°C for 24 h. Following incubation, the colour and intensity of colonies on each plate were counted according to the manufacturer's protocol. Presumptive *E. coli* hydrolyses the chromogenic substrate to produce purple colonies, and metallic blue colonies indicated other faecal coliforms (e.g. *Klebsiella* spp., *Enterobacter* spp., and *Citrobacter* spp.) (Figure II in Appendix), the term presumptive other faecal coliforms (OFCs) excluded *E. coli*. Cultivable plates yielding corresponding to 1-200 colonies were used for colony enumeration (Rizzo et al., 2014; Yuan et al., 2014). Quality control strains of *E. coli* (NCTC 13353) and OFCs (e.g. *Klebsiella pneumoniae* NCTC 13368 and *Enterobacter aerogenes* NCTC 10006) were used (see Figure III in the Appendix).

From seven sampling times (sampling 1 to 6 and 11), colonies of presumptive *E. coli* or OFCs isolate were picked randomly and streaked using sterile 1 µl loop onto Chromogenic Primary UTI agar to obtain pure culture, and incubated at 37°C for 24 h. Each pure single colony was cultured onto nutrient agar plate (Oxoid, CM0003) and incubated at 37°C for 24 h. Some isolates were stored in Microbank™ vials (Pro-lab Diagnostics) in accordance with the manufacturer's protocol at -70°C for further investigation.

3.3.4 Identification of presumptive *E. coli* and OFCs

3.3.4.1 Biochemical test by oxidase reaction

From Microbank™ vials, isolates of presumptive *E. coli* and OFCs were transferred to nutrient agar plates to perform oxidase test (biochemical test) and to extract DNA for further analysis. An oxidase strip test (40560, Sigma-Aldrich) was used for detection of the cytochrome oxidase enzyme activity. A small amount of pure culture was swabbed using 10 µl sterile loop from nutrient agar and smeared on the test area, and after 10 seconds (sec) it gave either a negative reaction (no colour change) as coliform bacteria (oxidase-negative) or positive reaction (dark purple-blue) (Figure IV in the Appendix) as *Pseudomonas aeruginosa*, according to the manufacturer's protocol. All isolates identified as oxidase-negative were stored in Microbank™ vials at -70°C for further analysis. The quality control *E. coli* NCTC 13353 was used as an oxidase negative control.

3.3.4.2 PCR *uidA* and *lacZ* genes

Oxidase-negative isolates were DNA-extracted by a procedure described by Chandra & Goswami (2014) and Dallenne et al., (2010). In this procedure, a small amount of a single pure colony of *E. coli* or OFCs grown on nutrient agar was transferred using 10 µl sterile loop into 200 µl of

autoclaved distilled water in an Eppendorf tube and vortexed to get a uniform suspension. The bacterial cells were lysed by heating the suspension at 95°C for 10 minutes (min). The cellular debris was removed by centrifugation at 12,000 rpm for 5 min. The upper aqueous phase was transferred to a new tube of Eppendorf and stored at -20°C until it was used as the template DNA.

The *uidA* (β-glucuronidase enzyme) and *lacZ* (β-galactosidase enzyme) genes have been used in PCR for identification *E. coli* and OFCs, respectively (Bej et al., 1990b). The primers used for detection of *uidA* and *lacZ* are listed in Table 3.1.

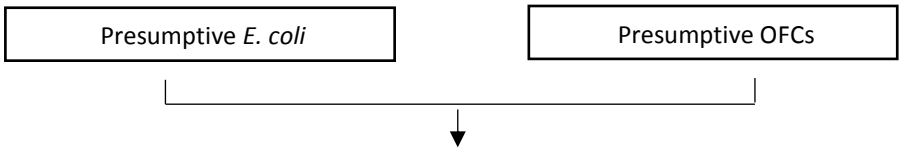
Table 3.1 *UidA* and *lacz* Genes, primer sequences and amplicon sizes

Organism	Gene	Primer code	Primer name	Primers sequence (5'-3')	Amplicon (bp)	Ref.
E. coli	*uidA*	*uidA*-F	UAL-754	AAAACGGCAAGAAAAAGCAG	147	Bej et al., 1991b
		uidA-R	UAR-900	ACGCGTGGTTACAGTCTTGCG		
OFCs	*lacZ*	*lacZ*-F	ZL-1675	ATGAAAGCTGGCTACAGGAAGGCC	264	Bej et al., 1990b
		lacZ-R	ZR-2025	GGTTTATGCAGCAACGAGACGTCA		

The identification of *E. coli* and OFCs was conducted in three phases, as explained below.

3.3.4.2.1 Multiplex PCR reaction

Multiplex PCR reaction was performed as illustrated in Figure 3.2 using a 50 µl reaction mixture consisting of 15 µl (0.6x) BioMix Red (Bioline USA), 1 µl (UAL-754) *uidA*-F, 1 µl (UAL-900) *uidA*-R, 1 µl (ZL-1675) *lacZ*-F, 1 µl (ZR-2025) *lacZ*-R (10 pmol ml^{-1} of each primer), 29 µl Water Molecular Biology Reagent (W4502, Sigma-Aldrich) and 2 µl DAN template. PCR amplification was conducted under the following PCR conditions: initial denaturation at 95°C for 3 min, preceding a loop of 30 cycles of denaturation at 94°C for 1 min, followed by annealing at 50°C for 1 min, and extension at 50°C for 1 min. Final elongation would occur at 72°C for 7 min in a thermal cycler (DNA Engine Tetrad 2 Peltier Thermal Cycler, BIO RAD) (Bej et al., 1991a; Bej et al. 1990a).



Multiplex PCR	PCR condition	PCR Reagent	Volume (µl)
Initial denaturation	95°C for 3 min	Water	29
Cycles	30	BioMix Red	15
Denaturation	94°C for 1 min	Primer (*uidA*-F)	1
Annealing	50°C for 1 min	Primer (*uidA*-R)	1
Extension	50°C for 1 min	Primer (*lacZ*-F)	1
Final steps	72°C for 7 min	Primer (*lacZ*-R)	1
Gel (2.5% agarose)10% NBS	5ul DNA - 25 min/100V	DNA template	2
		Total	50

Figure 3.2 Schematic identification of multiplex PCR for *uidA* and *lacZ* genes

3.3.4.2.2 Optimisation of non-specific bands results by single PCR

The conditions and reaction volume of the PCR were optimised to detect genes, where non-specific bands or error results were obtained from the multiplex PCR amplification. To confirm those results, two single PCR amplification assays were carried out targeting the *uidA* and *lacZ* gene. Concentrated DNA samples were diluted to roughly 20-30 ng, and absorbance was measured using a spectrophotometer (NanoDrop 1000 Spectrophotometer, Thermo Scientific). The concentration of the DNA was calculated as follows:

$$\frac{\textbf{Required final concentration of DNA (20 ng µl}^{-1}\textbf{) × 100 µl (final vol)}}{\textbf{Actual concentration of stock DNA (ng µl}^{-1}\textbf{)}} = \textbf{Vol of stock to add (µl)}$$

Diluted DNA was stored at -20°C until used as the template DNA.

PCR was performed for *uidA* by using a 50 µl reaction mixture consisting of 15 µl and 25 µl (1x) BioMix Red (Bioline), 1 µl *uidA*-F primer, 1 µl *uidA*-R primer, 31 and 21 µl Water Molecular Biology Reagent (W4502, Sigma-Aldrich) and 2 µl DAN template, as shown in Figure 3.3.

The single PCR for *lacZ* was carried out using a 50 µl reaction mixture consisting of 15 µl (0.6x) BioMix Red (Bioline), 1 µl (ZL-1675) *lacZ*-F, 1 µl (ZR-2025) *lacZ*-R, 31 µl Water Molecular Biology Reagent (W4502, Sigma-Aldrich), and 2 µl DAN template.

Single PCR amplification for *uidA* and *lacZ* genes was performed, with conditions as follows: initial denaturation step at 95°C for 3 min; 40 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min and extension at 70°C for 1 min. Final elongation would occur at 72°C for 7 min in a thermal cycler.

PCR-amplified fragments (5 µl aliquots) were separated on 2.5% (w/v) agarose gels. Agarose gels were run in 1x-TBE buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 8.0) at 100V for 25 min. The gels were stained with 10% of SafeView Nucleic Acid (NBS Biologicals) and visualized under UV light (Molecular Imager® Gel Doc™ XR System, Bio-Rad). *E. coli* (NCTC 13353), *Klebsiella pneumoniae* (NCTC 13368) were used as positive controls and distilled water was used as a negative control. All DNA of *uidA*-positive and *lacZ*-positive were stored at -20°C for further analysis.

False positive *E. coli*:

- Reduced DNA concentration (20-30 ng)
- Run single PCR

False positive OFCs:

- Reduced DNA concentration (20-30 ng)
- Run single PCR

Single PCR (*uidA*)	PCR condition
Initial denaturation	95°C for 3 min
Cycles	40
Denaturation	94°C for 1 min
Annealing	65°C for 1 min
Extension	70°C for 1 min
Final steps	72°C for 7 min
Gel (2.5% agarose) 10% NBS	2.5%- 5ul- 25 min/100V
PCR Reagent	Volume (µl)
Water	31 and 21
BioMix Red	15 and 25
Primer (*uidA*-F)	1
Primer (*uidA*-R)	1
DNA template	2
Total	50

Single PCR (*lacZ*)	PCR condition
Initial denaturation	95°C for 3 min
Cycles	40
Denaturation	94°C for 1 min
Annealing	65°C for 1 min
Extension	70°C for 1 min
Final steps	72°C for 7 min
Gel (2.5% agarose) 10% NBS	2.5%- 5ul- 25 min/100V
PCR Reagent	Volume (µl)
Water	31
BioMix Red	15
Primer (*lacZ*-F)	1
Primer (*lacZ*-F)	1
DNA template	2
Total	50

PCR negative *E. coli* excluded

PCR negative OFCs excluded

Figure 3.3 Schematic identification of single PCR for *uidA* and *lacZ* gene

3.3.4.3 DNA sequencing

3.3.4.3.1 *uidA* and *lacZ* genes

This study analysed a partial sequence (147 bp) of *uidA* gene, and (264 bp) of *lacZ* gene. DNA sequences, five *E. coli* strains (*n* = 2, multiplex PCR and *n* = 3, single PCR) and two OFCs strains (*n* = 1, multiplex PCR and *n* = 1, single PCR) were selected randomly to confirm those genes. PCR products were purified by PCR purification kit (QIAGEN, Germany) according to the manufacturer's protocol. DNA sequencing was performed by Tube Seq service (Eurofins Genomics). Each nucleotide sequence sample was compared with the known *uidA* and *lacZ* genes

sequence databases including NCBI (National Center for Biotechnology Information) using the nucleotide blastx (translated nucleotide to protein) programs (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.3.4.3.2 16S rRNA gene

This study used 16S rRNA gene to identify ESBL *E. coli* and ESBL OFCs isolates. The primers used for detection 16S rRNA gene were forward 27f (5' AGAGTTTGATCMTGGCTCAG 3'), reverse primer 1492r (5' TACGGYTACCTTGTTACGACTT 3') and amplicon sizes 1500pb (Frank et al., 2008). Amplification of the 16S rRNA gene was performed using a 50 µl reaction mixture consisting of 25 µl (1x) BioMix Red (Bioline USA), 1 µl (27f) 16S rRNA-F, 1 µl (1492r) 16S rRNA-R (10 pmol ml^{-1} of each primer), 21 µl Water Molecular Biology Reagent (W4502, Sigma-Aldrich) and 2 µl DAN template (Frank et al., 2008). The PCR program consisted of the following steps run on a PCR condition: initial denaturation at 95°C for 2 min, preceding a loop of 30 cycles of denaturation at 94°C for 30 sec, followed by annealing at 50°C for 1 min, and extension at 72°C for 2 min. Final elongation would occur at 72°C for 10 min in a thermal cycler (DNA Engine Tetrad 2 Peltier Thermal Cycler, BIO RAD) (Frank et al., 2008). PCR-amplified fragments (3 µl aliquots) were separated on 0.8% (w/v) agarose gels. Agarose gels were run in 1x-TBE buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 8.0) at 100V for 25 min. The gels were stained with 10% of SafeView Nucleic Acid (NBS Biologicals) and visualized under UV light (Molecular Imager® Gel Doc™ XR System, Bio-Rad). *E. coli* (NCTC 13353), *Klebsiella pneumoniae* (NCTC 13368) were used as positive controls and distilled water was used as a negative control.

PCR products were purified and sequenced by Macrogen; (https://dna.macrogen.com/eng/). Each nucleotide sequence sample was compared with the known 16S rRNA gene sequence databases including NCBI (National Center for Biotechnology Information) using 16S ribosomal RNA sequences by blastn program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

3.3.5 Screening ESBLs

There are three ways to detect ESBLs, including (i) double disc synergy test, (ii) combination disc test and (iii) gradient ESBL strips, but a combination disc test is recommended by the Clinical Laboratory Standard Institute guidelines due to a low cost and do not require critical disc spacing (Public Health England, 2016). In this study, verification of the ESBL-producing phenotype of

identified colonies was by the combination disk method, as described by the UK Standards for Microbiology Investigations (Public Health England, 2016). *E. coli* and OFCs confirmed isolates were sub-cultured on nutrient agar plates, and incubated for 24 h at 37°C. Colonies were picked from the agar plates, and suspended in normal saline (0.9% NaCl), and adjusted to 0.5 McFarland standard. The bacterial suspension was spread on Mueller-Hinton agar plates (PP0963; E&O Laboratories Ltd.) using a sterile swab stick, allowed to dry, and impregnated with antibiotic disk. The screening for ESBL was performed using both the antibiotics Cefpodoxime (CPD) 10 µg and Cefpodoxime-clavulanic acid (CPD-CV) (Oxoid Ltd) 10/1 µg placed on Mueller-Hinton agar. CPD for detection of ESBL production may be used to indicate ESBL producers (resistance to 3rd generation cephalosporins), and strains that hyper-produce AmpC or K1 enzymes (Livermore et al., 2001).

After incubation at 37°C for 24 h, the diameter of the inhibition zone around the two disks and the difference between them were measured twice (Figure 3.4). ESBL production was defined as positive when there was an increase of ≥ 5 mm in the zone around the disk containing clavulanic acid compared the zone of corresponding disks without clavulanic acid. In each batch, *E.coli* (NCTC 13353), *Klebsiella pneumoniae* (NCTC 13368) were used as an ESBL -positive control and *E.coli* (NCTC 10418) as an ESBL-negative control, respectively, as recommended by Public Health England (2016).

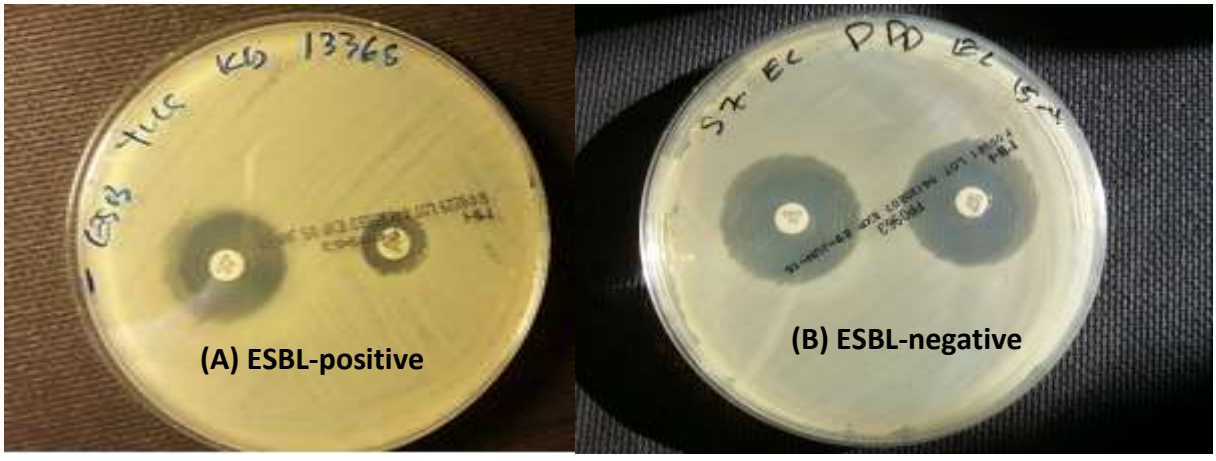


Figure 3. 4 Detection of ESBL showing, by combination disc test, using Cefpodoxime (CPD) 10 µg and Cefpodoxime-clavulanic acid 10/1 µg, (A) ESBL-producing strain and (B) ESBL-negative strain as described by Public Health England (2016)

3.3.6 Detection of ESBLs genes

Genotypic characterization of ESBL -producers was determined by multiplex PCR assay using primers as described by Dallenne et al., (2010) targeting *bla*$_{CTX-M}$ groups as *bla*$_{CTX-M}$ group 1, *bla*$_{CTX-M}$ group 2, *bla*$_{CTX-M}$ group 9 and *bla*$_{CTX-M}$ group 8/25, and *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA}$ genes (Table 3. 2).

Table 3.2 Gene target, primer sequences and amplicon sizes for multiplex PCR (I) and (II) (Dallenne et al., 2010)

PCR	Target gene(s)	Primer name	Sequences (5'-3')	Size (bp)
Multiplex PCR (I)	*bla*$_{CTX-M}$ group 1	CTXM1- F CTXM1- R	TTAGGAARTGTGCCGCTGYA CGATATCGTTGGTGGTRCCAT	688
	bla$_{CTX-M}$ group 2	CTXM2- F CTXM2- R	CGTTAACGGCACGATGAC CGATATCGTTGGTGGTRCCAT	404
	bla$_{CTX-M}$ group 9	CTXM9- F CTXM9- R	TCAAGCCTGCCGATCTGGT TGATTCTCGCCGCTGAAG	561
	bla$_{CTX-M}$ group 8/25	CTXM8/25- F CTXM8/25- R	AACRCRCAGACGCTCTAC TCGAGCCGGAASGTGTYAT	326
Multiplex PCR (II)	*bla*$_{TEM}$	TEM- F TEM- R	CTTTCCGTGTCGCCCTTATTC CGTTCATCCATAGTTGCCTGAC	800
	bla$_{SHV}$	SHV- F SHV- R	AGCCGCTTGAGCAAATTAAAC ATCCCGCAGATAAATCACCAC	713
	bla$_{OXA}$	OXA- F OXA - R	GGCACCGATTCAACTTTCAAG GACCCCAAGTTTCCTGTAAGTG	564

PCR reactions were performed using the recommended procedure by Dallenne et al. (2010) in a 25 µl reaction mixture which consisted of 12.5 µl BioMix Red (Bioline), forward and reverse primers (10 pmol μl^{-1}), Water Molecular Biology Reagent (W4502, Sigma-Aldrich) and to 1 µl (20-30 ng) of template DNA, as illustrated in Table 3.3.

Table 3.3 Multiplex PCR reactions for *bla*$_{CTX-M}$ groups, and *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA}$

A - Multiplex PCR (I) *bla*$_{CTX-M}$ groups

PCR reaction	Volume (µl)
Water	10.2
BioMix Red	12.5
CTXM1 F	0.2
CTXM1 R	0.1
CTXM2 F	0.1
CTXM2 R	0.1
CTXM9 F	0.2
CTXM9 R	0.2
CTXM8/25 F	0.2
CTXM8/25 R	0.2
DNA template	1
Total	25

B - Multiplex PCR (II) *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA}$

PCR reaction	Volume (µl)
Water	10.3
BioMix Red	12.5
TEM F	0.2
TEM R	0.2
SHV F	0.2
SHV R	0.2
OXA F	0.2
OXA R	0.2
DNA template	1
Total	25

The amplification protocol of *bla*$_{CTX-M}$ groups, and *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA}$ genes with oligonucleotide primers was carried out in a thermal cycler as follows: initial denaturation step (94°C, 10 min) followed by 30 cycles of denaturation (94°C, 40 sec), annealing (60°C, 40 sec) and extension (72°C, 20 sec), and a single final extension of 7 min at 72°C (Dallenne et al., 2010).

After PCR amplification, amplified products were visualized using 2.0% agarose gel electrophoresis. The gel consisted of 1x-TBE buffer (0.09 M Tris-borate, and 0.002 M EDTA, pH 8.0) and stained using 10% of Safe View Nucleic Acid (NBS Biologicals). Products were run at 100V for 30 min and visualized under UV light (Molecular Imager® Gel Doc™ XR System, Bio-Rad). *E.coli* (NCTC 13353) as *bla*$_{CTX-M-15}$ (group 1), *E.coli* (NCTC 13462) as *bla*$_{CTX-M-2}$ (group 2), *Enterobacter cloacae* (NCTC 13463) as *bla*$_{CTX-M-9}$ (group 9), *E.coli* (NCTC 13463) as *bla*$_{CTX-M-8}$ (group 8/25), *Klebsiella pneumoniae* (NCTC 13368) as *bla*$_{SHV-18}$ (SHV), *E.coli* (NCTC 11560) as *bla*$_{TEM-1}$ (TEM) and due to the difficulty in obtaining a *bla*$_{OXA}$ control, but *bla*$_{OXA}$ sample was sequenced; accession number ACI29956 (100% identity) as *bla*$_{OXA-1}$ (OXA) were used in the PCR run as ESBL-positive controls and *E. coli* (NCTC 10418) as ESBL-negative control.

3.3.7 Statistical analyses

Data were analysed using IBM SPSS Statistics 22 and Excel 2013. For comparison of means for more than two groups, number of *E. coli* and OFCs between influent (raw sample wastewater), primary sediment tank, secondary aeration tank and effluent were evaluated via Kruskal-Wallis one-way Analysis of Variance (ANOVA) (*P*-value <0.05). Mann-Whitney *U* test was used to compare mean concentrations between *E. coli* and OFCs via two sites of WWTP and a significance level of *P*<0.05. The relationship between the numbers of ESBL-producing bacteria carrying antibiotic resistance genes at different stage points was assessed.

3.4 Results

3.4.1. Enumeration of *E. coli* and OFCs

Over eleven sampling times, the overall mean of presumptive *E. coli* and OFCs were significantly different between the four stages of the treatment plant (Kruskal-Wallis one-way ANOVA test, *P* <0.05). The mean number of presumptive *E. coli* were higher than OFCs at WWTP. The mean of *E. coli* recovered from influent to sediment tank were not statistically different (Mann-Whitney test, *P* =0.086); however, there was a significant decrease in the mean of OFCs recovered (Mann-Whitney test, *P* =0.009). The mean of *E. coli* and OFCs recovered from the primary sediment tank to the aeration tank remained relatively stable (no significant difference, Mann-Whitney test, *P* >0.05). However, numbers of both bacteria did decrease significantly (by 99.9% for each) between the influent and effluent (Mann-Whitney *U* test, *P* <0.05) (Figure 3.5). As a proportion of initial input, 0.08% (232 CFU ml^{-1}) of *E. coli* and 0.07% (132 CFU ml^{-1}) of OFCs were released to the environment, after the treatment (see Table I in the Appendix).

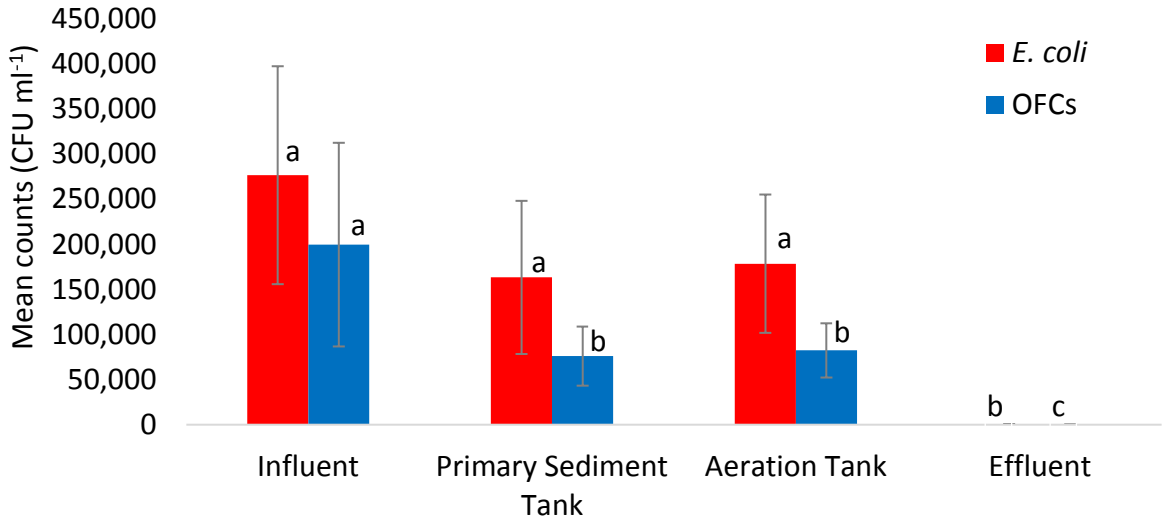


Figure 3.5 Mean (± SEM) presumptive counts of *E. coli* and other faecal coliform (OFCs) at each stage of the WWTP. Letters denote the significant differences (Mann-Whitney *U* test, $P<0.05$) between *E. coli* and OFCs at each stage

3.4.2 Bacterial confirmation

Over seven sampling times (sampling 1st to 6th and 11th), 155 presumptive *E. coli* and 152 OFCs were isolated randomly (see Table II in the Appendix).

Table 3.4 Bacteria isolates confirmed by oxidase and PCR (*uidA* & *lacZ* genes) tests

Sample site	Organism	No. isolated	Confirmatory tests			Total
			Oxidase (-ve)	Multi-PCR	Non-specific bands (single-PCR)	
Influent	*E. coli*	41	32	17	15	32
	OFCs	39	41	26	12	38
Primary Sediment Tank	*E. coli*	40	35	13	20	33
	OFCs	39	37	21	14	35
Aeration Tank	*E. coli*	38	34	9	23	32
	OFCs	41	36	22	13	35
Effluent	*E. coli*	36	29	13	13	26
	OFCs	33	30	12	16	28
Total isolates	*E. coli*	155	130	52	71	123
	OFCs	152	144	81	55	136

As in Table 3.4, biochemical (oxidase) tests confirmed 130 out of 155 (83.9%) as *E. coli* and 144 out of 152 (94.7%) as OFCs. Further testing by multiplex PCR was employed to confirm the presence of *uidA* and *lacZ* genes. This found that the size of the *uidA* and *lacZ* were 147 and 264 bp (Figure 3.6). The results of multiplex PCR confirmed 52 out of 130 to be *E. coli*, and 81 out of 144 to be OFCs. There were 71 and 55 non-specific bands for *E. coli* and OFCs, respectively. Afterwards, the concentration of each DNA templet strain was reduced to 20-30 ng and optimised for single PCR under standard PCR conditions. Single PCR detected the *uidA* gene for *E. coli* (Figure 3.7) and *lacZ* gene for OFCs (Figure 3.8). Some *E. coli* strains (*uidA* gene) were found difficult to detect by single PCR. After optimising the reaction conditions such as by increasing the BioMix Red volume to 25 µl in a 50 µl mixture, the single PCR yielded the expected band for the *uidA* gene (Figure 3.9).

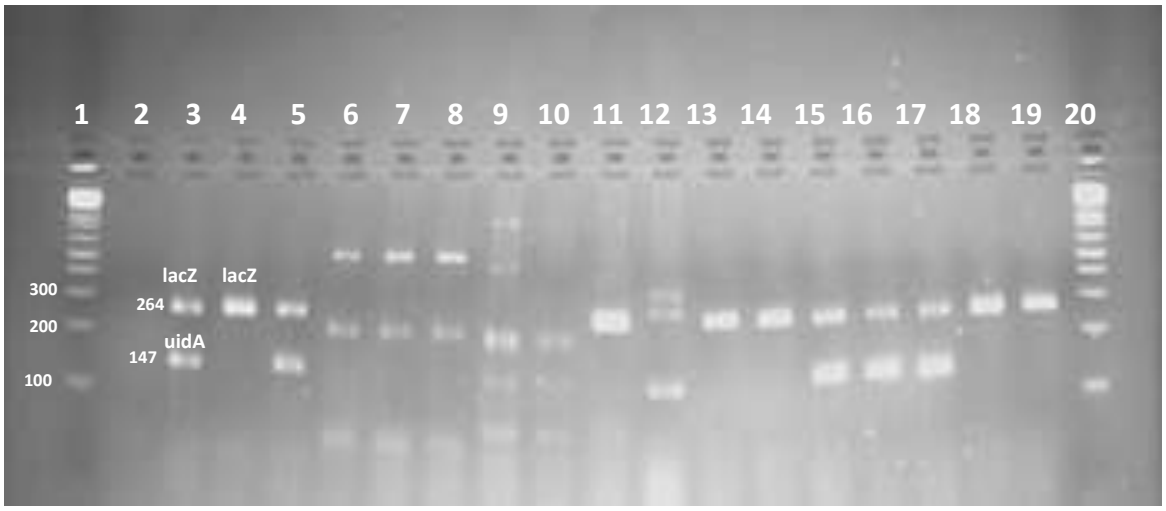


Figure 3.6 Multiplex PCR assay for *E. coli* and OFCs. Lanes: **1** and **20**, Marker 1, kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific); **2**, control negative (distilled water): **3**, *E. coli* NCTC 13353 as control positive for *uidA* gene and *lacZ* gene; **4**, OFCs as *Klebsiella pneumonia* NCTC 13368 as control positive for *lacZ* gene; **5**, **15** to **17** *E. coli* strains; **6** to **8**, and **12** false positive *E. coli*; **9** and **10** false positive OFCs; **11**, **13**, **14**, **18**, **19** OFCs strains, with the PCR conditions of initial denaturation at 95°C for 3 min, preceding a loop of 30 cycles of denaturation at 94°C for 1 min, followed by annealing at 50°C for 1 min, and extension at 50°C for 1 min. Final elongation would occur at 72°C for 7 min

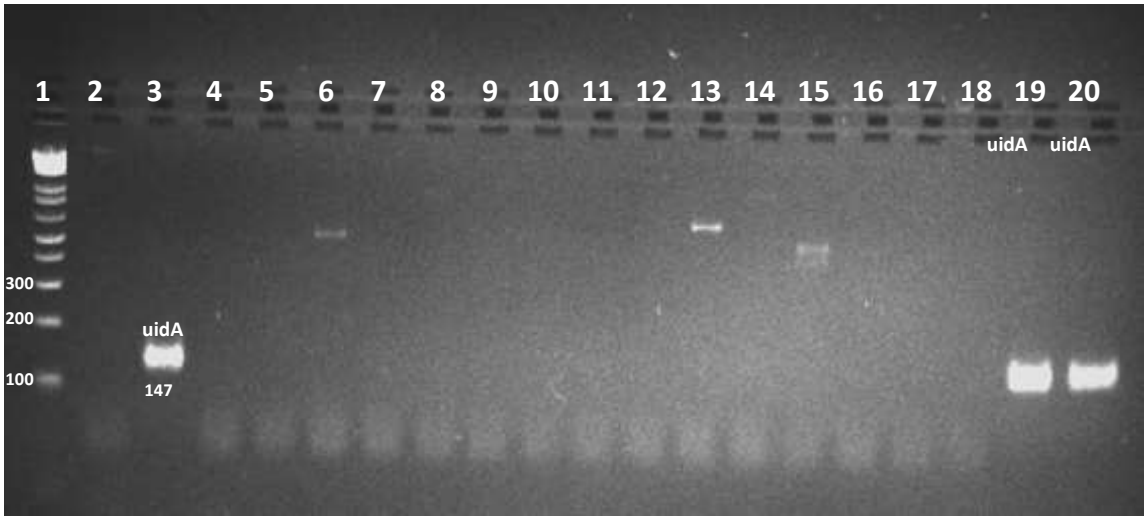


Figure 3.7 Single PCR assay for *E. coli* (*uidA* gene). Lanes: **1**, Marker 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific); **2**, control negative (distilled water): **3**, *E. coli* NCTC 13353 as positive control for *uidA* gene; **4** to **18** false positive *E. coli*; **19** and **20** *E. coli* strains, with the PCR conditions of initial denaturation at 95°C for 3 min, preceding a loop of 40 cycles of denaturation at 94°C for 1 min, followed by annealing at 65°C for 1 min, and extension at 70°C for 1 min. Final elongation would occur at 72°C for 7 min. Using a 50 µl reaction mixture consisting of 15 µl (0.6x) BioMix Red, 1 µl uidA-F, 1 µl uidA-R, 31 µl Water Molecular Biology Reagent (W4502, Sigma-Aldrich) and 2 µl DAN template

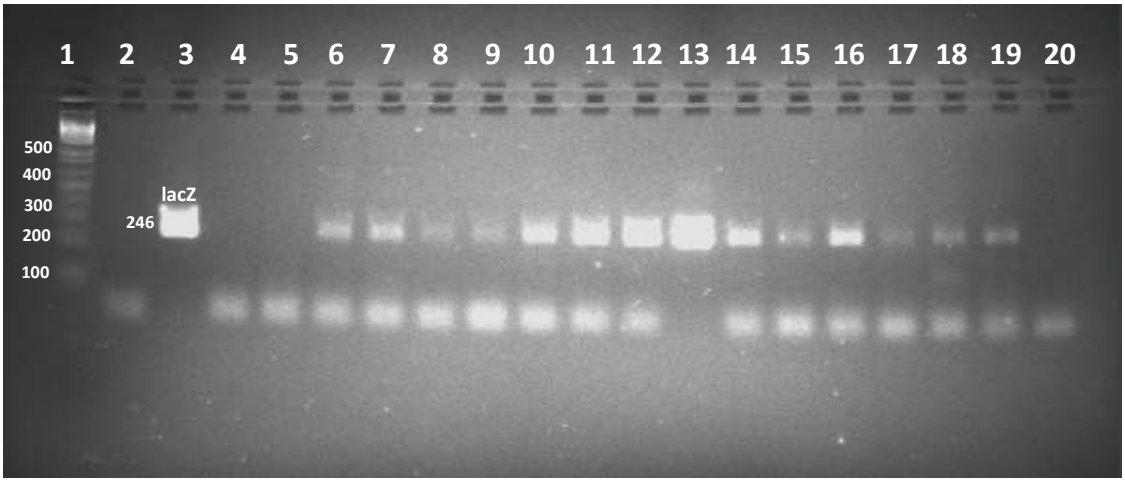


Figure 3.8 Single PCR assay for OFCs (*lacZ* gene). Lanes: **1**, Marker 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific); **2**, control negative (distilled water): **3**, OFCs as *Klebsiella pneumonia* NCTC 13368 as control positive for *lacZ* gene; **4**, **5** and **20**, strain negative; **6** to **19**, OFCs strains, with the PCR condition. initial denaturation at 95 °C for 3 min; 40 cycles of denaturation at 94 °C for 1 min, annealing at 65°C for 1 min and extension at 70°C for 1 min. Final elongation would occur at 72°C for 7 min

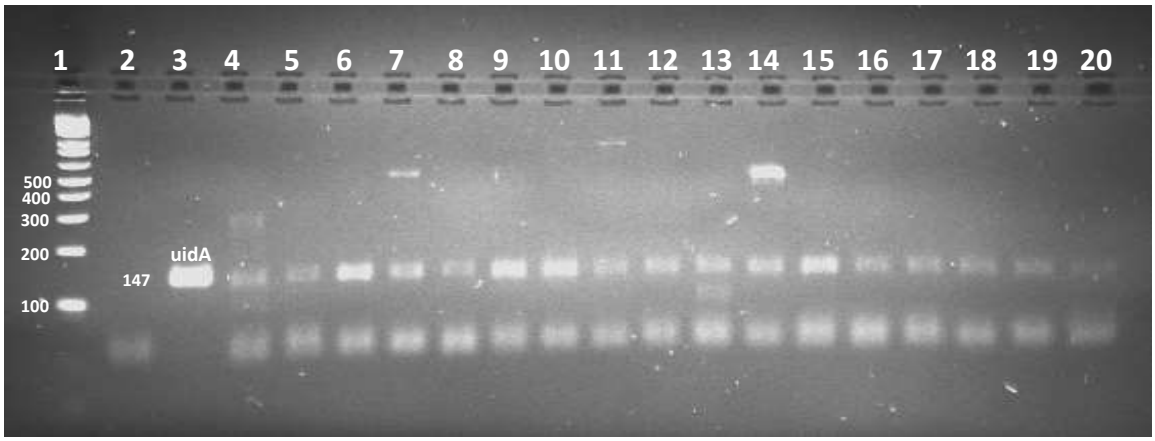


Figure 3.9 Single PCR assay for *E. coli* (*uidA* gene). Lanes: **1**, Marker 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific); **2**, control negative (distilled water): **3**, *E. coli* NCTC 13353 as control positive for *uidA* gene; **4** to **20**, *E. coli* strains, with the PCR condition initial denaturation step at 95°C for 3 min; 40 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min and extension at 70°C for 1 min. Final elongation would occur at 72°C for 7 min. Using a 50 µl reaction mixture consisting of 25 µl (1x) BioMix Red, 1 µl uidA-F, 1 µl uidA-R, 21 µl Water Molecular Biology Reagent (W4502, Sigma-Aldrich) and 2 µl DAN template

After the colonies were confirmed by morphological, biochemical and PCR tests, additional confirmation was carried out by sequencing some of the selected DNA samples (five *uidA* (*E. coli*) and two *lacZ* (OFCs) genes). Nucleotide sequences identified *uidA* and *lacZ* genes with the following accession number: CCM12452, AAR13951, and ABO70154 for *E. coli*, and KTG74013 for OFCs as illustrated in Table 3.5.

Table 3.5 Result of nucleotide sequences of *uidA* and *LacZ* genes selected from multiplex and single PCR randomly

PCR	Sample	Organism identified based via blastx	Accession	Identity (%)
Multiplex	1-*uidA*	*E. coli* (*uidA*)	CCM12452	100
	2-*uidA*	*E. coli* (*uidA*)	AAR13951	100
	3-*lacZ*	*Klebsiella pneumonia* (*lacZ)*	KTG74013	97.06
Single	4-*uidA*	*E. coli* (*uidA*)	ABO70154	100
	5-*uidA*	*E. coli* (*uidA*)	CCM12452	100
	6-*uidA*	*E. coli* (*uidA*)	ABO70154	100
	7-*lacZ*	*Klebsiella pneumonia* (*lacZ)*	KTG74013	98.57

Following PCR, 32 presumptive *E. coli* and sixteen presumptive OFC isolates gave negative results, and hence were excluded from further analysis. Nucleotide sequence analysis of 16S rRNA gene (based on *uidA* PCR) identified four *E. coli* strains (accession number NR_114042) and one *Enterobacter cancerogenus* (accession number NR_116756.1; Table 3.6). For the 17 ESBL OFC (based on *lacZ* PCR) isolates, all were confirmed as *Enterobacter* spp., *Kluyvera* spp., *Raoultella* spp. and *Citrobacter* spp. (Table 3.6).

Table 3. 6 Nucleotide sequences of 16S rRNA gene in five *E. coli* and seventeen OFCs of ESBL isolates at different times and sites

ID number of strain	Time collecting - site	NCBI (16S rRNA)		
		Organism	Accession	Identity (%)
1-*E. coli*	2nd - Influent	*E. coli*	NR_114042	99.17
2-*E. coli*	3rd – Primary Sediment Tank	*Enterobacter cancerogenus*	NR_116756	99.86
3-*E. coli*	3rd – Effluent	*E. coli*	NR_114042	99.79
4-*E. coli*	3rd – Effluent	*E. coli*	NR_114042	99.79
5-*E. coli*	6th - Primary Sediment Tank	*E. coli*	NR_114042	99.79
1-OFCs	1st - Influent	*Enterobacter ludwigii*	NR_042349	99.79
2-OFCs	1st - Influent	*Enterobacter ludwigii*	NR_042349	99.79
3-OFCs	1st - Influent	*Kluyvera cryocrescens*	NR_114108	99.17
4-OFCs	2nd - Influent	*Kluyvera ascorbata*	NR_114589	98.88
5-OFCs	3rd - Primary Sediment Tank	*Enterobacter ludwigii*	NR_042349	99.60
6-OFCs	4th - Primary Sediment Tank	*Raoultella ornithinolytica*	NR_044799	99.30
7-OFCs	4th - Primary Sediment Tank	*Raoultella ornithinolytica*	NR_114502	99.90
8-OFCs	5th - Primary Sediment Tank	*Raoultella ornithinolytica*	NR_114502	99.69
9-OFCs	6th - Influent	*Raoultella ornithinolytica*	NR_044799	99.90
10-OFCs	6th - Primary Sediment Tank	*Kluyvera ascorbata*	NR_114589	98.79
11-OFCs	6th - Primary Sediment Tank	*Citrobacter freundii*	NR_028894	99.11
12-OFCs	11th - Influent	*Raoultella ornithinolytica*	NR_044799	99.81
13-OFCs	11th - Influent	*Raoultella terrigena*	NR_037085	100
14-OFCs	11th - Influent	*Raoultella ornithinolytica*	NR_044799	99.90
15-OFCs	11th - Effluent	*Raoultella ornithinolytica*	NR_044799	99.90
16-OFCs	11th - Effluent	*Raoultella ornithinolytica*	NR_114502	99.69
17-OFCs	11th - Effluent	*Raoultella ornithinolytica*	NR_114502	99.90

3.4.3 ESBL phenotypes and genotypes

The overall prevalence of confirmed ESBL phenotypes was 4 *E. coli* and 18 OFCs. Their prevalence in ESBL-producing *E. coli* was 1/32 in influent, 1/33 in primary sediment tank, and 2/26 in effluent.

For ESBL-producing OFCs, 8/38 were recovered in influent, 7/35 in primary sediment tank, and 3/28 in effluent. No ESBL genes were found at the aeration tank stage (Table 3.7).

Table 3.7 Number of phenotypic and genotypic isolates detected ESBL genes in different site samples

Sample site	Organism	Total no. isolated	ESBLs isolates	ESBLs prevalence						
				bla$_{CTX-M}$ group				*bla*$_{TEM}$	*bla*$_{SHV}$	*bla*$_{OXA}$
				1	2	8/25	9			
Influent	*E. coli*	32	1	1	-	-	-	-	-	1
	OFCs	38	8	1	1	-	-	2	5	-
Primary Sediment Tank	*E. coli*	33	1	1	-	-	-	1	-	-
	OFCs	35	7	1	1	-	-	-	5	-
Aeration Tank	*E. coli*	32	0	-	-	-	-	-	-	-
	OFCs	35	0	-	-	-	-	-	-	-
Effluent	*E. coli*	26	2	2	-	-	-	2	-	-
	OFCs	28	3	-	-	-	-	-	3	-
Total	*E. coli*	123	4	4	-	-	-	3	-	1
	OFCs	136	18	2	2	-	-	2	13	-

To estimate the number of ESBLs released per day via the effluent, the following steps were taken:

1. The percentage of confirmed positive bacteria was multiplied by the mean counts of bacteria.
2. The mean counts of positive bacteria were multiplied by the percentage confirmed as ESBLs isolates.
3. This number was multiplied with the outflow per day (24.5 million litres).

This approach estimated approximately 300 billion each of both ESBL-producing *E. coli* and OFCs are released per day into the Menai Strait, as showed in Table 3.8.

Table 3. 8 Estimated ESBLs producers in *E. coli* and OFCs at Effluent site of Bangor's WWTP. [A] is mean counts of presumptive bacteria, and [B] is mean counts of positive bacteria

	E. coli	**OFCs**
[A]Mean CFU ml^{-1}	232	132
Number of presumptive isolates	36	33
Confirmed positive isolates (%)	26 (72.2%)	28 (84.8%)
[B]Mean CFU ml^{-1}	168	112
Confirmed positive ESBLs isolates (%)	2 (7.7%)	3 (10.7%)
ESBLs CFU ml^{-1}	12.936 ≈ 13	11.984 ≈ 12
ESBLs CFU l^{-1}	13,000	12,000
ESBLs CFU l day^{-1}	318,500,000,000	294,000,000,000

After optimising the amplification conditions for each assay, expected-bands of $bla_{CTX\text{-}M}$ groups, bla_{SHV}, bla_{TEM} and bla_{OXA} genes were obtained from control bacteria and sample strains. For example, multiplex PCR for $bla_{CTX\text{-}M}$ groups *E. coli* are shown in Figure 3.10 and for OFCs in Figure 3.11, and multiplex PCR for bla_{SHV}, bla_{TEM} and bla_{OXA} genes for OFCs are shown in Figure 3.12.

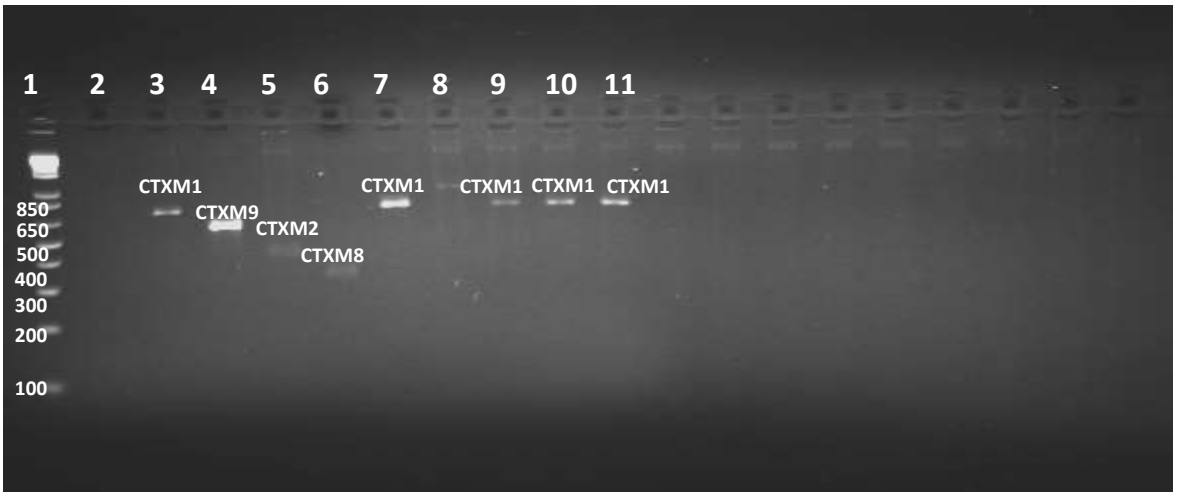


Figure 3.10 Multiplex PCR assay for *bla*$_{CTX-M}$ groups for *E. coli*. Lanes: **1**, Marker 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific); **2**, ESBL-negative control (*E. coli* NCTC 10418): **3**, *E. coli* (NCTC 13353) as *bla*$_{CTX-M-15}$ (group 1); **4**, *Enterobacter cloacae* (NCTC 13463) as *bla*$_{CTX-M-9}$ (group 9); **5**, *E. coli* (NCTC 13462) as *bla*$_{CTX-M-2}$ (group 2); **6**, *E. coli* (NCTC 13463) as *bla*$_{CTX-M-8}$ (group 8/25); **7**, and **9** to **11**, *E. coli* carried *bla*$_{CTX-M}$ group 1; **8**, *E. coli* negative *bla*$_{CTX-M}$ groups

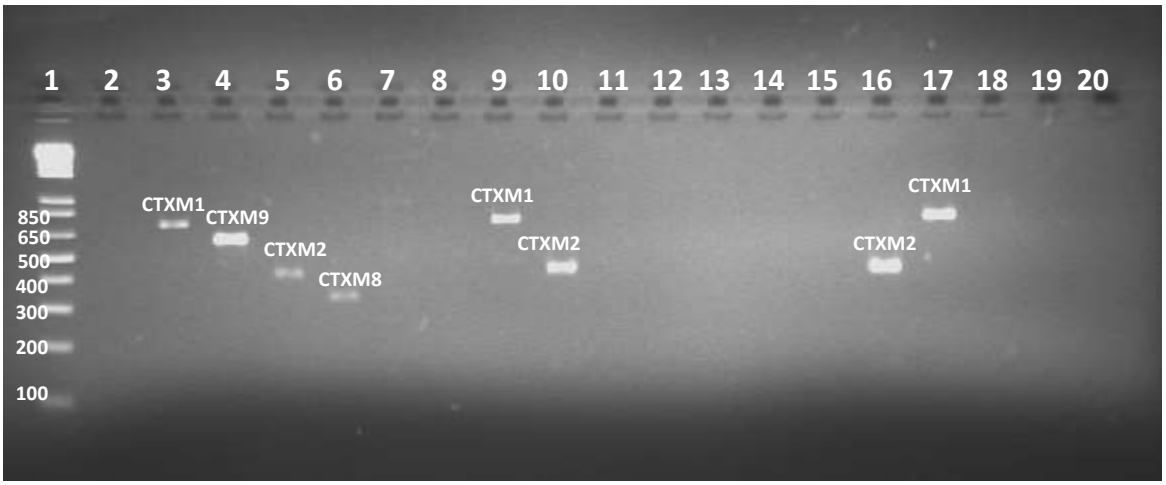


Figure 3.11 Multiplex PCR assay for *bla*$_{CTX-M}$ groups for OFCs. Lanes: **1**, Marker 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific); **2**, ESBL-negative control (*E. coli* NCTC 10418); **3**, *E. coli* (NCTC 13353) as *bla*$_{CTX-M-15}$ (group 1); **4**, *Enterobacter cloacae* (NCTC 13463) as *bla*$_{CTX-M-9}$ (group 9); **5**, *E.coli* (NCTC 13462) as *bla*$_{CTX-M-2}$ (group 2); **6**, *E.coli* (NCTC 13463) as *bla*$_{CTX-M-8}$ (group 8/25); **7**, **8**, **11** to **15,** and **18** to **20**, OFCs negative *bla*$_{CTX-M}$ groups; **9** and **17**, *E. coli* carried *bla*$_{CTX-M}$ group 1; **10** and **16**, OFCs carried *bla*$_{CTX-M}$ group **2**; **7**, **8**, **11** to **15**, **18** to **20** OFCs negative *bla*$_{CTX-M}$ groups

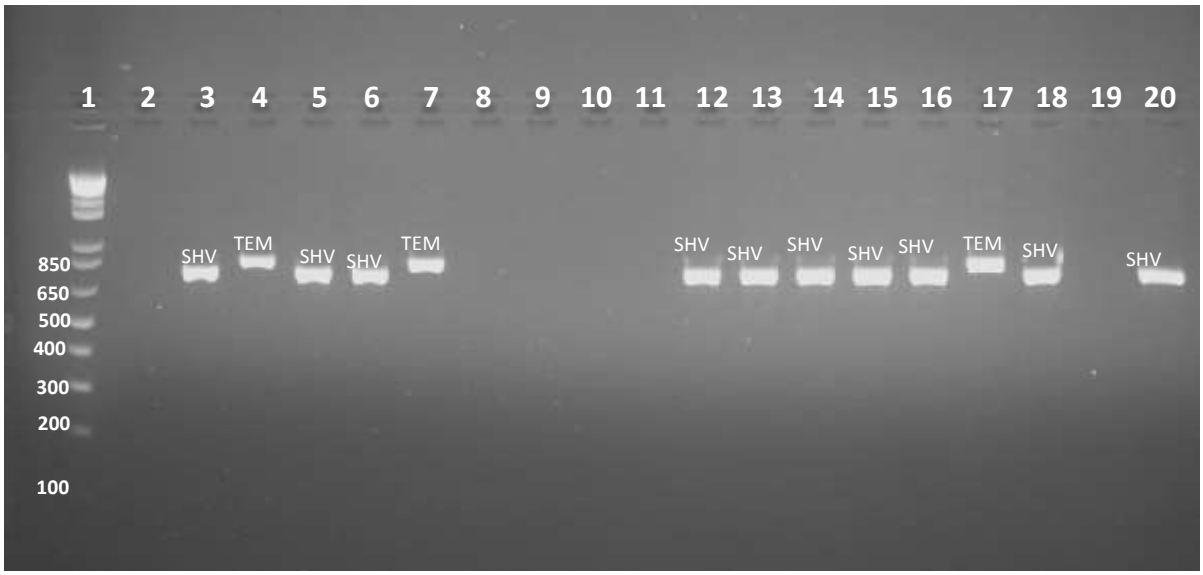


Figure 3.12 Multiplex PCR assay for *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{OXA}$ genes for OFCs. Lanes: **1**, Marker 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific); **2**, ESBL-negative control (*E. coli* NCTC 10418): **3**, *Klebsiella pneumoniae* (NCTC 13368) as *bla*$_{SHV-18}$ (SHV); **4**, *E. coli* (NCTC 11560) as *bla*$_{TEM-1}$ (TEM); **5** to **6**, **12** to **16**, **18**, **and 20**, OFCs *bla*$_{SHV}$;**7** and **17**, OFCs *bla*$_{TEM}$; **8** to **11**, and **19**, OFCs negative *bla*$_{SHV}$ *bla*$_{TEM}$ and *bla*$_{OXA}$ genes

At the influent site, one ESBL *E. coli* isolate was detected that carried two *bla* genes as *bla*$_{CTX-M}$ group1 and *bla*$_{OXA}$, while of eight ESBL OFC isolates, seven of them contained a single *bla* gene as *bla*$_{CTX-M}$ group 2 (*n* = 1), *bla*$_{TEM}$ (*n* = 1) and *bla*$_{SHV}$ (*n* = 5), and one isolate carried three *bla* genes (*bla*$_{CTX-M}$ group 1, *bla*$_{CTX-M}$ group 2 and *bla*$_{TEM}$). At the primary sediment tank stage, one ESBL-*E. coli* isolates carried two genes *bla*$_{CTX-M}$ group 1 and *bla*$_{TEM}$ (*n* = 1), while seven ESBL OFCs isolates harboured a single *bla* gene as *bla*$_{CTX-M}$ group1 (*n* = 1), *bla*$_{CTX-M}$ group 2 (*n* = 1) and *bla*$_{SHV}$ (*n* = 5). At the effluent stage, two ESBL *E. coli* isolates carried two genes *bla*$_{CTX-M}$ group 1 (*n* = 2) and *bla*$_{TEM}$ (*n* = 2), while three ESBL OFCs isolates harboured a single *bla* gene as *bla*$_{SHV}$ (*n* = 3).

The detected ESBL gene among four ESBL *E. coli* strains were identified as *bla*$_{CTX-M}$ group 1 in 4 (influent *n* = 1, primary sediment tank *n* = 1, and effluent *n* = 2), *bla*$_{TEM}$ in 3 (primary sediment tank *n* = 1 and effluent *n* = 2) and *bla*$_{OXA}$ in 1 (influent *n* = 1), isolates. Eighteen ESBL-producing OFCs carried gene coding as *bla*$_{SHV}$ in 13 (influent *n* = 5, primary sediment tank *n* = 5, and effluent *n* = 3), *bla*$_{TEM}$ in 2 at influent, *bla*$_{CTX-M}$ group 1 in 2 (influent *n* = 1 and primary sediment tank *n* = 1), and *bla*$_{CTX-M}$ group 2 gene in 2 isolates (influent *n* = 1 and primary sediment tank *n* = 1).

3.5 Discussion

Tertiary treatment process for wastewater is supposed to remove 99% of microorganisms (Amos et al., 2014; Baquero et al., 2008). The majority of bacteria within sewage water (raw wastewater) are human-derived faecal coliforms such as *E. coli* and OFCs (Malham et al., 2014). Others have found the number of faecal coliforms usually contained in raw influent to be 10^6–10^8 100 ml^{-1} (George et al., 2002). During wastewater treatment process, when wastewater moves from primary treatment to secondary treatment, there could be change in the bacterial population but tertiary or advanced treatment can eliminate most of the bacteria and components (Bouki et al., 2013). Traditional treatment plants (e.g. secondary treatment plant) are less efficient in removing organic matter such as suspended solids, and reducing biological and chemical oxygen demand. They typically reduce microbial populations by approximately 90%, which some have argued is not sufficient when the treated water is released to the environment (Mounaouer and Abdennaceur, 2015). In this study, between the influent and effluent stages (post-UV disinfection), approximately 99.9% of both *E. coli* and OFCs had been removed. However, hundreds of *E. coli* and OFCs per ml of effluent were discharged into the Menai Strait. Reinthaler et al. (2003) found a similar rate of *E. coli* released to the environment. There are many factors that could influence the efficiency of UV unit process, such as inappropriate UV dose, loads of suspended solids, turbidity and flocs (Hassen et al., 2000). Therefore, microbes could pass through UV lamps without destruction and then have capability to repair DNA deterioration (Mounaouer and Abdennaceur, 2015).

In this study, more colonies of *E. coli* were found than OFCs. This is because WWTP is predominantly human wastes, and *E. coli* is a common bacterium found in human faeces (Perkins et al., 2014) and the most representative genus of faecal contamination (Gerardi, 2006). This study found that bacterial populations fluctuated at different stages. For instance, there was a significant decrease in the number of OFCs recovered between the influent and the sediment tank; however, this may also partly reflect the sampling methodology adopted. I.e. it was the liquid portion of the sediment tank that was sampled, and given that bacteria tend to attach to organic matter within the water column (Malham et al., 2014), the results for the sediment tank stage may have been different had all fractions of the wastewater been sampled.

A drawback of the present study design was that triplicate samples for each site collection cultured in non-duplicate plate gave a high standard deviation. This is probably due to various

diversity of bacteria in each container of the total samples. Furthermore, samples were collected over eleven times, but isolates from only seven sampling times (1 to 6 and 11 sampling) were picked randomly for further analyses due to the limited availability of Microbank vials; though in hindsight, glycerol could have been used. In addition, an unequal number of isolates were recovered at different sampling events. Further, the media used, Chromogenic Primary UTI, has been developed to recover bacteria from clinical, rather than environmental, samples; which may have reduced specificity given that the latter samples are likely to have greater microbial diversity. This may reflect the variable proportion of presumptive colonies that were subsequently confirmed as positive by PCR (123/155; 79% and 136/152; 95% for *E. coli* and OFCs, respectively). It may also underestimate bacterial numbers due to cells entering a viable but non-cultureable (VBNC) state.

All faecal coliforms including *E. coli* and OFCs are oxidase negative, therefore this was used as a confirmatory test. In addition, a molecular method was used for strain confirmation by using *uidA* and *lacZ* gene (Bej et al., 1990). In this study, multiplex PCR did not always detect these genes. A recent study in Spain found detection of β-glucuronidase enzyme (*uidA*) in *E. coli* by PCR, and was difficult to identify because this enzymatic activity readily changes traits during the growth on media and temperature of incubation (Molina et al., 2015). Another reason might be the very high concentration of DNA samples (range 400-2500 ng) in our study. With reduced DNA concentration (20-30 ng) and optimised PCR reaction conditions, the expected bands were obtained. Furthermore, five of *E. coli* and two OFCs of those selected randomly were confirmed strains by sequence analysis. Overall, 123 out of 139 presumptive *E. coli* and 136 out of 146 presumptive OFCs random isolates were confirmed by biochemical (oxidase) test and molecular methods.

To detect all types of ESBLs ($bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} and bla_{OXA}), this study used cefpodoxime (CPD) (3rd generation cephalosporin antibiotic) as it is the best indicator for the identification of ESBL-producers (Swarna et al., 2015), though it is less specific than a combination of ceftazidime, cefotaxime. However, cefpodoxime with clavulanate combination discs can be successfully used for all *Enterobacteriaceae* except *Enterobacter* spp. and *Citrobacter freundii* to distinguish ESBL producers from strains that hyperproduce either AmpC or K1 enzymes (Public Health England, 2016). This study did not identify all strains isolated to species level and therefore we cannot be 100% accurate if some of the OFCs organisms were *Enterobacte*r spp. or *Citrobacter freundii*.

However, this study confirmed the identities of *E. coli* and OFCs of ESBLs strains by 16S rRNA gene sequencing. The results showed their identities were confirmed 4 *E. coli* ESBLs and 18 OFCs ESBLs. Further study is required to confirm ESBLs by DNA sequencing as recommended by Monstein et al. (2009), because some *K. oxytoca* isolates hyperproduce K1 enzymes as well as resistant to cefpodoxime, leading to confusion with ESBL (Public Health England, 2016).

WWTPs are regarded as one of the main sources of contamination of environmental compartments with ARB. In this study, overall, the number of phenotypic ESBL in OFCs was higher than *E. coli* (about 18 vs 4). However, the numbers of ESBLs genotypes detected were too low to enable robust statistical analysis, therefore should be interpreted with some caution. For future work, sampling over a longer timeframe may generate more data for statistical analyses. However, the low prevalence could be because Treborth WWTP serves a relatively small population (about 23,000 inhabitants). Alouache et al., (2014) compared ESBL presence in *E. coli* and *Klebsiella pneumoniae* in urban wastewater in Algeria; the outcome showed the numbers of ESBL-producing *Klebsiella pneumoniae* were higher than ESBL *E. coli.* The WWTP that they studied served was much larger than in our study (approximately 75,000 inhabitants), but the volume treated was lower (15,000,000 l day^{-1}), meaning that concentrations (and the likelihood of recovering ESBLs) would be greater. This could explain why they isolated 19 ESBLs out of 24 from influent (79%), and 21 out 24 isolates from effluent (87.5%) for *E. coli* and *Klebsiella pneumonia*.

In the present study, genotypic testing found no ESBL-producing *E.coli* and OFCs were detected at the aeration tank, probably because the number of isolates were small (*n*=32 *E. coli*, and *n*=35 OFCs). However, post aeration, the number of isolates actually increased (Table 3.7). This could be due to the dissemination of antibiotic resistance genes between bacteria by horizontal gene transfer (HGT) that could result in the re-appearance of ESBL isolates at the effluent stage, which would require further study. This study estimated the number of ESBL producers released to the environment to be 13 *E. coli* and 12 OFCs per ml. Given the outflow of 24.5 million litres per day, this equates to approximately 300 billion CFU per day each of both ESBL-producing *E. coli* and OFCs that are released into the Menai Strait. Berglund et al. (2015) and Rizzo et al. (2013) explored that WWTPs do not always efficiently remove ARB and MGEs, and have, therefore, been considered as potential 'hotspots' for dissemination and development of antibiotic resistance. Amine (2013) found that the proportion of ESBL (bla_{TEM}, bla_{SHV} and bla_{CTX-M}) -producing Gram-

negative strains was slightly reduced from 69.8 % in influent to 57.7% in effluent of WWTP. This might be probably due to transferred resistance genes to other bacteria strains by HGT.

Genotypic identification found that *bla*$_{CTX-M}$ group 1 (4/4) and *bla*$_{TEM}$ genes (3/4) were found in *E. coli* at WWTP, while *bla*$_{SHV}$ genes (13/18) were isolated in OFCs. A number of studies have found the most frequently ESBL-producing *E. coli* released via WWTP (treated water) to the environment was *bla*$_{CTX-M}$ group 1 (*bla*$_{CTX-M-15}$). Blaak and co-workers (2014) monitored the dissemination of ESBL producing *E.coli* at four WWTPs and found *bla*$_{CTX-M}$ to be the most prevalent gene at effluent points. In the UK, Livermore and Hawkey (2005) pointed out that the most common ESBL producer associated with *E. coli* in clinical settings was *bla*$_{CTX-M-15}$ (*bla*$_{CTX-M}$ group 1) and with *Klebsiella pneumoniae* was *bla*$_{TEM}$ and *bla*$_{SHV}$.

The ESBL-producing *E. coli* or OFCs detected in this study could be carrying a single resistance gene or more than one gene (2-3) with a smaller number of isolates. These results suggest that the prevalence of ESBL-producing bacteria in this study is similar to the few other reports (Amine, 2013). Everage et al. (2014) confirmed the presence of antibiotic resistance in all stages of treatment process, from raw to treated wastewater, and also could probably transfer resistance genes between bacteria by conjugation or transduction mechanisms. In addition, another study has proved that bacterial resistance to antibiotics was detected in all stages of wastewater treatment process, and even after UV disinfection (Yuan et al., 2014).

3.6 Conclusions

This study demonstrates that ESBL-producing faecal coliforms (*E. coli* and OFCs) are present in Bangor's WWTP, and a considerable number (approximately 300 billion each) are subsequently released per day into the Menai Strait. Tertiary treatment processes that contain UV disinfection do not therefore eliminate all ARB. Further work is therefore necessary to improve the effectiveness of wastewater treatment to reduce the level of AMR input to the environment.

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Chapter 4: Experiment 2

The impact of societal demographic changes on the diversity of ESBL-producing *Enterobacteriaceae* in a wastewater treatment plant

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4.1 Abstract

Bacterial resistance to antibiotics has become a threat to public health. During the last decade, the *Enterobacteriaceae* family that produce Extended Spectrum Beta Lactamase (ESBLs) enzymes such as *bla*$_{CTX-M}$ groups (1, 2, 8/25, 9), *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{OXA}$ have rapidly increased in many countries. Wastewater treatment plants (WWTPs) represent a potential point for the spread of ESBL-producing *Enterobacteriaceae* in the environment. Large changes to human populations due to different social and climatic events could exacerbate this issue. This work compared the presence of ESBL-producing faecal coliform bacteria in Bangor's WWTP, before and after the students' arrival during the university's "welcome week". Over a five-week period (two weeks before students' arrival for starting academic year and three weeks thereafter), water samples were collected twice a week, from the influent, primary sedimentation tank, aeration tank and treated wastewater throughout the WWTP. Counts of *Escherichia coli* (*E. coli*) and other faecal coliforms (OFCs) were performed on selective (Chromogenic Primary UTI) agar. Bacterial isolates were identified by biochemical and PCR tests. ESBL producers were identified by combination disc method. *bla* genes were identified by PCR and sequenced to identify type. Results showed the mean counts of *E. coli* and OFCs before welcome week were higher than after. Out of the total found before welcome week, a number of *E. coli* were ESBL-producers (7/208), and a similar number were found after students had arrived (6/238). However, in OFCs, the number of ESBLs increased after the arrival of students (18/155 before, and 26/220 thereafter). In addition, the diversity of *bla* resistance genes was increased (in both *E.coli*; 4 vs 6 and OFCs; 8 vs 10 genes) after the arrival of students. The new *bla* genes were detected in WWTP after the arrival of students; as *bla*$_{CTX-M-14,}$ (*bla*$_{CTX-M-15}$ + *bla*$_{OXA-1}$) and (*bla*$_{CTX-M-27}$ + *bla*$_{OXA-1}$) found in *E. coli* and as (*bla*$_{TEM-1}$ + *bla*$_{SHV-2}$), (*bla*$_{TEM-19}$ + *bla*$_{SHV-12}$ + *bla*$_{OXA-1}$), (*bla*$_{TEM-120}$ + *bla*$_{SHV-12}$ + *bla*$_{OXA-1}$) and *bla*$_{SHV-12}$ found in OFCs. Overall, this study found that while the large population changes did raise the frequently in OFCs at WWTP, but the diversity and new of *bla* resistance genes was increased genes (in both *E.coli* and OFCs) after the arrival of students. Further work is needed to determine how large demographic changes can affect the cycling and dissemination of antibiotic resistance to and from wastewater treatment systems.

4.2 Introduction

Globally, infections caused by antibiotic resistant bacteria (ARB) have been shown to be an increasing cause of mortality and morbidity to society (Ventola, 2015; WHO, 2015). This issue needs immediate attention as recent estimates predict that globally, it could lead to around 10 million deaths every year by 2050 and cost up to $100 trillion for treatment (Neill, 2014).

The most significant class of therapeutic choice for the treatment of infections in both human and veterinary medicine is in the Beta lactam (β-lactam) group of antibiotics (Madigan et al., 2012); counting for approximately 60% of the total antibiotics used (Pitout and Laupland, 2008a). As a result of their misuse, bacteria have created a variety of mechanisms to survive exposure to some β-lactam antibiotics (Galán et al., 2013) , including 1) change of antibiotic target site of penicillin binding proteins (PBPs), 2) moving the drug out of the cell by efflux pumps, or 3) production of enzymes that cause degradation of antibiotic activity (Galán et al., 2013).

Production of enzymes is the most common mechanism in *Enterobacteriaceae* to disable β-lactams. Encoded beta-lactamase enzymes can confer high levels of resistance by opening the amide bond in the β-lactam ring, enabling the bacteria to break down multi-groups of β-lactam (Nüesch-Inderbinen et al., 2013). One of the most important enzymes of production is Extended spectrum β-lactamase enzymes (ESBLs), which have the capability to hydrolyse and cause high resistance to penicillins, cephalosporins (e.g. cefotaxime, ceftriaxone, ceftazidime), monobactams (e.g. aztreonam), and carbapenems (e.g. imipenem, meropenem, and ertapenem) but not the cephamycins (e.g. cefoxitin and cefotetan) (Pitout and Laupland, 2008a). *bla*$_{\text{CTX-M}}$ groups (1, 2, 8/25, 9), *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$ and *bla*$_{\text{OXA}}$ enzymes have rapidly become the most important ESBLs, in many countries, during the last decade (Nüesch-Inderbinen et al., 2013). Although the *bla*$_{\text{CTX-M}}$ groups, *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$, are related to Amber class A (ESBL) or Bush group 2be, the *bla*$_{\text{OXA}}$ gene is related to class D or group 2d (Kar et al., 2015), as described in Chapter 2 in Section 2.3.1, but also confer resistance to cephalosporins as *bla*$_{\text{OXA-1}}$ (Sugumar et al., 2014). The *bla*$_{\text{CTX-M}}$ genes share 40% amino acid identity with the *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$ genes (Monstein et al., 2007). Those genes are considered the most common among antibiotic-resistant *Enterobacteriaceae* (Mnif et al., 2013).

ESBL-producing *Enterobacteriaceae* are most commonly identified in community settings, often in urinary tract infections (UTIs) (Östholm-Balkhed et al., 2013). *Enterobacteriaceae* can transfer enzyme genes from pathogenic bacterium to other non-pathogens via horizontal gene transfer

(HGT), with several genotypes of *bla*$_{CTX-M}$ groups and other *bla* genes as *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{OXA}$, which have been evolved by mutations in plasmid-mediated enzymes (Nüesch-Inderbinen et al., 2013).

ESBL-producing *Enterobacteriaceae* have appeared rapidly in different geographic regions (Weisenberg et al., 2012). Global travellers such as medical tourists, migrants, employees and overseas students could play a risk factor to dissemination of ESBLs between countries (Barlam and Gupta, 2015). A study demonstrated that from 300 million visitors who visit contaminated areas around the world, about 20% of them are then colonised by resistant faecal bacteria (Kantele et al., 2015). Potential reasons for colonisation with ESBL-producing *Enterobacteriaceae* during travel include consumption of contaminated foods (von Wintersdorff et al., 2014), poor hygiene, direct contact with people (Kantele et al., 2015), and a contaminated environment (Barlam and Gupta, 2015). Therefore, it is not surprising that traveller's faeces is considered as a potential reservoir of ARB, with both receiving and transferring their genes (Rolain, 2013).

It is well established that wastewater treatment plants (WWTPs) are a fundamental sources of ARB and play an important role in promoting their growth and dissemination in the wider environment (Wellington et al., 2013; Gao, Munir and Xagoraraki, 2012; Novo et al., 2013; Rizzo et al., 2013). However, it is unknown whether large demographic changes might increase the presence of ESBL producing *Enterobacteriaceae* in WWTP and their transfer to the environment. The aim of this work was to compare the prevalence and diversity of ESBL-producing *Enterobacteriaceae* in Bangor's WWTP before and after the arrival of students to the city.

4.3 Material and Methods

4.3.1 Study setting

Bangor, north Wales, UK is a small city with a population of approximately 16,500 people (Cyngor Bangor Council, 2015). The student population is approximately 11,000, and of these, there were approximately 1800 international students in the University in 2015-16 (Bangor University, personal communication), originating from 102 across the world; e.g., 1210 students from Asia, 252 students from Africa, 247 students from America, 22 students from the EU and 6 students from Australia (see Table III in the Appendix). Most of these students arrive in Bangor over a space of a few days or weeks, before the new academic year. As a result, the local WWTP is likely to be subject to sudden and considerable changes in microbial inputs. The WWTP serves Bangor,

Menai Bridge, Bethel and Felinheli areas (Welsh Water, personal communication), which have approximately 23,000 inhabitants (Rana, 2015) and processes > 24.5 million litres of raw wastewater per day (approximately 300 l s^{-1}).

4.3.2 Sample collection

Sampling took place in September and October 2015, over a period of five weeks, two weeks before the arrival of students ("welcome week"), and three weeks after. Triplicate samples were collected biweekly from four sampling sites: influent wastewater, primary sedimentation tank, aeration tank and effluent wastewater (post-UV disinfection). Each sample was poured into 50 ml sterile containers, with each sampling occurring between 9.30 am and 12.00 pm. The schematic diagram of the WWTP and sampling sites are illustrated in Figure 4.1 and Figure I in the Appendix. Furthermore, weather conditions (sunny or rainfall) and levels of the storm tank (either empty or contained water reflecting rainfall status) were recorded during sample collection. Samples were transferred to the laboratory within 1-2 hour (h) of collection, and microbiological analysis was performed immediately thereafter.

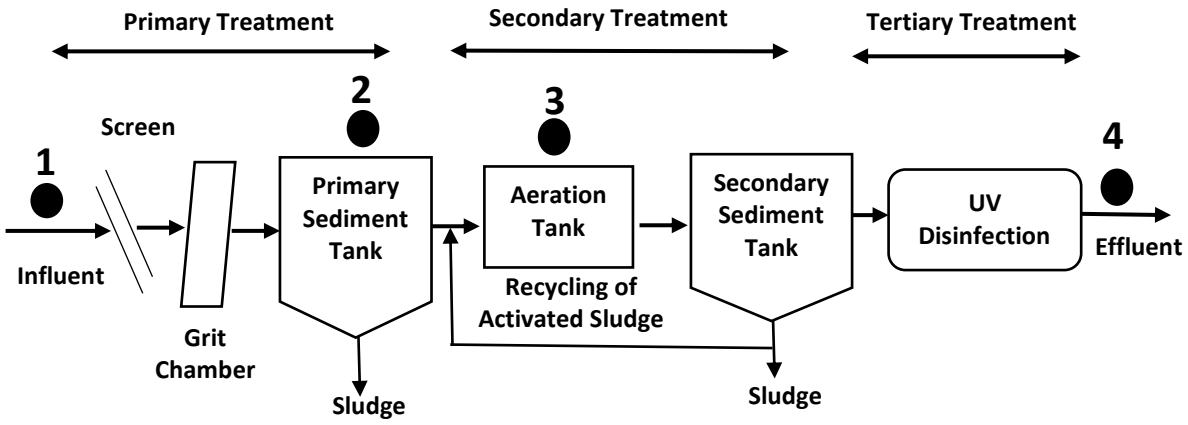

Figure 4.1 Samples were collected from points 1, 2, 3, and 4 along the WWTP (Naidoo and Olaniran, 2013)

4.3.3 Counting and isolation of presumptive *E. coli* and OFCs

The bacterial count was prepared as previously described in Chapter 3 in Section 3.3.3.

For isolation of presumptive *E. coli* and OFCs, two weeks before and two weeks after the arrival of students, colonies of *E. coli* or OFCs were picked randomly and streaked using sterile 1 µl loop onto Chromogenic Primary UTI agar to obtain pure culture, and incubated at 37°C for 24 h. Each pure single colony was cultured onto nutrient agar plate (Oxoid, CM0003) and stored in Microbank™ vials (Pro-lab diagnostics).

4.3.4 Identification of *E. coli* and OFCs

4.3.4.1 Oxidase test

As previously described in Chapter 3 in Section 3.3.4.1.

4.3.4.2 PCR confirmation

As previously described in Chapter 3 in Section 3.3.4.2.

4.3.4.2.1 Multiplex PCR

As previously described in Chapter 3 in Section 3.3.4.2.1.

4.3.4.2.2 Non-specific bands optimisation by single PCR

As previously described in Chapter 3 in Section 3.3.4.2.2.

4.3.4.3 Selecting DNA for sequence analysis

4.3.4.3.1 Sequencing *uidA* and *lacZ* genes

For the DNA sequences, four *E. coli* strains (*n* = 1, multiplex PCR and *n* = 3, single PCR) and two OFCs strains (*n* = 1, multiplex PCR and *n* = 1, single PCR) were selected randomly to confirm those genes.

Protocol *uidA* and *lacZ* genes sequencing was described previously in Chapter 3 in Section 3.3.4.3.1.

4.3.4.3.2 Sequencing 16S rRNA gene

This study used 16S rRNA gene sequencing to identify 15 *E. coli* ESBLs and 42 OFCs ESBLs isolates. The protocol of PCR 16S rRNA gene and sequencing were described previously in Chapter 3 in Section 3.3.4.3.2.

4.3.5 Screening ESBL

As previously described in Chapter 3 in Section 3.3.5.

4.3.6 Detection of the β-lactamase (*bla*) genes through multiplex PCR

Two multiplex PCR assays determined the genotypic characterization of ESBL-producers. ESBLs-positive isolates from the phenotypic screening test were analysed for the presence of genes targeting bla_{CTX-M} groups (bla_{CTX-M} group 1, bla_{CTX-M} group 2, bla_{CTX-M} group 9 and bla_{CTX-M} group 8/25), and bla_{TEM}, bla_{SHV} and bla_{OXA} genes, by multiplex PCR as described previously in Chapter 3 in Section 3.3.6.

4.3.7 Sequence analysis of *bla* genes

The family of bla_{CTX-M} (bla_{CTX-M} group 1, bla_{CTX-M} group 2 and bla_{CTX-M} group 9) and others *bla* genes (bla_{TEM}, bla_{SHV} and bla_{OXA} genes) were characterised by direct sequencing of PCR products to identify the nearest neighbour specific *bla* gene.

All bla_{CTX-M} genes were detected by single PCR except the bla_{CTX-M} group 9 among OFCs were detected by multiplex PCR. PCR products were purified by PCR purification kit (QIAGEN, Germany). Sequencing bla_{CTX-M} genes was performed by Tube Seq service (Eurofins Genomics) (https://www.eurofinsgenomics.eu/), while others *bla* genes (bla_{TEM}, bla_{SHV} and bla_{OXA} genes) were purified and sequenced by Macrogen; (https://dna.macrogen.com/eng/).

Each nucleotide sequence (partial DNA) sample was compared with the known gene sequences databases (non-redundant protein sequences (nr)) and genetic code (Bacteria) using the translated nucleotide to protein (blastx) by BLAST (NCBI) program.

4.3.8 Phylogenetic tree of *bla* genes

To increase confidence in the *bla* gene sequence samples (DNA) and GenBank genes, a phylogenetic tree was constructed. Multiple sequence alignment (Clustal W - Codons) and reference sequences from GenBank were classified in different types on the phylogenetic tree by using MEGA 7 (MEGA - Molecular Evolutionary Genetics Analysis) software program using the Neighbour-Joining method, Poisson correction model, bootstrap method, and the rates among used uniform via the program.

4.3.9 Statistical analyses

Data analysis was performed using IBM SPSS Statistics 22 and Excel 2013. The total *E. coli* and OFCs were expressed as mean ± SE (CFU ml^{-1}) at each sampling point of the WWTP. The results at the various sampling points from two different periods (before and after students' arrival) were evaluated by non-parametric tests (Kruskal-Wallis one-way ANOVA) because of non-normal

distribution. *P*-values <0.05 were considered statistically significant. Mann-Whitney *U* test was used to compare mean concentrations before (influent) and after (effluent) treatment over the two study periods (before / after student arrival) and a significance level of *P*<0.05. The number of ESBL-producing *E. coli* and OFCs were also compared across the different sites (influent, primary sediment tank, aeration tank and effluent).

4.4 Results

Over the study period, 40 wastewater samples were collected in triplicates from the four sites of the treatment plant: two weeks before the arrival of students (influent *n* = 4, primary sediment tank *n* = 4, aeration tank *n* = 4, effluent *n* = 4), and three weeks thereafter (influent *n* = 6, primary sediment tank *n* = 6, aeration tank *n* = 6, effluent *n* = 6). After the arrival of students, high rainfall events corresponded with two sampling times (5th and 9th) and the storm tank was full on the 10th sample collection (Figure 4.2).

Figure 4.2 Storm tank before (left) and after (right) heavy rainfall

4.4.1 Isolation and confirmation of *E. coli* and OFCs

Over the four-week period, a representative sample of 700 presumptive *E. coli* (before the arrival of students *n* = 293 and after *n* = 407) and 437 presumptive OFCs (*n* = 195 before students' arrival, and *n* = 242 afterwards) isolates were randomly picked for confirmatory biochemical test and PCR, as summarised in Table 4.1 (see Table IV in the Appendix). Multiplex PCR was used on oxidase-negative bacteria for confirmation of *E. coli* (*uidA* and *lacZ* genes), as shown in Figure 4.3 and OFCs (*lacZ* gene) in Figure 4.4.

Table 4.1 Numbers of bacteria isolated at WWTP sites and confirmed by oxidase and PCR (*uidA* & *lacZ* genes) over two weeks before and two weeks after arrival of students

Sampling site	Arrival of students	***E. coli***		**OFCs**	
		Isolates	Confirmed by Oxidase test and PCR	Isolates	Confirmed by Oxidase test and PCR
Influent	Before	77	62	53	37
	After	90	67	69	67
Primary Sediment Tank	Before	71	62	49	42
	After	99	63	53	43
Aeration Tank	Before	68	31	57	46
	After	106	33	59	54
Effluent	Before	77	53	36	30
	After	112	75	61	56
Total	Before	293	208	195	155
	After	407	238	242	220
Total		700	**446 (63.7%)**	437	**375 (85.8%)**

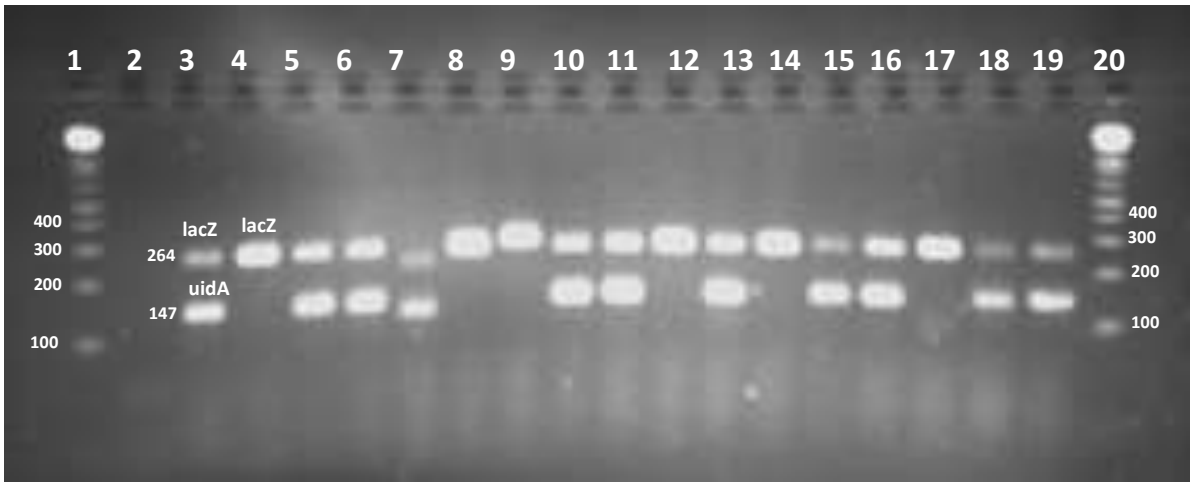

Figure 4.3 *E. coli* multiplex PCR assay for *uidA* and *lacZ* genes. Lanes: **1** and **20**, Marker 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific); **2**, control negative (distilled water): **3**, *E. coli* NCTC 13353 as control positive for *uidA* and *lacZ* genes; **4**, OFCs as *Klebsiella pneumoniae* NCTC 13368 as control positive for *lacZ* gene; **5** to **7**, **10** to **11**, **13**, **15** to **16**, and **18** to **19**, *E. coli* strains; **8**, **9**, **12**, **14** and **17**, *E. coli* strain negatives, with the PCR condition initial denaturation at 95°C for 3 min, preceding a loop of 30 cycles of denaturation at 94°C for 1 min, followed by annealing at 50°C for 1 min, and extension at 50°C for 1 min. Final elongation occurred at 72°C for 7 min

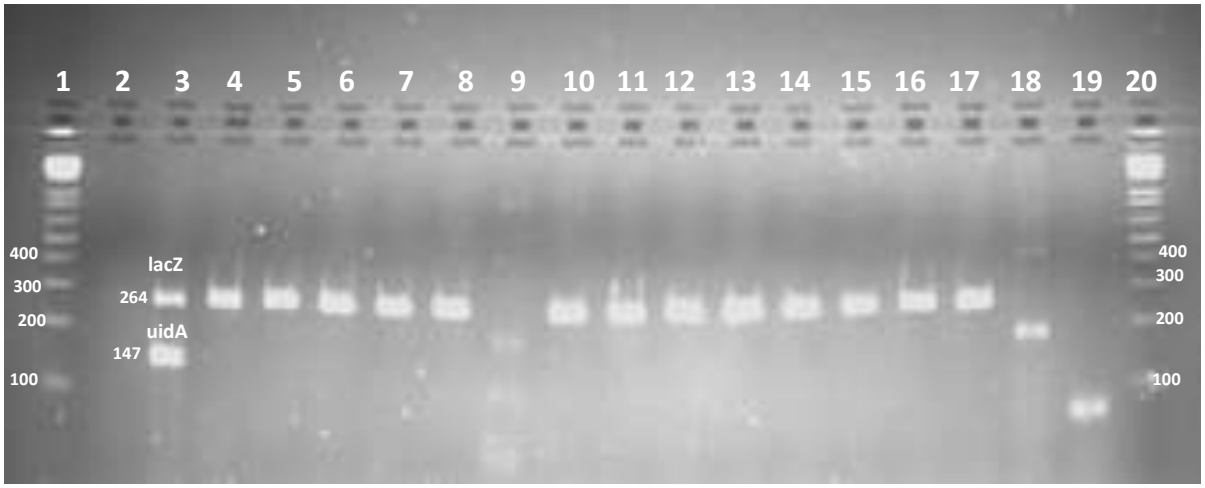

Figure 4.4 OFCs multiplex PCR assay for *uidA* and lacZ genes. Lanes: **1** and **20**, Marker 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific); **2**, control negative (distilled water): **3**, *E. coli* NCTC 13353 as control positive for *uidA* and *lacZ* genes; **4**, OFCs as *Klebsiella pneumoniae* NCTC 13368 as control positive for *lacZ* gene; **5** to **8**, **10** to **17**, OFCs; **9**, **18**, and **19** strain negatives, with the PCR condition initial denaturation at 95°C for 3 min, preceding a loop of 30 cycles of denaturation at 94°C for 1 min, followed by annealing at 50°C for 1 min, and extension at 50°C for 1 min. Final elongation occurred at 72°C for 7 min

Non-specific bands (negative results from multiplex PCR) found from the DNA of *E. coli* and OFCs were re-run as a single PCR. After reducing the concentration of DNA template strain and optimising for single PCR under standard PCR conditions, the *uidA* gene for *E. coli* (Figure 4.5) and *lacZ* gene for OFCs (Figure 4.6) were detected.

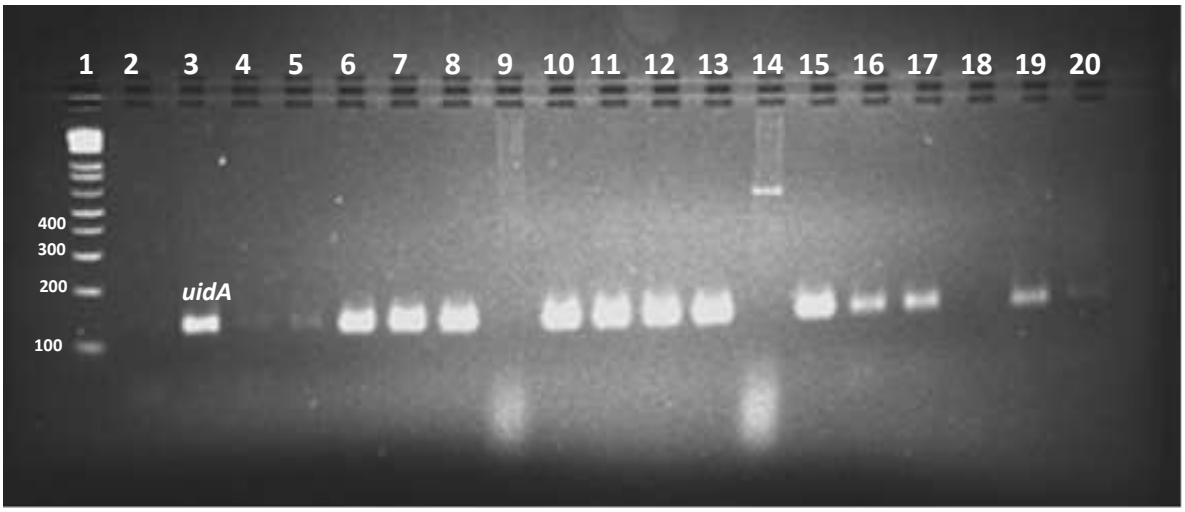

Figure 4.5 *E. coli* single PCR assay for *uidA* gene. Lanes: **1**, Marker 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific); **2**, control negative (distilled water): **3**, *E. coli* NCTC 13353 as control positive for *uidA* gene; **4** to **8, 10** to **13, 15** to **17** and **19** to **20** *E. coli* strains; **9**, **14**, and **18** strain negatives, with the PCR condition initial denaturation at 95°C for 3 min, preceding a loop of 40 cycles of denaturation at 94°C for 1 min, followed by annealing at 65°C for 1 min, and extension at 70°C for 1 min. Final elongation occurred at 72°C for 7 min. Using a 50 µl reaction mixture consisting of 15 µl (0.6x) BioMix Red, 1 µl uidA-F, 1 µl uidA-R, 31 µl Water Molecular Biology Reagent (W4502, Sigma-Aldrich) and 2 µl DAN template

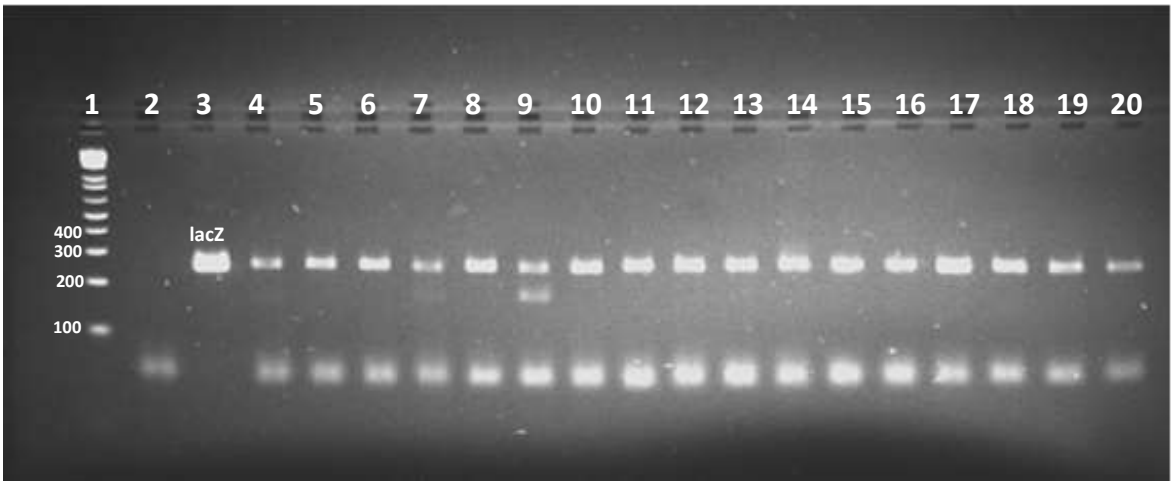

Figure 4.6 OFCs single PCR assay for *lacZ* gene. Lanes: **1**, Marker 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific); **2**, control negative (distilled water): **3**, OFCs as *Klebsiella pneumoniae* NCTC 13368 as control positive for *lacZ* gene; **4** to **6**, **8**, **10** to **20** OFCs strains; **7** and **9** OFCs strains because of the same position, with the PCR condition initial denaturation at 95 °C for 3 min; 40 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min and extension at 70 °C for 1 min. Final elongation occurred at 72 °C for 7 min

Some *E. coli* strains (*uidA* gene) were found to be difficult to detect by single PCR. After optimising the reaction condition such as by increasing the 2 x BioMix Red volume to 25 µl in total a 50 µl mixture, the single PCR detected the expected band for the *uidA* gene (Figure 4.7).

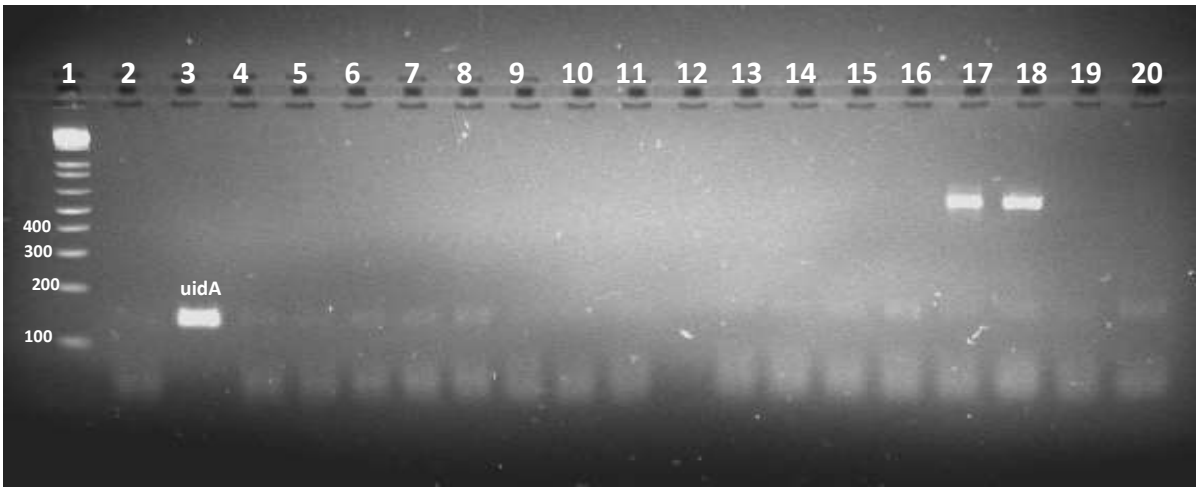

Figure 4.7 *E. coli* single PCR assay for *uidA* gene. Lanes: **1**, Marker 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific); **2**, control negative (distilled water): **3**, *E. coli* NCTC 13353 as control positive for *uidA* gene; **4** to **8**, **13** to **20** *E. coli* strains; **9** to **12**, negative strains, with the PCR condition initial denaturation at 95°C for 3 min, preceding a loop of 40 cycles of denaturation at 94°C for 1 min, followed by annealing at 65°C for 1 min, and extension at 70°C for 1 min. Final elongation occurred at 72°C for 7 min. Using a 50 µl reaction mixture consisting of 25 µl (1x) BioMix Red, 1 µl uidA-F, 1 µl uidA-R, 21 µl Water Molecular Biology Reagent (W4502, Sigma-Aldrich) and 2 µl DAN template

In this study, multiplex PCR did not always detect *uidA* and *lacZ* genes. This is consistent with study Molina et al., (2015), which have suggested that *uidA* gene is has quite difficult to detect by PCR, this is because enzymatic activity readily changes traits during the growth on media and temperature of incubation. Additional confirmation was used by sequencing some DNA samples selected randomly including four *uidA* (*E. coli*) genes (*n* = 1; multiplex and *n* = 3; single PCR) and two *lacZ* (OFCs) genes (*n* = 1; multiplex and *n* = 1; single PCR). Nucleotide sequences were identified by BLAST (using blastx) *uidA* and *lacZ* genes with the following accession number: CCM12452 for *E. coli*, PLM87621and PCD70119 for OFCs as illustrated in Table 4.2.

Table 4.2 Matched DNA sequences (*uidA* and *lacZ* genes) with GenBank database NCBI using blastx to identify strain at WWTP sites selected randomly from multiplex and single PCR

PCR	Time collecting – site (gene – strain)	Organism identified based via blastx	Accession	Identity (%)
Multiplex	4th – Primary sediment tank (*uidA* – *E. coli*)	*E. coli* (*uidA*)	CCM12452	100
	9th – Aeration tank (*lacZ* – OFCs)	*Klebsiella pneumoniae* (*lacZ*)	PLM87621	100
Single	1st – Influent (*uidA* – *E. coli*)	*E. coli* (*uidA*)	CCM12452	100
	6th – Primary sediment tank (*uidA* – *E. coli*)	*E. coli* (*uidA*)	CCM12452	100
	6th – Effluent (*uidA* – *E. coli*)	*E. coli* (*uidA*)	CCM12452	100
	3rd – Aeration tank (*lacZ* – OFCs)	*Klebsiella pneumoniae* (*lacZ*)	PCD70119	100

Following PCR, nucleotide sequence analysis of 16S rRNA gene (based on *uidA* PCR) identified thirteen *E. coli* strains (accession number NR_114042) and two *Citrobacter freundii* (accession number NR_028894; Table 4.3). For the 42 ESBL OFC (based on *lacZ* PCR) isolates, all were confirmed as *Raoultella* spp., *Klebsiella* spp., *Kluyvera* spp. and *Enterobacter* spp. (Table 4.4).

Table 4. 3 Nucleotide sequences of 16S rRNA gene in *E. coli* ESBLs isolated at different times and sites

No.	Time collecting – site (gene – strain)	NCBI (16S rRNA)		
		Organism	Accession	Identity (%)
1	1st - Primary Sediment Tank (*uidA* – *E. coli*)	*E. coli*	NR_114042	97.91
2	2nd - Influent (*uidA* – *E. coli*)	*E. coli*	NR_114042	97.64
3	2nd - Influent (*uidA* – *E. coli*)	*E. coli*	NR_114042	97.64
4	2nd – Primary Sediment Tank (*uidA* – *E. coli*)	*E. coli*	NR_114042	97.94
5	2nd – Primary Sediment Tank (*uidA* – *E. coli*)	*E. coli*	NR_114042	97.94
6	2nd – Primary Sediment Tank (*uidA* – *E. coli*)	*E. coli*	NR_114042	99.79
7	3rd - Influent (*uidA* – *E. coli*)	*E. coli*	NR_114042	98.94
8	3rd - Influent (*uidA* – *E. coli*)	*Citrobacter freundii*	NR_028894	99.90
9	3rd – Effluent (*uidA* – *E. coli*)	*Citrobacter freundii*	NR_028894	99.90
10	5th – Effluent (*uidA* – *E. coli*)	*E. coli*	NR_114042	98.68
11	5th – Effluent (*uidA* – *E. coli*)	*E. coli*	NR_114042	99.58
12	6th – Effluent (*uidA* – *E. coli*)	*E. coli*	NR_114042	98.46
13	7th – Effluent (*uidA* – *E. coli*)	*E. coli*	NR_114042	99.79
14	8th – Aeration Tank (*uidA* – *E. coli*)	*E. coli*	NR_114042	99.69
15	8th – Effluent (*uidA* – *E. coli*)	*E. coli*	NR_114042	99.79

Table 4. 4 Nucleotide sequences of 16S rRNA gene in the 42 OFCs ESBLs isolated at different times and sites

No.	Time collecting – site (*lacZ* – OFCs)	NCBI (16S rRNA)		
		Organism	Accession	Identity (%)
1	1st - Influent	*Raoultella ornithinolytica*	NR_044799	99.79
2	1st - Influent	*Raoultella ornithinolytica*	NR_114502	99.90
3	1st - Primary Sediment Tank	*Raoultella terrigena*	NR_114503	98.86
4	1st - Primary Sediment Tank	Klebsiella variicola	NR_025635	99.90
5	1st - Primary Sediment Tank	*Raoultella terrigena*	NR_114503	100
6	1st - Primary Sediment Tank	*Raoultella ornithinolytica*	NR_114502	99.79
7	1st - Primary Sediment Tank	*Raoultella ornithinolytica*	NR_114502	100
8	1st - Aeration Tank	*Raoultella ornithinolytica*	NR_114502	100
9	2nd - Aeration Tank	*Raoultella ornithinolytica*	NR_044799	99.90
10	3rd - Effluent	*Kluyvera cryocrescens*	NR_114108	99.90
11	4th - Influent	*Raoultella ornithinolytica*	NR_044799	99.90
12	4th - Influent	*Klebsiella grimontii* strain	NR_159317	99.79
13	4th - Influent	*Raoultella ornithinolytica*	NR_114502	100
14	4th - Influent	*Raoultella ornithinolytica*	NR_114502	90.90
15	4th - Effluent	*Raoultella ornithinolytica*	NR_044799	99.90
16	4th - Effluent	*Raoultella ornithinolytica*	NR_114502	90.90
17	5th - Influent	*Raoultella ornithinolytica*	NR_114502	90.79
18	5th - Aeration Tank	*Klebsiella grimontii* strain	NR_159317	99.80
19	5th - Effluent	*Klebsiella grimontii* strain	NR_159317	99.79
20	6th - Influent	*Klebsiella grimontii* strain	NR_159317	99.79
21	6th - Influent	*Klebsiella grimontii* strain	NR_159317	99.79
22	6th - Influent	*Klebsiella grimontii* strain	NR_159317	99.79
23	6th - Influent	*Raoultella ornithinolytica*	NR_114502	99.59
24	6th - Influent	*Raoultella ornithinolytica*	NR_114502	99.90
25	6th - Influent	*Enterobacter ludwigii*	NR_042349	99.90
26	6th - Influent	*Klebsiella grimontii* strain	NR_159317	99.80
27	6th - Influent	*Raoultella ornithinolytica*	NR_044799	99.50
28	6th - Influent	*Klebsiella grimontii* strain	NR_159317	99.29
29	6th - Primary Sediment Tank	*Raoultella ornithinolytica*	NR_114502	99.90
30	6th - Primary Sediment	*Raoultella ornithinolytica*	NR_044799	99.36
31	6th - Primary Sediment Tank	*Kluyvera cryocrescens*	NR_114108	99.69
32	6th - Effluent	*Raoultella ornithinolytica*	NR_044799	99.61
33	6th - Effluent	*Raoultella ornithinolytica*	NR_044799	99.90
34	7th - Influent	*Kluyvera cryocrescens*	NR_114108	99.90
35	7th - Aeration Tank	*Klebsiella grimontii*	NR_159317	100
36	7th - Aeration Tank	*Raoultella terrigena*	NR_114503	99.48
37	7th - Effluent	*Raoultella ornithinolytica*	NR_044799	99.80
38	7th - Effluent	*Klebsiella grimontii* strain	NR_159317	99.80
39	8th - Aeration Tank	*Raoultella terrigena*	NR_114503	98.55
40	8th - Aeration Tank	*Raoultella terrigena*	NR_114503	98.46
41	8th - Aeration Tank	*Raoultella ornithinolytica*	NR_044799	100
42	8th - Aeration Tank	*Raoultella ornithinolytica*	NR_114502	99.90

4.4.2 *E. coli* and OFCs counts

Overall, 63.7% (446/700) of the presumptive isolates were confirmed as *E. coli.* For OFCs, 85.8% (375/437) of the presumptive isolates were confirmed (Table 4.1). Based on confirmed bacteria, this study accepted 63.7% of *E. coli* and 85.8% of total OFCs over total count as seen in Table V in the Appendix.

Over the course of the study, mean counts of presumptive *E. coli* and OFCs were significantly higher before the arrival of students (Kruskal-Wallis one-way ANOVA, $P<0.05$) (Figure 4.8). At each sampling point, significantly greater *E. coli* and OFCs were recovered before student arrival (Mann-Whitney test, $P<0.05$), except for OFC at the effluent stage, where the difference was not statistically significant.

By the tertiary treatment stage (UV-disinfection) of the plant, the number of *E. coli* and OFCs had reduced (Mann-Whitney test, $P<0.05$) by approximately 98.9% for *E. coli* and 99.2% for OFCs, relative to numbers at the initial stage (Figure 4.8). At the effluent site, the mean of *E. coli* increased significantly after students arrived (Mann-Whitney *U* test, $P<0.05$) from 1.12×10^3 to 1.92×10^3 CFU ml^{-1}, respectively. However, the mean of OFCs were not significantly different in both periods (Mann-Whitney *U* test, $P>0.05$) (before 8.04×10^2, after 1.08×10^3 CFU ml^{-1}).

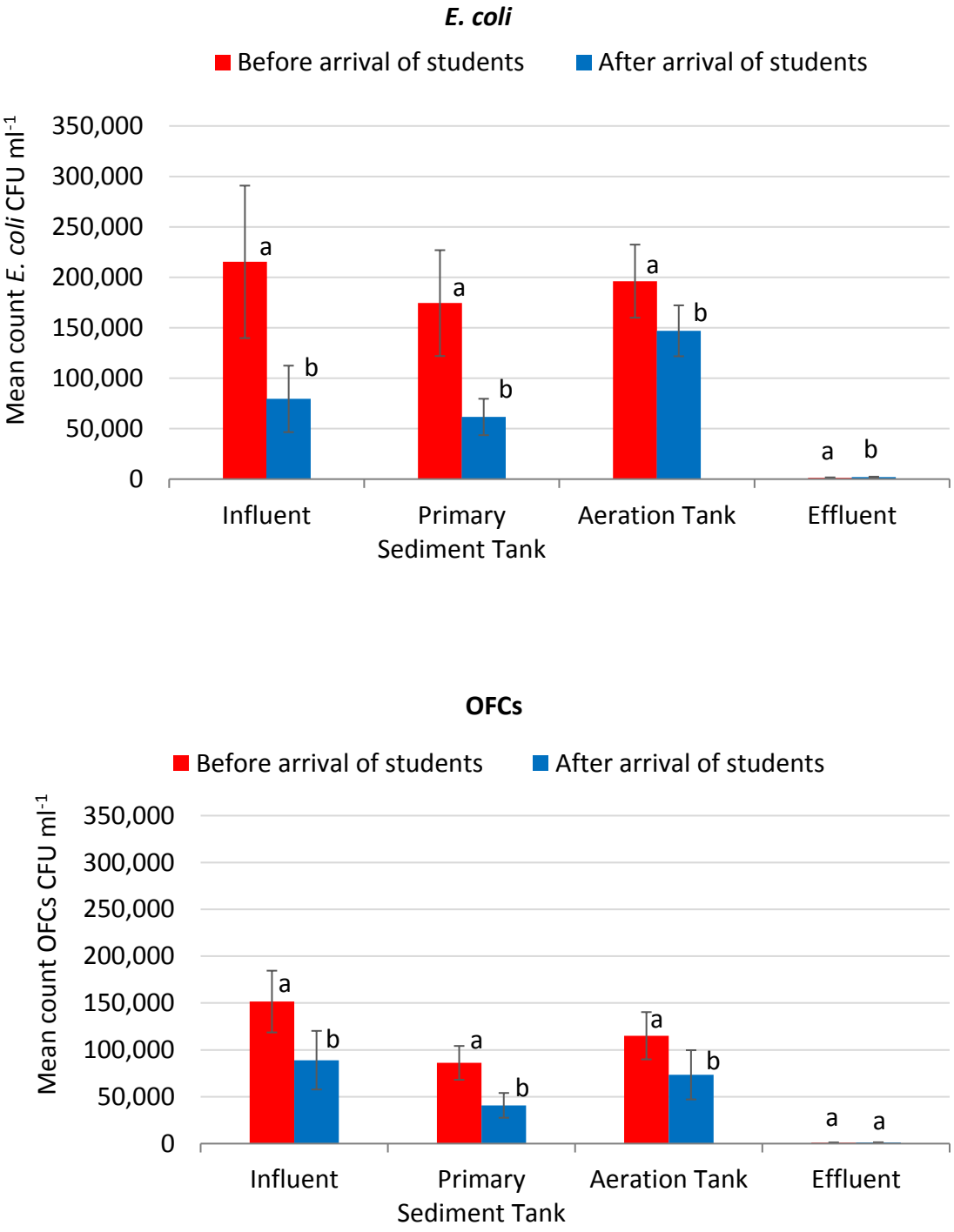

Figure 4.8 Mean counts (±SEM) of presumptive *E. coli* and OFCs before and after the arrival of students at several sites of WWTP. Letters denote the significant differences in bacterial numbers before / after student arrival for that sampling point (Mann-Whitney *U* test, $P<0.05$). For *E. coli*, numbers were significantly greater before student arrival than after arrival, except for the effluent site, where the opposite effect was seen

4.4.3 Detection of ESBL-producers

The results of phenotypic and genotypic analysis to determine the numbers of recovered bacteria that were ESBL-producers is summarised in Table 4.5.

Table 4.5 Number of ESBL-producing bacterial isolates and diversity of the bla gene obtained at WWTP sites before and after student arrival

	Influent		Primary Sediment Tank		Aeration Tank		Effluent		Total		Total number of isolates
	Before	After	Before	After	Before	After	Before	After	Before	After	
E. coli											
Single or multi-resistant gene type / No. ESBL out of total isolated	3/62	0/67	4/62	0/63	0/31	1/33	0/53 (0)	5/75	7/208	6/238	13/446
bla$_{CTX-M-15}$	-	-	3	-	-	-	-	1	3	1	4
bla$_{CTX-M-27}$	1	-	-	-	-	-	-	1	1	1	2
bla$_{CTX-M-1}$	2	-	-	-	-	-	-	-	2	-	2
bla$_{CTX-M-14}$	-	-	-	-	-	1	-	-	-	1	1
bla$_{CTX-M-15}$ + *bla*$_{TEM-1}$	-	-	1	-	-	-	-	1	1	1	2
bla$_{CTX-M-15}$ + *bla*$_{OXA-1}$	-	-	-	-	-	-	-	1	-	1	1
bla$_{CTX-M-27}$ + *bla*$_{OXA-1}$	-	-	-	-	-	-	-	1	-	1	1
Number of *bla* gene detected	2	-	2	-	-	1	-	5	4	6	-
OFCs											
Single or multi-resistant gene type / No. ESBL out of total isolated	7/37	11/67	5/42	3/43	2/46	7/54	4/30	5/56	18/155	26/220	44/375
bla$_{CTX-M-3}$	-	1	-	1	-	-	1	-	1	2	3
bla$_{TEM-1}$	1	-	1	-	-	-	1	-	3	-	3
bla$_{TEM-19}$	-	-	3	-	1	2	-	-	4	2	6
bla$_{TEM-120}$	2	1	-	-	-	1	-	1	2	3	5
bla$_{TEM-1}$ + *bla*$_{SHV-2}$	-	2	-	-	-	-	-	1	-	3	3
bla$_{TEM-1}$ + *bla*$_{SHV-12}$	1	2	1	-	-	1	-	1	2	4	6
bla$_{TEM-19}$ + *bla*$_{SHV-2}$	1	-	-	-	-	1	-	-	1	1	2
bla$_{TEM-19}$ + *bla*$_{SHV-12}$ + *bla*$_{OXA-1}$	-	1	-	-	-	-	-	-	-	1	1
bla$_{TEM-120}$ + *bla*$_{SHV-12}$ + *bla*$_{OXA-1}$	-	-	-	-	-	1	-	-	-	1	1
bla$_{SHV-2}$	1	1	-	1	-	-	2	1	3	3	6
bla$_{SHV-12}$	1	3	-	1	1	1	-	1	2	6	8
Number of *bla* gene detected	6	7	3	3	2	6	3	5	8	10	-

4.4.3.1 Phenotypic detection

Among the phenotypes, this study found that the prevalence of ESBL carrying *E. coli* was lower than ESBL-producing OFCs isolates (Table 4.5). Seven out of 208 isolates of *E. coli* obtained before the arrival of students were found to be ESBL-producers, while six out of 238 isolates were obtained after student arrival. As Table 4.5 shows before the arrival of students, prevalence of ESBL-producing *E. coli* in influent and primary sediment tank were three and four isolates out of 62 in both sites, while after the arrival of students, ESBL genes were not observed in the same sites. Before the arrival of students, no ESBL-producing *E. coli* were detected in the aeration tank and the effluent, whereas thereafter one isolate out of 33 in the aeration tank and five isolates out of 75 in effluent were found to be ESBL-producers.

As Table 4.5 illustrates the prevalence of ESBL-producing OFCs after the arrival of students were increased at the influent (from 7/37 to 11/67), aeration tank (from 2/46 to 7/54) and at the effluent (from 4/30 to 5/56) stages. However, the opposite effect was seen at the primary sediment tank (from 5/42 to 3/43).

4.4.3.2 Genotypic detection

Thirteen out of total *E. coli* and forty-four out of total OFCs as phenotypic-positive ESBL isolates were selected for genotypic analysis, as illustrated in Table 4.5. The results of multiplex PCR showed that ESBL genes were detected as *bla*$_{\text{CTX-M}}$ groups and other *bla* genes.

4.4.3.2.1 *bla*$_{\text{CTX-M}}$ groups

4.4.3.2.1.1 Multiplex PCR *bla*$_{\text{CTX-M}}$ groups

Results of multiplex *bla*$_{\text{CTX-M}}$ groups PCR identified the size of *bla*$_{\text{CTX-M}}$ group 1 and *bla*$_{\text{CTX-M}}$ group 9 to be 688 and 561 bp, respectively. Multiplex PCR showed the presence of various *bla*$_{\text{CTX-M}}$ groups on electrophoresis gel for *E. coli* strains, as illustrated in Figure 4.9. However, *bla*$_{\text{CTX-M}}$ group 8/25 and *bla*$_{\text{CTX-M}}$ group 2 were not detected among *E. coli* and OFCs.

The results found that *bla*$_{\text{CTX-M}}$ groups were present in 13/13 of *E. coli* isolates (n = 9; group 1 and n = 4; group 9), while 10/44 of OFC isolates had *bla*$_{\text{CTX-M}}$ groups (n = 3; group 1 and n = 7; group 9).

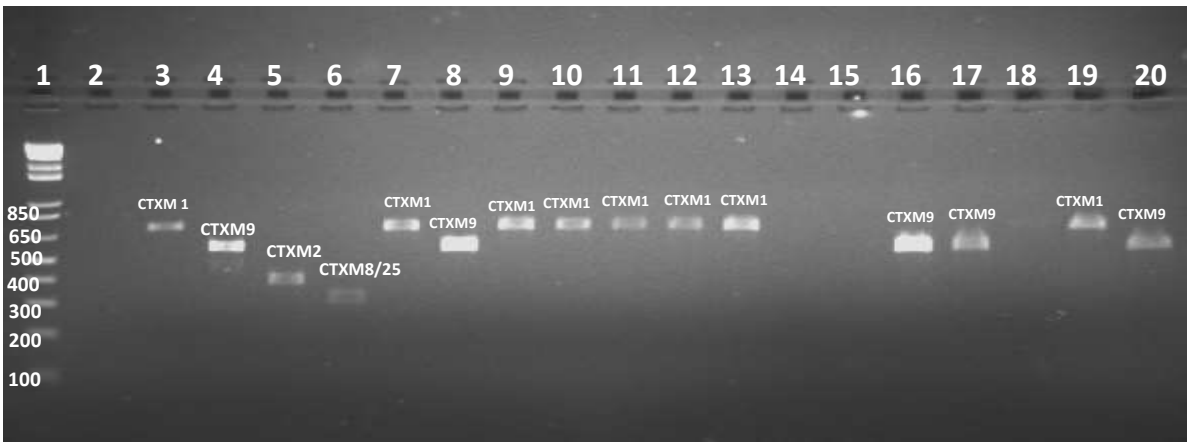

Figure 4.9 Multiplex PCR assay for *bla*$_{CTX-M}$ groups for *E. coli*. Lanes: **1**, Marker 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific); **2**, ESBL-negative control (*E. coli* NCTC 10418): **3**, *E. coli* (NCTC 13353) as *bla*$_{CTX-M-15}$ (group 1); **4**, *Enterobacter cloacae* (NCTC 13463) as *bla*$_{CTX-M-9}$ (group 9); **5**, *E. coli* (NCTC 13462) as *bla*$_{CTX-M-2}$ (group 2); **6**, *E. coli* (NCTC 13463) as *bla*$_{CTX-M-8}$ (group 8/25); **7**, and **9** to **13**, and **19**, *E. coli* carried *bla*$_{CTX-M-15}$ (group 1); **8**, **16**, **17**, and **20**, *E. coli* as *bla*$_{CTX-M-9}$ (group 9); **14**, **15** and**18**, *E. coli* negative *bla*$_{CTX-M}$ group

4.4.3.2.1.2 Partial DNA sequence analysis of *bla*$_{CTX-M}$

Twenty three strains (*n* = 13 *E. coli* and *n* = 10 OFCs) were confirmed as positive by PCR, carrying *bla*$_{CTX-M}$ groups (Table 4.5). The results of nucleotide sequences revealed that of the nine *E. coli* strains positive for the *bla*$_{CTX-M}$ group 1, two isolated from the influent carried *bla*$_{CTX-M-1}$, four from the primary sediment tank carried *bla*$_{CTX-M-15}$ and three from effluent carried *bla*$_{CTX-M-15}$ as seen in Table 4.6. However, three OFCs strains were positive for *bla*$_{CTX-M}$ group 1 carrying *bla*$_{CTX-M-3}$ at the influent, the primary sediment tank and effluent.

Of the four *E. coli* strains positive for the *bla*$_{CTX-M}$ group 9, one isolated from the influent carried *bla*$_{CTX-M-27}$, and one from the aeration tank carried *bla*$_{CTX-M-14}$ and two isolated from the effluent carried *bla*$_{CTX-M-27}$. By PCR technique, we found seven OFCs strains to be positive for *bla*$_{CTX-M}$ group 9, but subsequent sequencing found them to be false positives, with 99% similarity to *bla*$_{OXY-6-2}$ of *Klebsiella oxytoca* (accession number: NG_052696). PCR is one of the most commonly used technique to detect ESBL genes, but it is not always effective, especially for *bla*$_{CTX-M}$ genes. Therefore, we implemented an additional DNA sequencing for further confirmation, as suggested by Monstein et al. (2009).

bla$_{OXY}$ enzymes have been classified into six groups (*bla*$_{OXY-1}$ to *bla*$_{OXY-6}$) based on nucleotide acid sequence (González-López et al., 2009). It is known that *bla*$_{OXY}$ genes are the constitutive expression of a chromosomal class A β-lactamase, previously known as K1 or KOXY but now *bla*$_{OXY}$, due to the hyperproduction of the chromosomal β-lactamase, causing mutations in the promoter region (Fevre et al., 2005). In addition, *K. oxytoca* strains potentially carry *bla*$_{CTX-M}$ genes (Izdebski et al., 2015), but those strains may give a false positive result, because *bla*$_{OXY}$ enzymes in *K. oxytoca* can hydrolyse certain cephalosporins in the same way as ESBL enzymes, but, in fact, this is not due to *bla*$_{CTX-M}$ genes (Schmitt et al., 2007; Nilsson et al., 2007). Therefore, seven OFC isolates from group 9 were excluded from this study.

Table 4.6 Sequence analysis for *bla*$_{CTX-M}$ groups producing *E. coli* and OFCs by NCBI

No.	*bla*$_{CTX-M}$ groups	Time collecting	Sample site	ID-strain	Strain *bla*$_{CTX-M}$ type by NCBI	Accession No.	Identity (%)
1	1	2nd	Influent	2-*E. coli*	*E. coli bla*$_{CTX-M-1}$	NG_048897	100
2		3rd	Influent	6-*E. coli*	*E. coli bla*$_{CTX-M-1}$	NG_048897	99
3		1st	Primary sediment tank	1-*E. coli*	*E. coli bla*$_{CTX-M-15}$	KR186073	100
4		2nd	Primary sediment tank	3-*E. coli*	*E. coli bla*$_{CTX-M-15}$	KY927164	100
5		2nd	Primary sediment tank	4-*E. coli*	*E. coli bla*$_{CTX-M-15}$	KY927164	99
6		2nd	Primary sediment tank	5-*E. coli*	*E. coli bla*$_{CTX-M-15}$	AGO28148	97.98
7		6th	Effluent	7-*E. coli*	*E. coli bla*$_{CTX-M-15}$	KY927164	99
8		7th	Effluent	8-*E. coli*	*E. coli bla*$_{CTX-M-15}$	KY927164	99
9		8th	Effluent	9-*E. coli*	*E. coli bla*$_{CTX-M-15}$	KY927164	100
10	9	2nd	Influent	12-*E. coli*	*E. coli bla*$_{CTX-M-27}$	NG_048976	99
11		8th	Aeration tank	15-*E. coli*	*E. coli bla*$_{CTX-M-14}$	NG_048929	100
12		5th	Effluent	13-*E. coli*	*E. coli bla*$_{CTX-M-27}$	NG_048976	99
13		5th	Effluent	14-*E. coli*	*E. coli bla*$_{CTX-M-27}$	NG_048976	99
14	1	7th	Influent	18-OFCs	*Klebsiella oxytoca bla*$_{CTX-M-3}$	KU200455	99
15		6th	Primary sediment tank	17-OFCs	*Citrobacter koseri bla*$_{CTX-M-3}$	AB059404	99
16		3rd	Effluent	16-OFCs	*Citrobacter koseri bla*$_{CTX-M-3}$	AB059404	99
17	9	4th	Influent	22-OFCs	*Klebsiella oxytoca bla*$_{OXY-6-4}$	NG_052696	99
18		6th	Influent	24-OFCs	*Klebsiella oxytoca bla*$_{OXY-6-4}$	NG_052696	99
19		6th	Influent	25-OFCs	*Klebsiella oxytoca bla*$_{OXY-6-4}$	NG_052696	99
20		6th	Influent	26-OFCs	*Klebsiella oxytoca bla*$_{OXY-6-4}$	NG_052696	99
21		5th	Aeration tank	23-OFCs	*Klebsiella oxytoca bla*$_{OXY-6-4}$	NG_052696	99
22		7th	Aeration tank	27-OFCs	*Klebsiella oxytoca bla*$_{OXY-6-4}$	NG_052696	99
23		7th	Effluent	28-OFCs	*Klebsiella oxytoca bla*$_{OXY-6-4}$	NG_052696	99

4.4.3.2.2.3 Phylogenetic tree of *bla*$_{CTX-M}$

The results of phylogenetic tree showed 16 *bla*$_{CTX-M}$ genes were divided into two groups and showed similar degree between different groups with homologous sequences in GenBank (Figure 4.10). The most closely related sequences to the *bla*$_{CTX-M}$ group 1and *bla*$_{CTX-M}$ group 9 from *E. coli* and OFCs are shown. These two groups have become the most common group of *bla*$_{CTX-M}$ (Hartmann et al., 2012). Group 1 like *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-3}$ and *bla*$_{CTX-M-15}$ share only 99 to 100% similarity. For instance, *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-3}$ are of very close identity, but differ by a single amino acid substitution (Asp-240 → Gly) (Woodford et al., 2004). Similarly, *bla*$_{CTX-M-3}$ and *bla*$_{CTX-M-1}$ differ by four amino acid positions (V77A, D114N, S140A, and N288D) (Cantón et al., 2012). This study found that four of the *E. coli* strains belonged to *bla*$_{CTX-M}$ group 9; the difference between *bla*$_{CTX-M-27}$ and *bla*$_{CTX-M-14}$ are by amino acid substitution (D240G) (Bonnet et al., 2003).

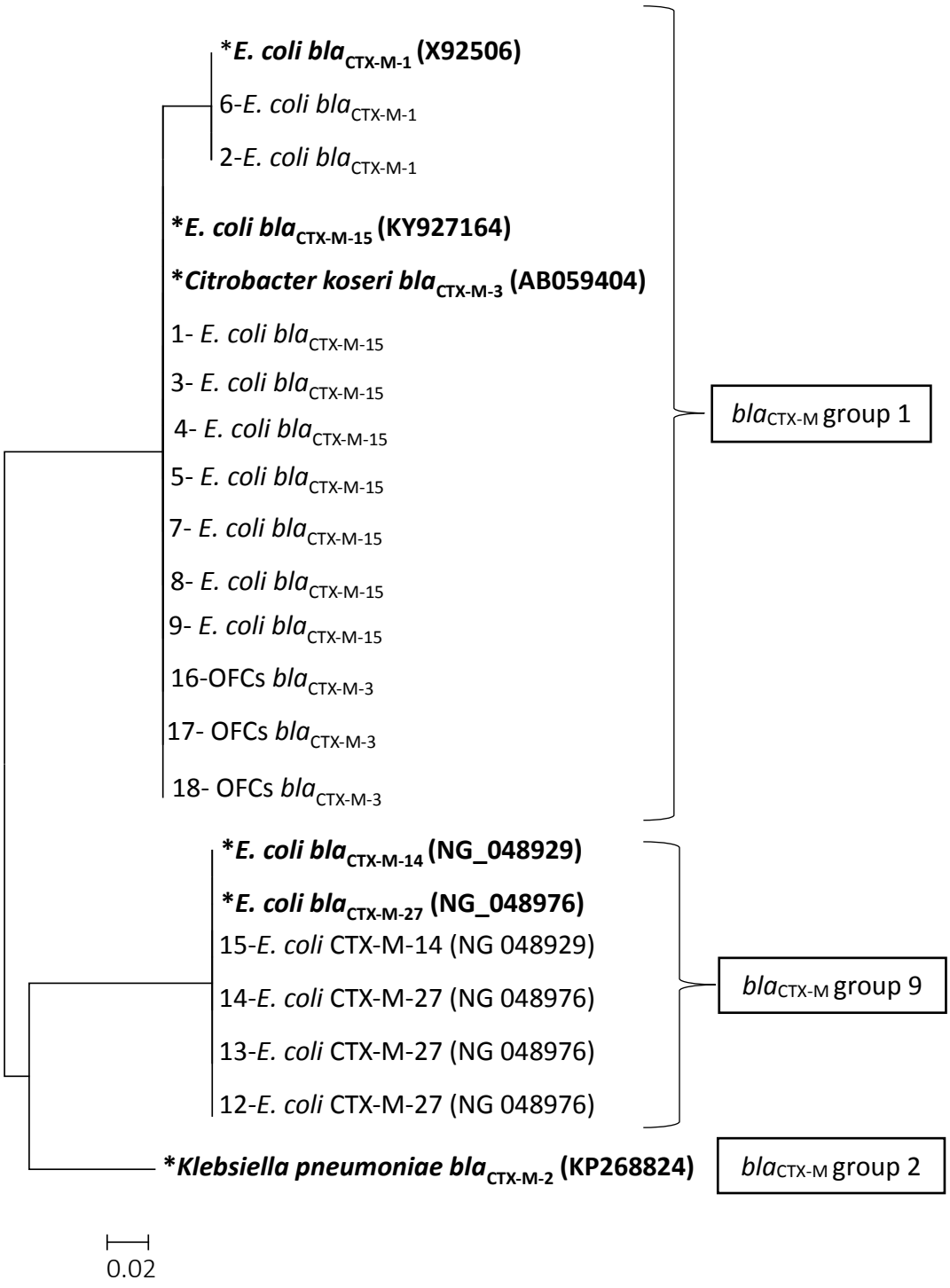

Figure 4.10 Dendrogram showing similarity among enzymes of the *bla*$_{CTX-M}$ linage in *E. coli* and OFCs strains (ID-strain *bla*$_{CTX-M}$ genotype) and GenBank references retrieved from database (*strain *bla*$_{CTX-M}$ genotype (accession number) and clustering of members of different *bla*$_{CTX-M}$ groups (1, 2 and 9 of *bla*$_{CTX-M}$ group), based on the Neighbhour-Joining method using MEGA7 software

4.4.3.2.2 Other *bla* genes (*bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA}$)

After optimising the amplification conditions, the results of other *bla* genes were present in 4 out of 13 *E. coli* strains (*n* = 2 *bla*$_{TEM}$ and *n* = 2 *bla*$_{OXA}$), and 42 out of 44 OFCs strains (*n* = 27 *bla*$_{TEM}$, *n* = 27 *bla*$_{SHV}$ and *n* = 2 *bla*$_{OXA}$). There was no *bla*$_{SHV}$ gene detected in *E. coli* at any stage of WWTP. Multiplex PCR (II) *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA}$ genes (other *bla* genes) were targeted 800, 713 and 564 pb, respectively, for example, as showed in Figure 4.11 for OFCs.

Figure 4.11 Multiplex PCR assay for *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{OXA}$ genes for OFCs. Lanes: **1**, Marker 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific); **2**, ESBL-negative control (*E. coli* NCTC 10418): **3**, *Klebsiella pneumoniae* (NCTC 13368) as *bla*$_{SHV-18}$ (SHV); **4**, *E. coli* (NCTC 11560) as *bla*$_{TEM-1}$ (TEM); **5 to 10**, **14**, and **16**, OFCs *bla*$_{TEM}$; **6** to **18**, and **20**, OFCs as *bla*$_{SHV}$; **6** and **8**, OFCs as *bla*$_{OXA}$; **19**, OFCs negative *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{OXA}$ genes

4.4.3.2.2.1 Partial DNA sequence analysis of *bla*$_{TEM}$

The results of nucleotide sequences of four *E. coli bla*$_{TEM}$ isolated showed similarity to *bla*$_{TEM-1}$ (100% identity) by NCBI as seen in Table 4.7. However, 25 isolates of OFCs -producing *bla*$_{TEM}$ were similar to *bla*$_{TEM-1, 19,}$ and $_{120}$, with between 99 to 100% homogenous identities.

Table 4. 7 Sequence analysis for *bla*$_{TEM}$ groups producing *E. coli* and OFCs by NCBI

Sample				NCBI (blastx)		
No.	Time collecting	Site	Isolate	Strain producing *bla* gene	Accession No.	Identity (%)
1	1st	Primary Sediment Tank	1-*E. coli*	*E. coli bla*$_{TEM-1}$	AHB36963	100
2	7th	Effluent	4-*E. coli*	*E. coli bla*$_{TEM-1}$	AEQ55215	100
3	3rd	Influent	2-*E. coli*	*Klebsiella pneumonia bla*$_{TEM-1}$	AWY04210	100
4	3rd	Effluent	3-*E. coli*	*Klebsiella pneumonia bla*$_{TEM-1}$	AWY04210	100
5	4th	Influent	11-OFCs	Gammaproteobacteria *bla*$_{TEM-19}$	WP_033560332	100
6	4th	Influent	12-OFCs	*Klebsiella pneumonia bla*$_{TEM-1}$	AVE02075	100
7	4th	Influent	13-OFCs	*Klebsiella oxytoca bla*$_{TEM-120}$	WP_063864806	100
8	4th	Influent	14-OFCs	*Klebsiella oxytoca bla*$_{TEM-120}$	WP_063864806	100
9	5th	Influent	15-OFCs	*Klebsiella oxytoca bla*$_{TEM-120}$	WP_063864806	100
10	6th	Influent	18-OFCs	Gammaproteobacteria *bla*$_{TEM-19}$	WP_033560332	100
11	6th	Influent	19-OFCs	*Morganella morganii bla*$_{TEM-1}$	ACX83572	100
12	6th	Influent	20-OFCs	*Morganella morganii bla*$_{TEM-1}$	ACX83572	99
13	6th	Influent	21-OFCs	*Klebsiella pneumonia bla*$_{TEM-1}$	AWY04210	100
14	6th	Influent	22-OFCs	*Klebsiella pneumonia bla*$_{TEM-1}$	ALP00857	100
15	1st	Primary Sediment Tank	5-OFCs	Gammaproteobacteria *bla*$_{TEM-19}$	WP_033560332	100
16	1st	Primary Sediment Tank	6-OFCs	*Klebsiella pneumonia bla*$_{TEM-1}$	AEM63746	100
17	1st	Primary Sediment Tank	7-OFCs	*Klebsiella pneumonia bla*$_{TEM-1}$	AEM63746	100
18	1st	Primary Sediment Tank	8-OFCs	Gammaproteobacteria *bla*$_{TEM-19}$	WP_033560332	100
19	1st	Primary Sediment Tank	9-OFCs	Gammaproteobacteria *bla*$_{TEM-19}$	WP_033560332	100
20	1st	Aeration Tank	10-OFCs	Gammaproteobacteria *bla*$_{TEM-19}$	WP_033560332	100
21	5th	Aeration Tank	16-OFCs	*Klebsiella oxytoca bla*$_{TEM-120}$	WP_063864806	100
22	7th	Aeration Tank	23-OFCs	*Klebsiella pneumonia bla*$_{TEM-1}$	AWY04200	100
23	7th	Aeration Tank	24-OFCs	Gammaproteobacteria *bla*$_{TEM-19}$	WP_033560332	99
24	8th	Aeration Tank	27-OFCs	Gammaproteobacteria *bla*$_{TEM-19}$	WP_033560332	100
25	8th	Aeration Tank	28-OFCs	Gammaproteobacteria *bla*$_{TEM-19}$	WP_033560332	100
26	8th	Aeration Tank	29-OFCs	*Klebsiella oxytoca bla*$_{TEM-120}$	WP_063864806	100
27	5th	Effluent	17-OFCs	*Klebsiella pneumonia bla*$_{TEM-1}$	AEM63746	100
28	7th	Effluent	25-OFCs	*Klebsiella oxytoca bla*$_{TEM-120}$	WP_063864806	99
29	7th	Effluent	26-OFCs	*Klebsiella pneumonia bla*$_{TEM-1}$	ATG30024	99

The results of phylogenetic tree found 29 *bla*$_{TEM}$ genes had closely related sequences to three types - *bla*$_{TEM-1}$ *bla*$_{TEM-19}$ and *bla*$_{TEM-120}$, and showed similarity to homologous sequences in GenBank (Figure 4.12). The *bla*$_{TEM-1}$ shared 100% identity in *E. coli* (n = 4), and 99–100% in OFCs (n = 10). In OFCs (n = 9), the *bla*$_{TEM-19}$ share 99–100% identity. However, *bla*$_{TEM-120}$ share with 100% identity in OFCs (n = 6). The results of phylogenetic tree also showed amino acid of *bla*$_{TEM-52}$, *bla*$_{TEM-3}$ and *bla*$_{TEM-26}$ were given other branches divided.

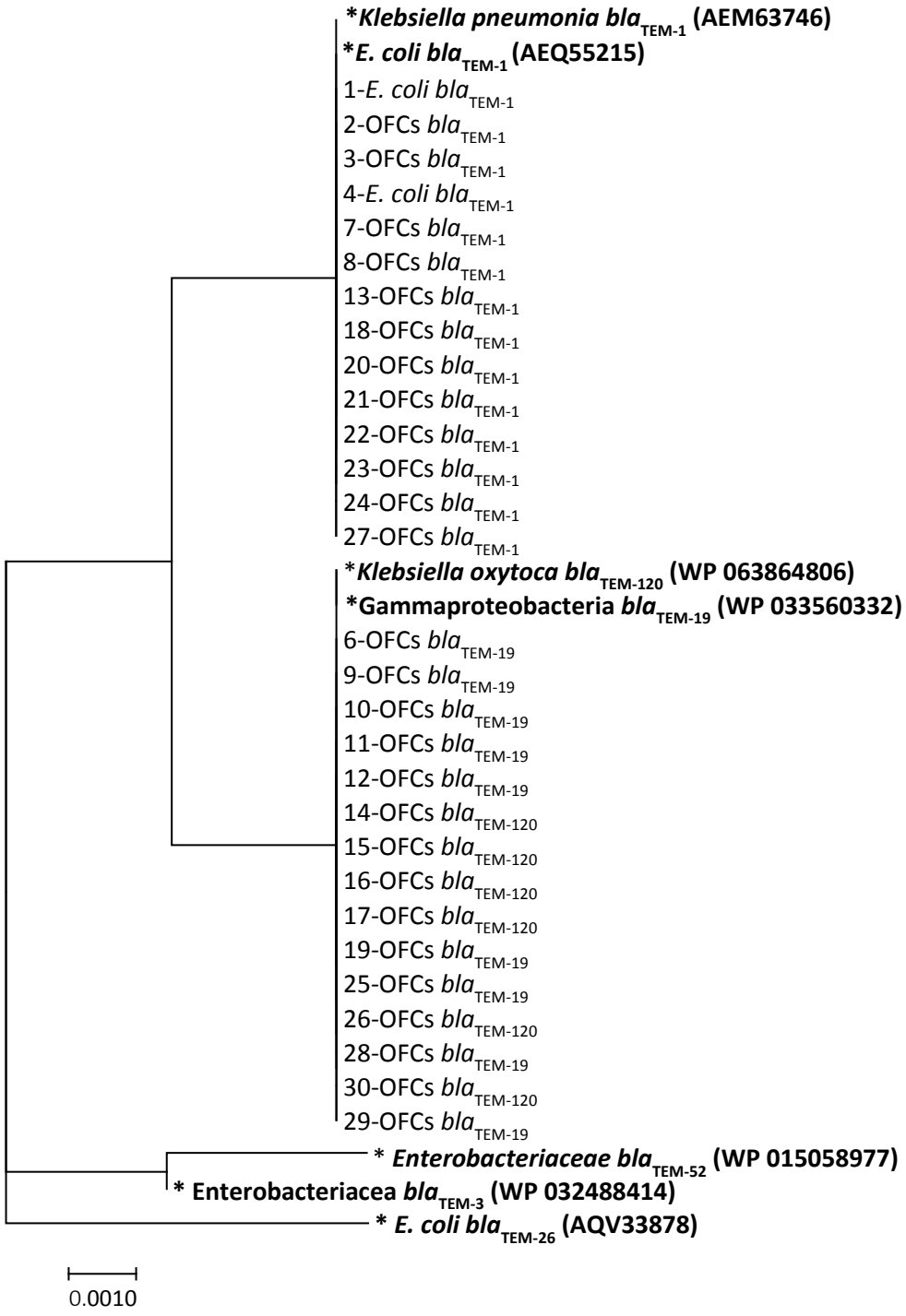

Figure 4. 12 Dendrogram showing similarity among enzymes of the bla_{TEM} linage in *E. coli* and OFCs strains (ID-strain bla_{TEM} genotype) and GenBank references retrieved from database (*strain bla_{TEM} genotype (accession number)) and clustering of members of different bla_{TEM} types, based on the Neighbour-Joining method using MEGA7 software

4.4.3.2.2.2 Partial DNA sequence analysis of *bla*$_{SHV}$

The results of nucleotide sequences of 27 OFCs *bla*$_{SHV}$ isolates were similar to *bla*$_{SHV-2, 11}$ and $_{12}$, with between 99–100% identities by NCBI as seen in Table 4.8.

Table 4. 8 Sequence analysis for *bla*$_{SHV}$ producing OFCs by NCBI

Sample				NCBI (blastx)		
No.	Time collecting	Site	ID-strain	Strain producing *bla*	Accession No.	Identity (%)
1	1st	Influent	1-OFCs	*Klebsiella pneumoniae bla*$_{SHV-2}$	AFN82059	100
2	1st	Influent	2-OFCs	*Klebsiella pneumoniae bla*$_{SHV-12}$	AHM26530	99
3	4th	Influent	5-OFCs	*Klebsiella pneumoniae bla*$_{SHV-2}$	AFN82059	100
4	4th	Influent	6-OFCs	*Acinetobacter* spp. *bla*$_{SHV-12}$	AHM26530	100
5	6th	Influent	11-OFCs	*Acinetobacter* spp. *bla*$_{SHV-12}$	AHM26530	100
6	6th	Influent	12-OFCs	*Klebsiella pneumoniae bla*$_{SHV-2}$	AFN82059	100
7	6th	Influent	13-OFCs	*Acinetobacter* spp. *bla*$_{SHV-12}$	AHM26530	100
8	6th	Influent	14-OFCs	*Acinetobacter* spp. *bla*$_{SHV-12}$	AHM26530	99
9	6th	Influent	15-OFCs	*Klebsiella pneumoniae bla*$_{SHV-2}$	AFN82059	100
10	6th	Influent	16-OFCs	*Acinetobacter* spp. *bla*$_{SHV-12}$	AHM26530	100
11	6th	Influent	17-OFCs	*Klebsiella pneumoniae bla*$_{SHV-2}$	AFN82059	100
12	6th	Influent	18-OFCs	*Klebsiella variicola bla*$_{SHV-2}$	CEL88822	100
13	6th	Influent	19-OFCs	*Acinetobacter* spp. *bla*$_{SHV-12}$	AHM26530	100
14	1st	Primary Sediment Tank	3-OFCs	*Klebsiella pneumoniae bla*$_{SHV-12}$	ABD93869	100
15	6th	Primary Sediment Tank	20-OFCs	*Klebsiella pneumoniae bla*$_{SHV-2}$	AFN82059	100
16	6th	Primary Sediment Tank	21-OFCs	*Acinetobacter* spp. *bla*$_{SHV-12}$	AHM26530	100
17	2nd	Aeration Tank	4-OFCs	*Klebsiella pneumoniae bla*$_{SHV-12}$	AHM26530	100
18	5th	Aeration Tank	9-OFCs	*Acinetobacter* spp. *bla*$_{SHV-12}$	AHM26530	100
19	7th	Aeration Tank	24-OFCs	*Acinetobacter* spp. *bla*$_{SHV-12}$	AHM26530	100
20	7th	Aeration Tank	25-OFCs	*Klebsiella pneumoniae bla*$_{SHV-2}$	AFN82059	100
21	8th	Aeration Tank	27-OFCs	*Acinetobacter* spp. *bla*$_{SHV-12}$	AHM26530	99
22	4th	Effluent	7-OFCs	*Klebsiella pneumoniae bla*$_{SHV-2}$	AFN82059	100
23	4th	Effluent	8-OFCs	*Klebsiella pneumoniae bla*$_{SHV-2}$	AFN82059	100
24	5th	Effluent	10-OFCs	*Klebsiella pneumoniae bla*$_{SHV-2}$	AFN82059	100
25	6th	Effluent	22-OFCs	*Acinetobacter* spp. *bla*$_{SHV-12}$	AHM26530	99
26	6th	Effluent	23-OFCs	*Klebsiella pneumoniae bla*$_{SHV-2}$	AFN82059	100
27	7th	Effluent	27-OFCs	*Acinetobacter* spp. *bla*$_{SHV-12}$	AHM26530	100

The results of phylogenetic tree found 27 *bla*$_{SHV}$ genes in OFCs were closely related sequences to three types, e.g. *bla*$_{SHV-2}$ and *bla*$_{SHV-12}$, and showed similarity to homologous sequences in GenBank (Figure 4.13). The *bla*$_{SHV-2}$ (*n* = 12) share with 99–100% and the *bla*$_{SHV-12}$ (*n* = 15) share 100- 99% identity. The results of phylogenetic tree showed amino acid of *bla*$_{SHV-18}$ and *bla*$_{SHV-16}$ were given other branches divided.

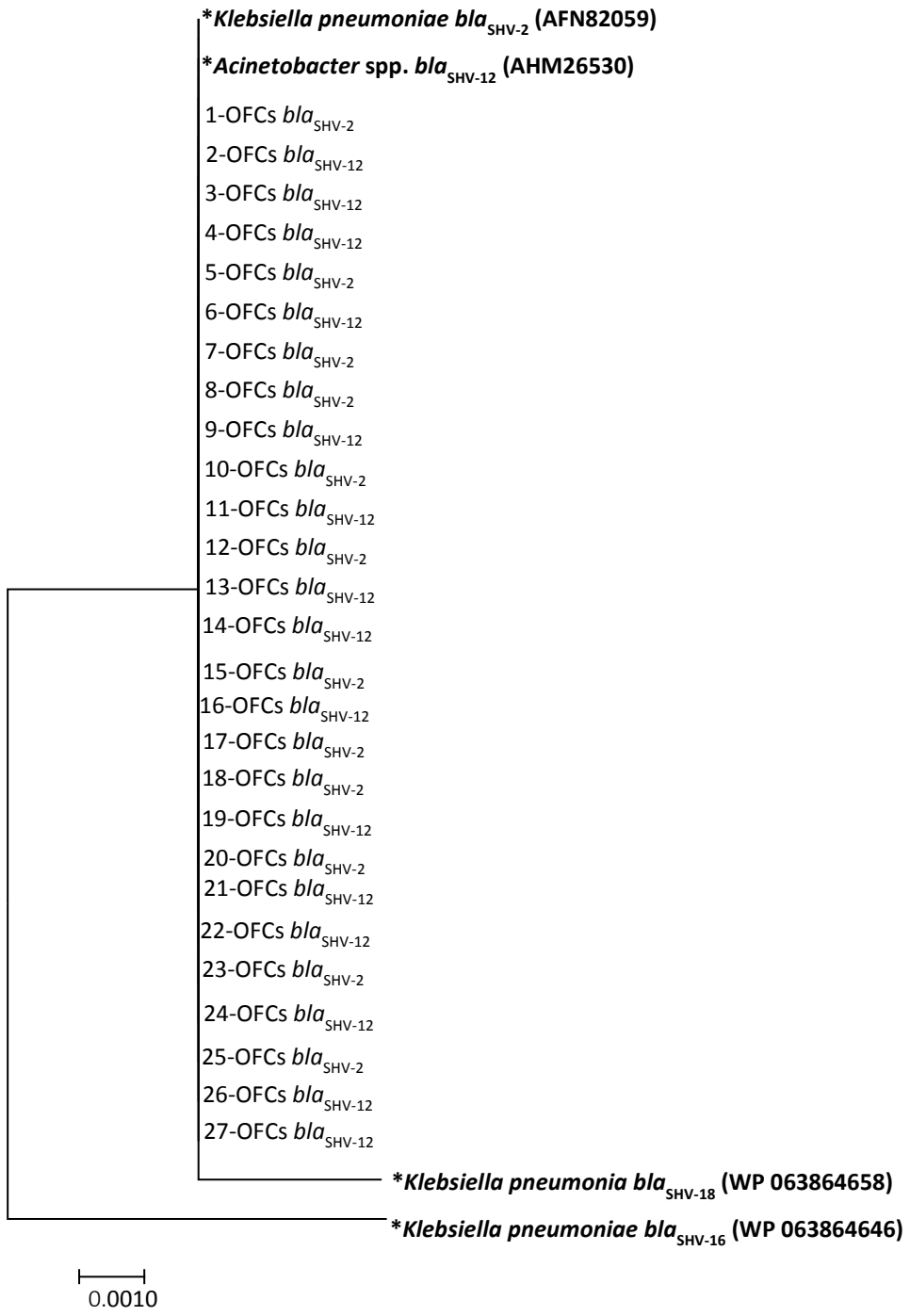

Figure 4. 13 Dendrogram showing similarity among enzymes of the *bla*$_{SHV}$ linage in *E. coli* and OFCs strains (ID-strain *bla*$_{SHV}$ genotype) and GenBank references retrieved from database (*strain *bla*$_{SHV}$ genotype (accession number)) and based on the Neighbour-Joining method, using MEGA7 software

4.4.3.2.2.3 Partial DNA sequence analysis of *bla*$_{OXA}$

The results of nucleotide sequences of 4 *bla*$_{OXA}$ in two *E. coli* and two OFCs strains showed similarity to *bla*$_{OXA-1}$ (between 99–100% identities) by NCBI as seen in Table 4.9.

Table 4. 9 Sequence analysis for *bla*$_{OXA}$ producing OFCs by NCBI

sample				NCBI (blastx)		
No.	Time collecting	Site	isolate	Strain producing *bla*	Accession No.	Identity (%)
1	6th	Effluent	1-*E. coli*	*E. coli bla*$_{OXA-1}$	AJW28746	100
2	8th	Effluent	2-*E. coli*	*E. coli bla*$_{OXA-1}$	AFN82057	100
3	6th	Influent	5-OFCs	*Acinetobacter baumannii bla*$_{OXA-1}$	SCY69726	99
4	5th	Aeration Tank	4-OFCs	*Acinetobacter baumannii bla*$_{OXA-1}$	SCY69726	100

The results of phylogenetic tree found 4 *bla*$_{OXA}$ genes were closely related sequences to *bla*$_{OXA-1}$ and showed similarity to homologous sequences in GenBank (Figure 4.14). The *bla*$_{OXA-1}$ share with 99–100% identity in *E. coli* (*n* = 2) and OFCs (*n* = 2).

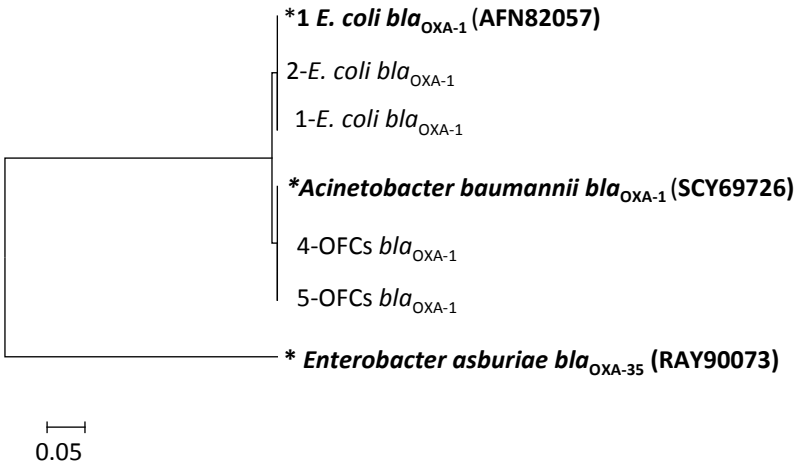

Figure 4. 14 Dendrogram showing similarity among enzymes of the *bla*$_{OXA}$ linage in *E. coli* and OFCs strains (ID-strain *bla*$_{TEM}$ genotype) and GenBank references retrieved from database (*strain *bla*$_{OXA}$ genotype (accession number)) and clustering of members of different *bla*$_{OXA}$ types, based on the Neighbour-Joining method using MEGA7 software

4.4.4 Prevalence of ESBL genes

At the influent site before the arrival of students, *bla*$_{CTX\text{-}M\text{-}1}$ and *bla*$_{CTX\text{-}M\text{-}27}$ genes were found in *E. coli*, while thereafter the *bla* gene was not detected (Table 4.5). At the primary sediment tank, four ESBL *E. coli* strains were detected before the arrival of students, with three of them carrying the *bla*$_{CTX\text{-}M\text{-}15}$ gene and one isolate carrying (*bla*$_{CTX\text{-}M\text{-}15}$ + *bla*$_{TEM\text{-}1}$). After the arrival of students, the *bla* gene was not observed. Although no *bla* type gene was detected before the arrival of students in the aeration tank, *bla*$_{CTX\text{-}M\text{-}14}$ was found thereafter. At the effluent site, there was no gene found in *E. coli* before the arrival of students, while *bla*$_{CTX\text{-}M\text{-}15}$, *bla*$_{CTX\text{-}M\text{-}27}$, (*bla*$_{CTX\text{-}M\text{-}15}$ + *bla*$_{TEM\text{-}1}$), (*bla*$_{CTX\text{-}M\text{-}15}$ + *bla*$_{OXA\text{-}1}$) and (*bla*$_{CTX\text{-}M\text{-}27}$ + *bla*$_{OXA\text{-}1}$) genes were observed at later stages.

In OFCs, before the arrival of students, *bla*$_{TEM\text{-}1}$, *bla*$_{TEM\text{-}120}$, (*bla*$_{TEM\text{-}1}$ + *bla*$_{SHV\text{-}12}$), (*bla*$_{TEM\text{-}19}$ + *bla*$_{SHV\text{-}2}$), *bla*$_{SHV\text{-}2}$ and *bla*$_{SHV\text{-}12}$ were found in isolates recovered from influent, but after the arrival of students, *bla*$_{CTX\text{-}M\text{-}3}$, (*bla*$_{TEM\text{-}1}$ + *bla*$_{SHV\text{-}2}$) and (*bla*$_{TEM\text{-}19}$ + *bla*$_{SHV\text{-}12}$ + *bla*$_{OXA\text{-}1}$) as new genes were discovered. At the primary sediment tank, *bla*$_{TEM\text{-}1}$, *bla*$_{TEM\text{-}19}$ and (*bla*$_{TEM\text{-}1}$ + *bla*$_{SHV\text{-}12}$) were detected before the arrival of students, while thereafter, *bla*$_{CTX\text{-}M\text{-}3}$, *bla*$_{SHV\text{-}2}$ and *bla*$_{SHV\text{-}12}$ were also detected. Before the students' arrival, only *bla*$_{TEM\text{-}19}$ and *bla*$_{SHV\text{-}12}$ were isolated from the aeration tank, but thereafter *bla*$_{TEM\text{-}19}$, *bla*$_{TEM\text{-}120}$, (*bla*$_{TEM\text{-}1}$ + *bla*$_{SHV\text{-}12}$), (*bla*$_{TEM\text{-}19}$ + *bla*$_{SHV\text{-}2}$), (*bla*$_{TEM\text{-}120}$ + *bla*$_{SHV\text{-}12}$ + *bla*$_{OXA\text{-}1}$) and *bla*$_{SHV\text{-}12}$ were recovered. In effluent, *bla*$_{CTX\text{-}M\text{-}3}$, *bla*$_{TEM\text{-}1}$ and *bla*$_{SHV\text{-}2}$ were recovered before the students' arrival, but after the arrival of students, *bla*$_{TEM\text{-}120}$, (*bla*$_{TEM\text{-}1}$ + *bla*$_{SHV\text{-}2}$), (*bla*$_{TEM\text{-}1}$ + *bla*$_{SHV\text{-}12}$, *bla*$_{SHV\text{-}2}$) and *bla*$_{SHV\text{-}12}$ were recovered.

Overall, the most frequently gene-producing *E. coli* was *bla*$_{CTX\text{-}M\text{-}15}$, followed by *bla*$_{TEM\text{-}1}$. However, the most frequently detected ESBL gene among OFCs before arrival of student was *bla*$_{SHV\text{-}2}$ and then *bla*$_{TEM\text{-}19}$, but *bla*$_{SHV\text{-}12}$ and then *bla*$_{TEM\text{-}1}$ predominated thereafter. Comparing before and after the arrival of students, there were no increased numbers of the *bla* genes in *E. coli* following the arrival of students, while there were greater numbers of *bla* genes recovered e.g. *bla*$_{CTX\text{-}M\text{-}3}$, *bla*$_{TEM\text{-}120}$, and (*bla*$_{TEM\text{-}1}$ + *bla*$_{SHV\text{-}12}$) in OFCs. However, the diversity of *bla* resistance genes was increased (in both *E.coli*; 4 vs 6 and OFCs; 8 vs 10 genes) after the arrival of students, as seen in Table 4.5.

In terms of new genes, the *bla* resistance genes was detected after the arrival of students in *E.coli* and OFCs isolates (Table 4.5). Sampling post students' arrival found previously undetected genes (*bla*$_{CTX\text{-}M\text{-}14}$, *bla*$_{CTX\text{-}M\text{-}15}$ + *bla*$_{OXA\text{-}1}$) and (*bla*$_{CTX\text{-}M\text{-}27}$ + *bla*$_{OXA\text{-}1}$) in *E. coli*, and (*bla*$_{TEM\text{-}1}$ + *bla*$_{SHV\text{-}2}$), (*bla*$_{TEM\text{-}19}$ + *bla*$_{SHV\text{-}12}$ + *bla*$_{OXA\text{-}1}$) and (*bla*$_{TEM\text{-}120}$ + *bla*$_{SHV\text{-}12}$ + *bla*$_{OXA\text{-}1}$) in OFCs.

4.5 Discussion

Studies have verified that WWTPs are a significant point of receiving ARB (Tao et al., 2016). However, it is unclear how changes in population may impact on WWTP systems and the release of ARB and their genes to the environment (Barlam and Gupta, 2015). Bangor, north Wales in UK, is a relatively small city, of which a significant proportion of the population are students. These arrive in a short span of time and from across the world. Therefore, WWTPs are subjected to sudden and considerable changes in microbial inputs. To the best of our knowledge, this study represents the first report of the presence of ARB through various stages of the WWTP at Bangor. The limitation of the present study was an unequal number of isolates were recovered before arrival of students and thereafter (293 vs 407 in *E. coli* and 195 vs 242 in OFCs) at different sampling events. Further, the media used, Chromogenic Primary UTI, has been developed to recover bacteria from clinical, rather than environmental, samples; which may have reduced specificity given that the latter samples are likely to have greater microbial diversity. This may reflect the variable proportion of presumptive colonies that were subsequently confirmed as positive by PCR (63.7% and 85.8% for *E. coli* and OFCs, respectively). Plating methods may also underestimate bacterial numbers due to cells entering a VBNC state. Funding limitations meant that this study selected ESBL isolates in *E. coli* and OFCs to confirm their identities by 16S rRNA sequencing, as opposed to all strains. The results identified that 13/15 as *E. coli* but all isolates of OFCs ESBLs confirmed as members of OFCs.

This study used best indicator for the identification of ESBL-producers as cefpodoxime (CPD) (3rd generation cephalosporin antibiotic) (Swarna et al., 2015), though it is less specific than a combination of ceftazidime, cefotaxime. However, cefpodoxime with clavulanate combination discs can be successfully used for all *Enterobacteriaceae* except *Enterobacter* spp. and *Citrobacter freundii* (Public Health England, 2016). This combination discs method gives either ESBL producers or AmpC or K1 enzymes due to the hyperproduction of the chromosomal β-lactamase (Fevre et al., 2005). Therefore, this study sequenced all ESBL isolates to identify their genes. This study found that the seven OFC ESBLs isolated from $bla_{\text{CTX-M}}$ group 9 were false ESBLs results such as bla_{OXY}.

The numbers of ESBLs genotypes detected in this study were too low to enable robust statistical analysis, e.g. to assess whether there were differences in the number of resistance genes carried ESBL-producers before/after the arrival of students. As described in Chapter 3 in Section 3.5,

sampling over a longer timeframe may generate more data for statistical analyses. It would also be of interest to see whether the size of the WWTP (and therefore the volume of wastewater treated) has any bearing on the likelihood of recovering ESBL-producers.

4.5.1 Prevalence of *E. coli*, OFCs and ESBL-producers

In this study, *E. coli* was found to be more prevalent in wastewater, compared to OFCs. *E. coli* is the most common bacteria in the intestine and excreted within faeces (Perkins et al., 2014). However, the numbers of ESBL-producing OFCs isolates were higher than ESBL-producing *E. coli*. This finding is consistent with the study reported in Algeria showing the number of *Klebsiella pneumoniae* carrying ESBL genes in WWTP were greater than *E. coli* (Alouache et al., 2014). Another study revealed that *Klebsiella pneumoniae* strains were the most frequent producers of ESBL than *E. coli* in human urinary tract infections (Gales et al., 2002). This may be because OFCs of course contain a variety of faecal coliform organisms.

The increased volume of inflow water from higher rainfall could impact on the effectiveness of UV treatment due to higher loads of suspended solids and hence greater turbidity, and reduced retention time (Hassen et al., 2000; Passerat et al., 2011; Mounaouer and Abdennaceur, 2015). These factors might lead to microbial survival post UV treatment; after which some bacteria have the capability to repair their DNA (Mounaouer and Abdennaceur, 2015). Rizzo et al. (2013) showed that the disinfection stage during wastewater treatment does not always efficiently remove ARB and resistance genes. Importantly, several studies have suggested that wastewater treatment plants should be redesigned to include a disinfection unit to halt the spread of antibiotic resistant bacteria and their genes in the environment (Biswal et al., 2014). It should be noted that a significant proportion of WWTP would not have the UV disinfection stage (tertiary treatment); therefore, the bacterial load being released in effluent might be considerably higher. Although not measured in this study, it is known that even where there is a significant reduction in bacterial numbers following treatment, their DNA could remain viable and the resistance genes integrated into other bacteria following release into the environment (McKinney and Pruden, 2012).

The mean numbers of *E. coli* and OFCs were significantly greater before the arrival of students than after their arrival. This might be a result of the heavy rain that occurred following student arrival, meaning that the number of *E. coli* and OFCs recovered per ml of wastewater was reduced due to a dilution effect. Although dilution could reduce the number per ml of sample, total

microbial load entering and leaving the WWTP could still be increased, should the volume of wastewater be greater under high rainfall periods. This relationship between microbial concentration, wastewater volume, and microbial load could be an area for further research.

The results showed that the UV disinfection significantly reduced the numbers of *E. coli* and OFCs before the arrival of students by 99.4%; these results are also consistent with those reported by Reinthaler et al. (2003). However, after the arrival of students, the effectiveness of UV disinfection was slightly reduced for *E. coli* and OFCs (97.5 and 98.7%, respectively).

4.5.2 Frequency and diversity of *bla* genes

The results from this study show that the frequency of *bla* genes after the arrival of students did not increase in *E. coli*, but did in OFCs as $bla_{\text{TEM-3}}$, $bla_{\text{TEM-120}}$, ($bla_{\text{TEM-1}}$ + $bla_{\text{SHV-12}}$) and $bla_{\text{SHV-12}}$.increased. The $bla_{\text{CTX-M-3}}$ gene was detected in the mid-1990s in Poland in *Klebsiella pneumoniae* and in Taiwan (Paterson and Bonomo, 2005). In a mini-review, Carattoli (2009) summarised that $bla_{\text{CTX-M-3}}$ had become distributed in Bulgaria, Croatia, France, Korea, Russia, Australia, Spain, Taiwan; whilst others have reported its prevalence in Algeria (Nedjai et al., 2013), the UK (Livermore et al., 2007) and Japan (Govinden et al., 2007). As seen in Table III in Appendix, students from all these countries arrived at Bangor University during the welcome week, therefore could affect the WWTP.

A study reported that $bla_{\text{CTX-M-15}}$ in *E. coli* is common worldwide, such as in India, Philippines, Singapore, Thailand, Malaysia, Vietnam, Australia, and New Zealand (Sheng et al., 2013). In this study, the ESBL gene most frequently detected *E. coli* was $bla_{\text{CTX-M-15}}$, and this could possibly be explained by the arrival of Asian students during welcome week (Table III in Appendix); though further studies are needed to validate this claim. In the UK, the first $bla_{\text{CTX-M-15}}$ gene recognised in *E. coli* were from visitors returning from India in the early 2000s (Woodford et al., 2004); this was also seen in a later study (Kuenzli et al., 2014). Hawkey (2015) pointed out that travellers to Asia and the Middle East are often associated with colonisation; with $bla_{\text{CTX-M-15}}$ attributed from India for *E. coli*. This finding is consistent with a number of studies that have researched $bla_{\text{CTX-M-15}}$-producing *E. coli* released via WWTPs to the environment (Bréchet et al., 2014; Rasheed et al., 1997; Amos et al., 2014b; Blaak et al., 2014).

Among OFCs, the numbers of bla_{SHV} (e.g. $bla_{\text{SHV-12}}$ and $bla_{\text{SHV-2}}$) and bla_{TEM} (e.g. $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-19}}$) genes in Bangor's WWTP increased after the arrival of students. Livermore and Hawkey (2005) stated that the most common ESBL genes in *Klebsiella pneumoniae* were bla_{SHV} and bla_{TEM}. In

1993-1995, the first *bla*$_{SHV-12}$ gene reported in *Klebsiella pneumonia* in Switzerland, whereas the *bla*$_{SHV-2}$ was detected in *Klebsiella ozaenae* from patient in Germany, in 1983 (Liakopoulos, et al., 2016). A study has reported that the global distribution of *Enterbacteriaceae*-producing *bla*$_{SHV-12}$ in Brazil, Tunisia, France, Italy, UK, Netherlands, Portugal, Taiwan, Bulagria, Ploland, Australia and China, while the *bla*$_{SHV-2}$ was found in France, Canada, China, Uruguay, Portugal and Netherlands (Liakopoulos, et al., 2016). In Kuwait, Mohmid and El-haliem (2013) found *bla*$_{SHV}$ and *bla*$_{TEM}$ are also most common in *E. coli* post of *bla*$_{CTX-M}$ genes. The *bla*$_{SHV-12}$ and *bla*$_{SHV-2}$ are most common genes of the *bla*$_{SHV}$ family detected (Villegas et al., 2008). They also have been reported in Japan (Chanawong et al., 2001). In Table III in Appendix, the numbers of the international students e. g. China, Kuwait, Switzerland, Taiwan, Canada and Australia could possibly be excreted those genes and released into the WWTP. Further research is necessary to assess this state.

In this study, an inconsistent pattern was seen in the numbers of ESBL-producing isolates at various stages of the WWTP before and after the arrival of students. The survival of bacterial populations during the various stages of the WWTP is likely to be affected due to interactions between microbial populations, and physiochemical changes during mixing. Greater work is needed to improve our understanding of bacterial dynamics in such environments; especially where antibiotic and antibacterial compounds are present (Al-Ahmad et al., 2009; Yuan et al., 2016).

Some of the isolates in this study were found to harbour a single resistant gene, but few isolates harboured multi-resistant genes (Table 4.5). This is similar to that reported by others, that ESBL-mediating plasmids are known to carry multi-resistant ESBL genes as *bla*$_{CTX-M}$, *bla*$_{SHV}$ and *bla*$_{TEM}$ (Amine, 2013) that can cause high-level resistance (Reinthaler et al., 2010). For instance, this study detected *Klebsiella pneumoniae* carrying *bla*$_{OXA}$ with *bla*$_{CTX-M-15}$ after the arrival of students. This finding is consistent with study reporting *Klebsiella pneumoniae* that frequently carried *bla*$_{CTX-M-15}$ was associated with *bla*$_{OXA}$ (Sugumar et al., 2014).

In this study, the diversity of prevalent *bla* genes after the arrival of students were increased in both *E. coli* and OFCs. Further, new genes were detected after the arrival of students in WWTP; with *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$ + *bla*$_{OXA-1}$ and *bla*$_{CTX-M-27}$ + *bla*$_{OXA-1}$ found in *E. coli* and (*bla*$_{TEM-1}$ + *bla*$_{SHV-2}$), (*bla*$_{TEM-19}$ + *bla*$_{SHV-12}$ + *bla*$_{OXA-1}$), (*bla*$_{TEM-120}$ + *bla*$_{SHV-12}$ + *bla*$_{OXA-1}$) and *bla*$_{SHV-12}$. In OFCs. It could be hypothesised that the significant influx of overseas students to the area (Section 4.2.1) may have introduced these genes. For instance, in *E. coli*, *bla*$_{CTX-M-14}$ gene is the most prevalent in China,

Taiwan and South Korea (Sheng et al., 2013). Overall, *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-14}}$ are the most common *bla*$_{\text{CTX-M}}$ genes found worldwide (Cantón et al., 2012), followed by *bla*$_{\text{CTX-M-3}}$ and *bla*$_{\text{CTX-M-1}}$ (Zhao and Hu, 2012). The *bla*$_{\text{CTX-M-27}}$ gene was frequently detected in Egypt and Tunisia in *Enterobacteriaceae* (Cheaito and Matar, 2014). Relatively little is known about the geographical distribution of the ESBL *bla*$_{\text{OXA}}$ gene (Peymani et al., 2014); although *bla*$_{\text{OXA-1}}$ has been reported in 1 to 10% of *E. coli* as described by Shaikh et al. (2015), and many types of *bla*$_{\text{OXA}}$ genes have been found in France and Turkey (Bradford, 2001). The *bla*$_{\text{OXA-1}}$ and *bla*$_{\text{CTX-M-1}}$ genes are most often associated together with *E coli* and detected a worldwide (Poirel et al., 2010). One study point out that those genes have found in the USA and Portugal and isolated from *E. coli* and *K. pneumonia* (Barguigua et al., 2011). The *bla*$_{\text{CTX-M-120}}$ has been reported in Canada (Mulvey et al., 2004).

The findings of our study suggest that people arriving from different parts of the world could play a role in the dissemination of antibiotic resistance in the environment, as previously suggested (Barlam and Gupta, 2015), while studies have verified that WWTPs are a significant point of receiving ARB into the environment (Tao et al., 2016). Whilst some of Bangor's residents originate from across the world, the arrival of international students to the city significantly increases the proportion of the population that come from China, India and the Middle East. As explained above, many of the resistant genes detected in the WWTP post-arrival of students are particularly prevalent in these regions, and this may explain why both a greater frequency and diversity of resistant genes were found after the arrival of students to Bangor. Once introduced, it is possible antibiotic resistant genes may be transferred between bacteria within sewage water by HGT mechanisms such as conjugation or transduction (Everage et al., 2014). Given that even tertiary treatment was not found to completely eradicate these bacteria, WWTP release new antibiotic resistant genes into the environment. Similar to how others have reported (Barlam and Gupta, 2015), our results suggest that movement of people between countries could exacerbate the dissemination of antibiotic resistance in the environment.

4.6 Conclusions

This study shows that the arrival of students coincided with an increase the numbers and diversity of *bla* genes in ESBL-producing OFCs at the receiving WWTP, with three new *bla* genes detected. For *E. coli*, although the number of isolates did not increase over the same timeframe,

they too showed greater diversity, also with three new *bla* genes detected. Further work is needed to determine how large demographic changes can affect the cycling and dissemination of ARB and their genes to and from wastewater treatment systems.

4.7 References

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Chapter 5: Experiment 3

The impact of sediment concentrations on the survival of *bla*$_{\text{CTX-M-15}}$-producing *E. coli* in wastewater released to seawater and freshwater

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5.1 Abstract

Antibiotic resistant bacteria (ARB) and their genes have become a public health issue. In particular, *bla*$_{CTX\text{-}M\text{-}15}$ produced by *E. coli* has rapidly become the most common type of Extended-Spectrum β-Lactamase enzyme (ESBLs) in many countries during the last decade, especially in the UK. Wastewater treatment plants (WWTPs) release large amounts of ESBL-producing bacteria into the environment. Thereafter, their survival is dependent on many factors, including shielding by sediments, which could protect them from inactivation by UV radiation. However, it is unclear how sediment concentrations may influence the survival of ARB in environmental water. The aim of this study was to assess the impact of sediment concentrations on the inactivation rate of *bla*$_{CTX\text{-}M\text{-}15}$ -producing *E. coli* released from human wastewater into seawater and freshwater. Microcosms containing either sea or freshwater with low, medium and high turbidity were inoculated with *bla*$_{CTX\text{-}M\text{-}15}$ -producing *E. coli* and then exposed to the average duration of UV radiation in winter (14 min) and in summer (4.5 h) in north Wales. Counts of *bla*$_{CTX\text{-}M\text{-}15}$ -producing *E. coli* were performed on duplicate Brilliance™ ESBL Agar plates, then the identified bacterial isolates and its gene (*bla*$_{CTX\text{-}M\text{-}15}$) confirmed by biochemical and PCR tests. Furthermore, the physiochemical properties of the waters (electrical conductivity, pH, phosphate, nitrate, total organic carbon and total organic nitrogen) were determined. The results showed that sediment concentration could be a potential impact on the survival of *bla*$_{CTX\text{-}M\text{-}15}$-producing *E. coli*, with greater survival under higher turbidity in both seawater and freshwater. In the first thirty minutes, the survival of *bla*$_{CTX\text{-}M\text{-}15}$-producing *E. coli* was reduced slightly (20-28%) at all turbidity levels in both waters, with the exception of freshwater of lower turbidity (45% reduction). However, after 4.5 h UV exposure, the survival was significantly reduced (85-100%) at all turbidity levels in sea and freshwater. Overall, the number of *bla*$_{CTX\text{-}M\text{-}15}$-producing *E. coli* recovered from the microcosms declined over 8 h, particularly so in seawater of lower turbidity. In conclusion, this study found that there was a positive relationship between sediment concentrations and survival of *bla*$_{CTX\text{-}M\text{-}15}$-producing *E. coli* in water. Survival in freshwater was greater than in seawater and under UV exposure rates, more typical of winter conditions. Further study is required to analyse how chemical and biological factors impact on survival of *bla*$_{CTX\text{-}M\text{-}15}$-producing *E. coli* post release from a WWTP into freshwater and seawater.

5.2 Introduction

Antibiotics such as those in the β-lactam (Beta lactam) group are widely used in clinical and community settings to treat human infections. Frequent bacterial exposure to β-lactam antibiotics, such as penicillins, carbapenem, monobactams and cephalosporins may cause bacteria to develop the ability to produce so-called Extended-Spectrum β-Lactamase enzymes (ESBLs), that disable the antibiotic effect on pathogens (Pitout et al., 2005; Samaha-kfoury & Araj, 2003).

Production of ESBLs is the most common resistance mechanism to inactivate antibiotic function by the hydrolysis of β-lactam antibiotics group (Pitout et al., 2005; Samaha-kfoury & Araj, 2003). ESBL-producing *Enterobacteriaceae* confer high-level resistance to oxyimino-cephalosporins including cefotaxime, aztreonam and ceftazidime (Branger et al., 2005; Pitout and Laupland, 2008). ESBL-producing *Enterobacteriaceae* have become particularly prominent, not only in hospitals, but also in community settings. As stated by U.S. Centres for Disease Control (CDC), ESBL-producing *Enterobacteriaceae* are deemed a ''serious threat to public health''. They estimated that 26,000 ESBL infections per year are resistant to antibiotics; causing 1,700 deaths , and each hospitalised case costing approximately $40,000 (CDC, 2013).

bla$_{\text{CTX-M}}$ groups are the most important ESBL enzymes that have been reported (Nüesch-Inderbinen et al., 2013). *bla*$_{\text{CTX-M}}$ enzymes are divided into several groups based on amino acid sequence similarity as *bla*$_{\text{CTX-M}}$ group 1, *bla*$_{\text{CTX-M}}$ group 2, *bla*$_{\text{CTX-M}}$ group 8/25 or (*bla*$_{\text{CTX-M}}$ group 8 and *bla*$_{\text{CTX-M}}$ group 25) and *bla*$_{\text{CTX-M}}$ group 9 (Bonnet, 2004). In particular, *bla*$_{\text{CTX-M-15}}$ belonging to group 1 is the most common enzyme produced by *E. coli*, with increasing spread in many countries over the last decade, especially in the UK (Hawkey, 2015).

Wastewater treatment plants (WWTPs) are known as hotspots of developing antibiotic resistant bacteria (ARB), leading to their growth and dissemination in the wider environment (Wellington et al., 2013; Gao, Munir and Xagoraraki, 2012; Novo et al., 2013; Rizzo et al., 2013). A study by Bréchet and colleagues (2014) in France calculated that treated wastewater containing more than 600 billion ESBL-producing *E. coli* were released daily in the receiving river. A study in the Netherlands by Blaak and co-workers (2014) found ESBL-producing *E. coli* in four areas of recreational waters derived from WWTPs; *bla*$_{\text{CTX-M-15}}$ enzymes were the majority of ESBL isolates detected.

When WWTP discharge pathogenic bacteria into the river or marine environments, the bacteria face many environmental challenges such as ultraviolet (UV) radiation derived from sunlight, a change in temperature, salinity, heavy metals, pH, aggregation, algal toxins, competition, predation, and viral lysis (Krstulovic et al., 2007), oxygen depletion and nutrient deficiencies (Whitman et al., 2004). Of these factors, turbidity i.e. the concentration of suspended sediments in water might play a crucial key for bacteria surivial within the water environment (Lawler et al., 2006a). Several published studies found that water sediments nearby a WWTP were a reservoir of ESBL-producing *Enterobacteriaceae* (Amos, Hawkey, Gaze, & Wellington, 2014; Lu et al., 2010). Pathogens have the capablity to attach to sediment particles in water (Malham et al., 2014), acting as a barrier to UV sunlight, and offering protection to bacteria from UV radiation (Malham et al., 2014; Lawler et al., 2006b). UV sunlight exposure is regarded as one of the most important factors in determining the death rate of microorganisms in surface waters (Walters et al., 2014).

However, it is unclear how suspended sediment concentrations influence the survival of ARB in fresh and seawater following their release from a WWTP (Malham et al., 2014). The aims of this study were (i) to assess the impact of the sediment concentration on the survival of *bla*$_{\text{CTX-M-15}}$-producing *E. coli* under UV exposure in seawater and freshwater derived from human wastewater and, (ii) to compare duration of survival in simulated winter and summer time.

5.3 Material and Methods

5.3.1 Study site

5.3.1.1 Seawater

Treborth WWTP (53°12’52”N, 4°11’28”W) serves the areas of Bangor, Menai Bridge, Bethel and Felinheli (Welsh Water, personal communication); an area of approximately 23,000 inhabitants (Rana, 2015). The WWTP discharged treated effluent at a permitted daily rate of 24.5 million l d^{-1} (300 l s^{-1}) to the Menai Strait, a narrow strip of sea between the island of Anglesey and the north Wales mainland (Figure 5.1).

5.3.1.2 Freshwater

Llanrwst, Conwy, has a secondary treatment plant (STP) that serves approximately 4,000 inhabitants (2011 census). The Llanrwst STP discharged treated effluent at a permitted daily rate of >2,419,100 l d^{-1} (28 l s^{-1}) to the river Conwy (Afon Conwy), eventually reaching the sea as a small estuary near the castled town of Conwy on the north Wales coast. The river has a relatively large drainage basin of approximately 590 km², which drains some of the highest mountains in the UK (FSC, 2018.) (Figure 5.2).

5.3.2 Sample collection

Water and sediment samples were collected downstream of both sites (Figures 5.1 and 5.2) (Figure V in Appendix). Triplicate water samples were collected from 15 cm below of the water surface, about 200 m downstream of the outlets, using sterile 1 litre glass bottles. Sediment samples were taken from the same location at both respective sites by hand spatula and then transferred into sterile 2 litre glass bottles. Samples were transported at 4°C to the laboratory within 1-2 hours (h).

Figure 5.1 Sample site collected from downstream of nearby treated water of Treborth WWTP in the Menai Strait, north Wales, UK (https://en.wikipedia.org/wiki/Bangor,_Gwynedd)

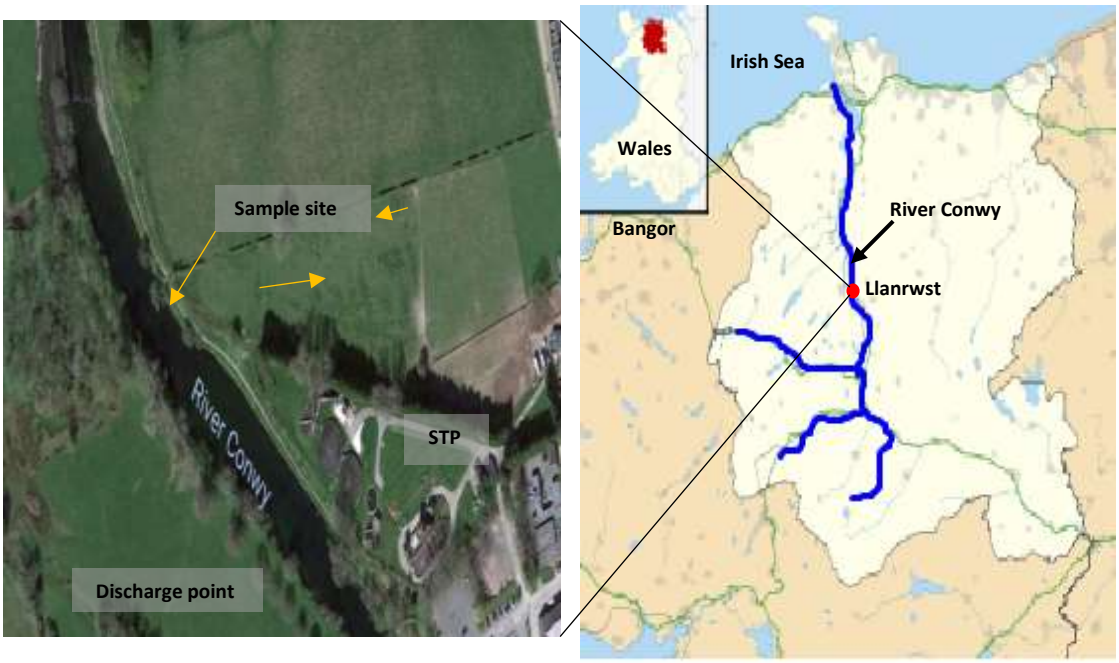


Figure 5.2 Sample site collected from downstream of nearby of Llanrwst STP, north Wales, UK

5.3.3 Characterisation of waters

All physiochemical analyses were performed directly on water samples. Electrical conductivity (EC) and pH were measured using EC meter (Jenway Ltd, Dummow, UK) and Hanna precision pH meter (Model pH 209), respectively. Phosphate (PO_4^{3-}) concentration was determined by the method of Murphy & Riley (1962), and nitrate (NO_3^-) by Downes (1978) and Mulvaney (1996) using a spectrophotometer (PowerWave XS, BioTek, USA). Concentrations of total organic carbon (TOC) and total organic nitrogen (TON) were determined by a C-N analyser (Multi N/C 2100s, AnalyticJena, Germany). The instruments used this study are shown in Figure VII in the Appendix. The physiochemical analytical procedures are explained in the Appendix (pages 186-188).

5.3.4 Microcosm equipment

5.3.4.1 Sediment culturing

Sediment samples were dried at 105°C for 6-12 h as seen in Figure VI in the Appendix, then large solid matters were removed by sieving to obtain fine sediment (size: 63 µm) and kept in sterile plastic bag at 4°C for further analysis.

An enrichment procedure was prepared, where a 5 g sub-sample of each sediment sample was mixed in triplicate with 15 ml of Tryptone Soya Broth (TSB; Oxoid) and shaken at 150 revolutions per minute (rpm) for 6 h at 37°C before streaking the broth onto duplicate Brilliance™ ESBL agar (Williams et al., 2007). Plates were then incubated at 37°C for 24-48 h, and the presence of *bla*$_{\text{CTX-M-15}}$-producing *E. coli* was confirmed by oxidase and PCR tests as described below (Section 5.3.6).

5.3.4.2 Preparation of turbid water

Triplicate water samples were refrigerated at 4°C for 6 h to allow for settling of sediment. For each water sample, 190 ml was transferred in three replicates to 250 ml sterilise glass bottles (Fisherbrand™ Borosilicate Glass Narrow Neck Laboratory Bottles), which represented three sediment concentrations.

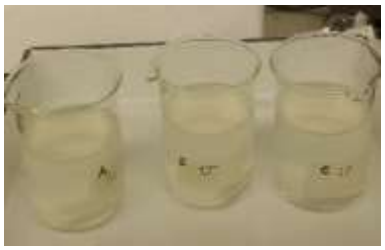
The standard of suspended sediment in surface water is 1 FTU (Formazin Turbidity Units) and ≤ 5 FTU in recreational water (Amalfitano et al., 2017). The U.S. Environmental Protection Agency and others have found the level of turbidity is 25 FTU for seawater and 50 FTU for freshwater (Mallin et al., 2009). Of course, suspended sediment concentrations will increase

during heavy rainfall. Lawler et al. (2006a) found that the highest turbidity in a river following a storm event was 500 FTU. For this study, it was decided to set up three different levels of water turbidity (suspended sediment) by adjusting a sample with an initial turbidity of 10 FTU for low turbidity (LT), 150 FTU for medium turbidity (MT) and 400 FTU for high turbidity (HT). Sediment concentrations were measured before and after adjustment using a turbidity meter (HANNA instruments, turbidity meter HI-93703). These were achieved by adding a sufficient amount of sediment (g) to 190 ml of water samples and then shaking by hand in order to dissolve sediments (Table VI in the Appendix).

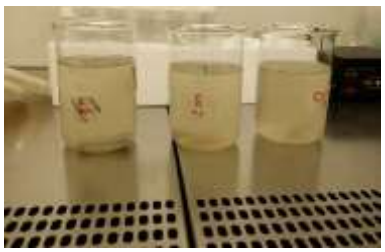
The mean turbidity of seawater was 2.47 FTU and 0.59 FTU for freshwater, after leaving for 6 h for settling of sediments. After adjustment, the turbidity for the different treatments for seawater were 11 FTU (low), 154 FTU (medium) and 409 FTU (high) (Figure 5.3 (A)), and for freshwater 12 FTU (low), 155 FTU (medium) and 415 FTU (high) (Figure 5.3 (B)).

To determine the presence or absence of ESBL-producing *E. coli* in water, 100 µl from each sample was spread onto duplicate Brilliance™ ESBL Agar plates, and then incubated at 37°C for 24-48 h.

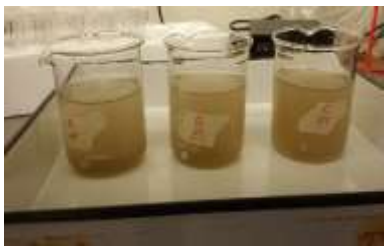
A. Seawater



LT (11 FTU)



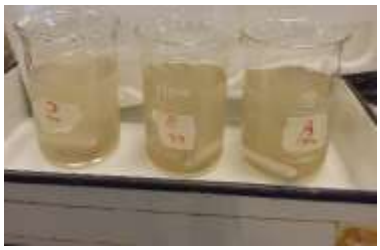
MT (154 FTU)



HT (409 FTU)

B. Freshwater

LT (12 FTU)



MT (155 FTU)

HT (415 FTU)

Figure 5.3 The difference between LT (low turbidity), MT (medium turbidity) and HT (high turbidity) in sea and freshwater

5.3.4.3 Preparation of inoculum

An inoculum was prepared from a fresh overnight culture (Nutrient broth; CM0001, Oxoid Ltd., Basingstoke, UK; 24 h, 37°C, 150 rpm) of control strain *bla*$_{\text{CTX-M-15}}$-producing *E. coli* (NCTC 13353) obtained from the National Collection of Type Cultures (NCTC), a Culture Collection of Public Health England (https://www.phe-culturecollections.org.uk/collections/nctc.aspx). A series of decimal dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) of samples were prepared as previously described in Chapter 3 in Section 3.3.3. Subsequently, a 100 µl volume of suspension was spread by L-shaped spreader over the entire surface of duplicate Brilliance™ ESBL Agar plates and incubated at 37°C for 24-48 h. Cell numbers were calculated from the mean CFUs obtained from duplicate plates at the appropriate dilution.

5.3.4.4 Inoculation of water samples

A 10 ml aliquot of inoculum was added to 190 ml of each water sample in a 250 ml sterilised beaker (Fisherbrand™ Squat Form Beakers) and the mixture was stirred vigorously for one

minute on the magnetic stirrer (HANNA instruments, Magnetic Mini-Stirrer; which consist of rapid mixing (100-1000 rpm)). A series of decimal dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of water samples were prepared as described above. Plates were incubated at 37°C for 24-48 h. The resulting CFUs provided enumeration of ESBL bacteria and represented as time zero (T_0).

5.3.4.5 Artificial sunlight design

The outdoor exposures were measured in winter and summer 2017 at Hiraethlyn, north Wales by the Centre for Ecology & Hydrology (see Table VII in the Appendix). They found that the average duration of UV radiation a day at a wavelength of 300-400 nm and UV radiation 40 W m^{-2} was equivalent to 14 min (33.6 kJ m^{-2}) in winter and 4.5 h (648 kJ m^{-2}) in summer (Kata Farkas, personal communication).

A separate experiment was run in three replicate samples for each turbidity level (6 cm depth water level in 250 ml beaker) and exposed UV light to simulate outdoor weather parameters in an Atlas Suntest XXL+FD containing xenon lamps filtered by daylight filters at wavelength range at 300-400 nm and the irradiance level was 40 W m^{-2}. The chamber temperature was set at 15°C. Samples were stirred slowly (100 rpm) by magnetic stirrer to maintain a constant turbidity level (Figure 5.4).

Samples were diluted as described above from each time, before exposure (T_0), and after 0.5 hour (h) ($T_{0.5}$), 1 h (T_1), 2 h (T_2), 3 h (T_3), 4.5 h ($T_{4.5}$), 6 h (T_6) and 8 h (T_8). Before enumeration, Brilliance™ ESBL Agar plates were incubated at 37°C for 24-48 h.

Where no colonies had grown on the media, an enrichment procedure was employed to test for the presence of *E. coli* $bla_{CTX\text{-}M\text{-}15}$, as described above (5.2.4.1). The detection limit of bacterial growth in conventional media is 1 CFU m^{-1}. Those samples that were negative by conventional plating but positive after enrichments were assigned a value equal to half the limit of detection (0.5 CFU m^{-1}), and colonies were confirmed as $bla_{CTX\text{-}M\text{-}15}$-producing *E. coli* by PCR.

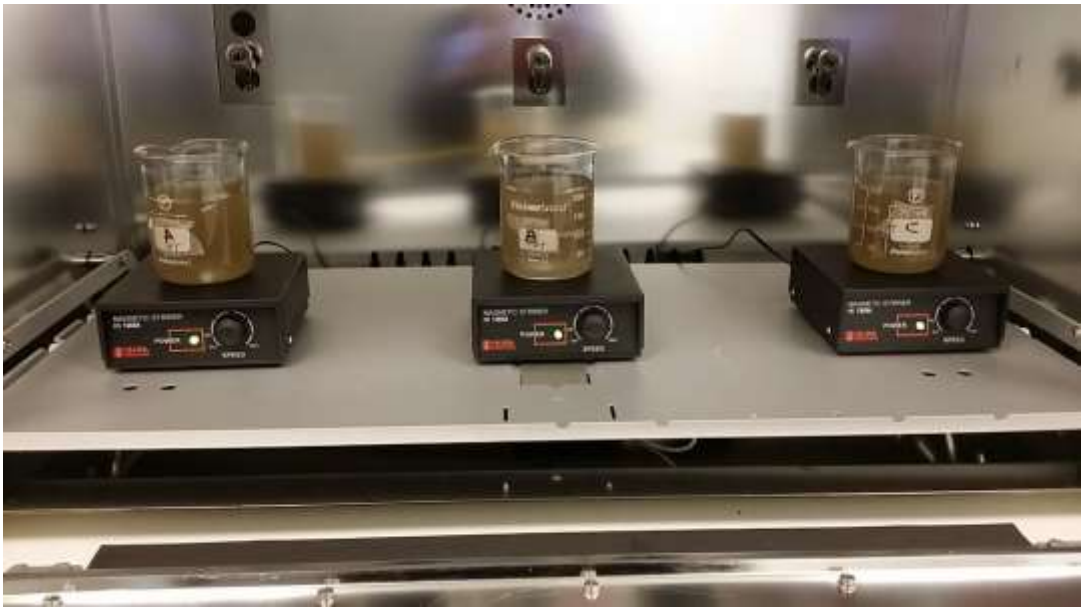


Figure 5.4 High turbid water samples stirred on the magnetic stirrer under the Suntest XXL+FD instrument

5.3.5 Isolation of *E. coli*

As the *E. coli* that grew on Brilliance™ ESBL agar (Figure 5.5) was not the same colour as recommended in the manufacture's protocol (see Figure VIII in the Appendix), further tests were conducted to confirm the colonies. Six pure single colonies of presumptive *E. coli* were picked randomly by sterile 1 µl loop from each time T_0, $T_{0.5}$, T_1, T_2, T_3, $T_{4.5}$, T_6 and T_8 of UV exposure and spread onto nutrient agar plate (Oxoid, CM0003) , then incubated at 37°C for 24 h to maintain for further analysis.

Figure 5.5 Brilliance™ ESBL Agar, with *E. coli* grown as dark green colonies

Over the course of the study, 241 presumptive *E. coli* isolates (seawater *n* = 115 and freshwater *n* = 126) were randomly picked, as illustrated in Table 5.1.

Table 5.1 Presumptive isolates of *E. coli* in three levels of turbidity from seawater and freshwater

Time (h)	Seawater			Freshwater		
	LT	MT	HT	LT	MT	HT
T_0	6	6	6	6	6	6
$T_{0.5}$	6	6	6	6	6	6
T_1	6	6	6	6	6	6
T_2	6	6	6	6	6	6
T_3	6	6	6	6	6	6
$T_{4.5}$	0	6	6	3	6	6
T_6	0	1	6	1	6	6
T_8	0	0	6	0	2	6
Total	30	37	48	34	44	48
Total	115			126		

5.3.6 Identification of *bla*$_{CTX-M-15}$-producing *E. coli*

5.3.6.1 *E. coli*

5.3.6.1.1 Oxidase test

As previously described in Chapter 3 in Section 3.3.4.1.

The quality control *E. coli* NCTC 13353 was used as an oxidase negative. All isolates were stored in Microbank™ vials (Pro-lab Diagnostics) at -70°C for further analysis.

5.3.6.1.2 PCR *uidA* confirmation

As previously described in Chapter 3 in Section 3.3.4.2, the concentration of DNA samples was diluted to roughly 20-30 ng, and absorbance was measured using a spectrophotometer (NanoDrop 1000 Spectrophotometer, Thermo Scientific). Diluted DNA was stored at -20°C until used as the template DNA.

The *uidA* (β-glucuronidase enzyme) has been used for identification *E. coli* (Bej et al., 1990). The primers used for detection of *uidA* are shown in Table 5.2.

Table 5. 2 Primer sequence and amplicon size for *uidA* gene (Bej et al., 1991)

Organism	Gene	Primer code	Primer name	Primer sequence (5'-3')	Amplicon (bp)
E. coli	*uidA*	*uidA*-F	UAL-754	AAAACGGCAAGAAAAAGCAG	147
		uidA-R	UAR-900	ACGCGTGGTTACAGTCTTGCG	

Single PCR amplification for *uidA* gene was performed under the following conditions: initial denaturation step at 95°C for 3 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 50°C for 1 min. Final elongation would occur at 72°C for 7 min in a thermal cycler.

PCR condition was conducted for *uidA* by using a 50 µl reaction mixture consisting of 25 µl (1x) BioMix Red (Bioline), 1 µl *uidA*-F primer, 1 µl *uidA*-R primer, 21 µl Water Molecular Biology Reagent (W4502, Sigma-Aldrich) and 2 µl DNA template.

PCR-amplified fragments (5 µl aliquots) were separated and run on 2.5% (w/v) agarose gels, as previously described in Chapter 3 in Section 3.3.4.2.2.

5.3.6.2 Detection of the *bla*$_{CTX-M-15}$ gene through a single-plex PCR

Genotypic characterization of ESBL-producers was determined by single-plex PCR assays. The isolates were analysed for the presence of genes targeting *bla*$_{CTX-M}$ group 1 as *bla*$_{CTX-M-15}$ gene, using primers described by Dallenne et al. (2010), as shown in Table 5.3.

Table 5.3 Gene target, primer sequences and amplicon sizes for *bla*$_{CTX-M}$ group 1 (Dallenne et al., 2010)

Target gene	Primer name	Sequences (5'-3')	Size (bp)
bla$_{CTX-M}$ group 1	CTXM1- F CTXM1- R	TTAGGAARTGTGCCGCTGYA CGATATCGTTGGTGGTRCCAT	688

PCR reactions were performed as described by Dallenne et al. (2010) in a 50 µl reaction mixture which consisted 25 µl (1x) BioMix Red (Bioline), forward and reverse primers (10 pmol/µl) 0.4 µl CTXM1-F primer, 0.2 µl CTXM1-R primer, Water Molecular Biology Reagent (W4502, Sigma-Aldrich) and to 1 µl (20-30 ng) of template DNA.

The amplification protocol of *bla*$_{CTX-M}$ group 1 gene and amplified products on agarose gel electrophoresis were as previously described in Chapter 3 in Section 3.3.6. *E.coli* (NCTC 13353) was used as a positive control for *bla*$_{CTX-M-15}$ (group 1), and *E. coli* (NCTC 10418) as an ESBL-negative control.

5.3.7 Statistical analysis

Evaluation of data was performed with IBM SPSS Statistics 22 and Excel 2013. Total *E. coli* was expressed as mean ± SD (CFU ml^{-1}) at the different hours (0, 0.5, 1, 2, 3, 4.5, 6 and 8) for each turbidity level. Significant differences between freshwater and seawater at three levels of turbidity were identified using non-parametric test (Kruskal-Wallis one-way ANOVA) because of non-normal distribution. *P*-values <0.05 were considered statistically significant. Mann-Whitney *U* test was used to compare two groups (seawater and freshwater) in low, medium and high turbidity levels over different time exposures and a significance level of $P<0.05$.

5.4 Results

5.4.1 Characterization of water

Physiochemical characteristics of water samples used in the study are displayed in Table 5.4.

Table 5.4 Chemical characteristics in the studied area, located in seawater (Menai Strait) and freshwater (river Conwy)

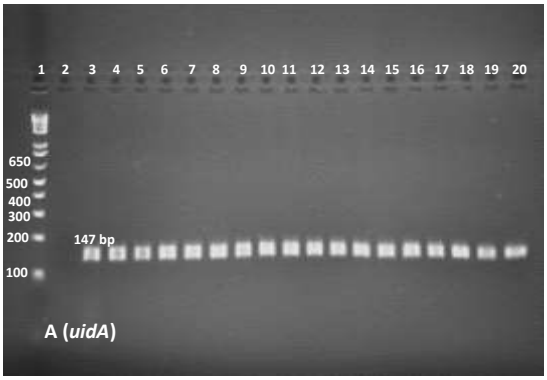
Parameter	Sample (mean ± SEM)	
	Seawater	Freshwater
pH	7.92 ± 0.04	7.41 ± 0.09
Electrical conductivity (EC) mS cm^{-1}	40.43 ± 0.09	0.10 ± 0.01
Total organic carbon (TOC) mg l^{-1}	4.47 ± 0.6	5.92 ± 1.1
Total organic nitrogen (TON) mg l^{-1}	0.57 ± 0.19	1.42 ± 0.19
Nitrate (NO_3^-) mg l^{-1}	0.2 ± 0.04	0.89 ± 0.01
Phosphate (PO_4^{3-}) mg l^{-1}	0.04 ± 0.03	0.02 ± 0.01

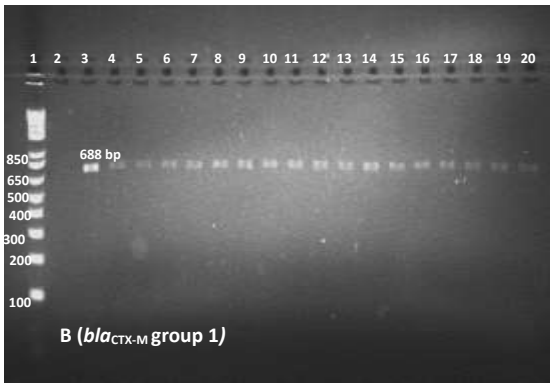
The average of pH in seawater and freshwater were 7.92 and 7.41, respectively (Table 5.4), showing within the standard scale (sea pH; 7.5-8.4 and river pH; 6.5-8.5) (CWT, 2004). Electrical conductivity was higher in seawater than freshwater. Nitrate, total organic carbon (TOC) and total organic nitrogen (TON) concentrations were higher in freshwater than seawater and phosphate concentration was slightly higher in seawater than freshwater. The results of pH, EC, nitrate, phosphate, TOC and TON are shown in Table VIII, Table IX, Figure IX, Figure X, Figure XI and Figure XII, respectively in the Appendix.

5.4.2 Confirmation of *E. coli* and their $bla_{CTX\text{-}M\text{-}15}$ genes

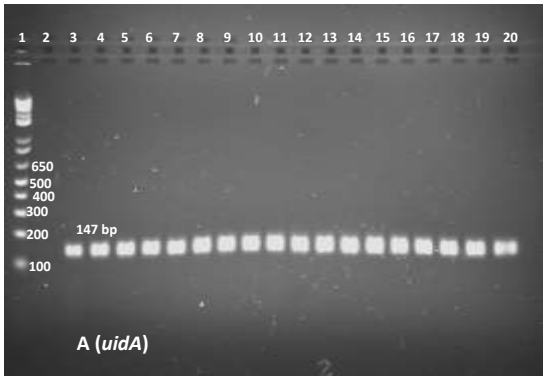
Over the whole study period, the results showed that 241 isolates (seawater *n* = 115 and freshwater *n* = 126) were confirmed as $bla_{CTX\text{-}M\text{-}15}$ *E. coli* by oxidase test and PCR, as shown for some isolates for seawater and freshwater in Figure 5.6 (A) *uidA* and (B) $bla_{CTX\text{-}M}$ group 1

Seawater





Freshwater



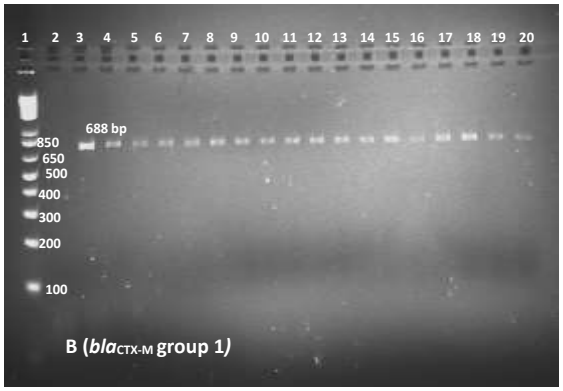


Figure 5.6 Single PCR assay for (A) *uidA* and (B) *bla*$_{\text{CTX-M-15}}$ genes in *E. coli* isolates from sea and freshwater samples, Lanes: **1**, Marker 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific); **2**, control negative (distilled water for *uidA* and *E. coli* NCTC 10418 for negative *bla*$_{\text{CTX-M-15}}$): 3, control positive as *E. coli* NCTC 13353 for *uidA* and *bla*$_{\text{CTX-M-15}}$ genes; **3** to **20** positive *E. coli* strains

5.4.3 Influence of turbidity on survival of *bla*$_{CTX-M-15}$-producing *E. coli*

Result showed that ESBL-producing *E. coli* were not present in the raw samples of waters and sediments before inoculation. After inoculation and UV exposure, mean counts of *bla*$_{CTX-M-15}$-producing *E. coli* between low, medium and high turbidity were significantly different in both types of water (Kruskal-Wallis one-way ANOVA, $P<0.05$) as illustrated in Figure 5.7.

Over the course of the experiment, the number of *E. coli* recovered from the microcosms in seawater and freshwater declined over time. In low turbidity, numbers of *E. coli* were reduced completely (zero CFU ml^{-1}) at $T_{4.5}$ in seawater, while in freshwater, *E. coli* were reduced below the detection limit at T_8, but were positive by enrichment. In water of medium turbidity, *E. coli* in seawater were reduced to below the detection limit at T_8, but 2.58 log CFU ml^{-1} were still recovered in freshwater at T_8. In water of high turbidity, the number of *E. coli* in both sea and freshwater were reduced to 2.38 and 5.39 log CFU ml^{-1}, respectively.

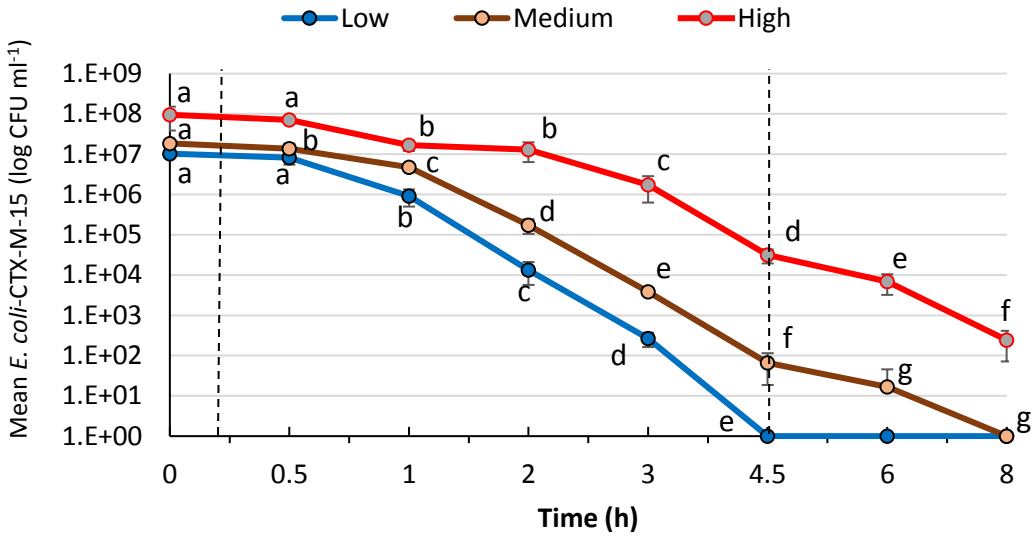
In water of low turbidity, the mean number of *bla*$_{CTX-M-15}$-producing *E. coli* did not significantly reduce between time T_0 and $T_{0.5}$ (Mann-Whitney test, $P>0.05$) in seawater (from 7.01 to 6.91 log CFU ml^{-1}), but in freshwater, there was a significant reduction ($P = 0.065$) (from 7.43 to 7.17 log CFU ml^{-1}). From $T_{0.5}$ to $T_{4.5}$, the number of *bla*$_{CTX-M-15}$-producing *E. coli* significantly decreased in seawater (zero log CFU ml^{-1}) ($P<0.05$). However, from T_1 to $T_{4.5}$ in freshwater, there was a significant decrease (from 6.74 to 2 log CFU ml^{-1}), then between $T_{4.5}$ and T_8, the decrease in numbers were not statistically significant (1.22 and zero log CFU ml^{-1}, respectively).

At medium turbidity, the mean number of *bla*$_{CTX-M-15}$-producing *E. coli* in the seawater was reduced significantly between T_0 and T_6 (from 7.27 to 1.22 log CFU ml^{-1}) ($P<0.05$), but from T_6 to T_8, there was no significant change ($P>0.05$). In freshwater, the mean number were significantly reduced from $T_{0.5}$ to T_2 (from 7.78 to 6.53 log CFU ml^{-1}) and T_3 to $T_{4.5}$ (from 6.29 to 4.09 log CFU ml^{-1}) ($P<0.05$).

At the higher levels of turbidity, the UV radiation did not significantly affect numbers of *E. coli* in seawater between T_0 to $T_{0.5}$ (from 7.98 to 7.85 log CFU ml^{-1}) and T_1 to T_2 (from 7.23 to 7.11 log CFU ml^{-1}) (Mann-Whitney test, $P>0.05$) but from $T_{0.5}$ to T_1 and T_2 to T_8, there was significant difference ($P<0.05$). In freshwater, there were no major differences from T_0 to T_3 in survival (from 7.99 to 7.53 log CFU ml^{-1}) ($P>0.05$) but from T_3 to T_8, the means were significantly different ($P<0.05$) (from 7.53 to 5.39 CFU ml^{-1}).

Results observed (Figure 5.7 in dash lines) that the UV radiation for simulated winter exposure (14 min) did not completely eliminate *bla*$_{CTX\text{-}M\text{-}15}$-producing *E. coli* at all turbidity levels in both waters. However, in summer (4.5 h), the UV exposure significantly reduced numbers of *bla*$_{CTX\text{-}M\text{-}15}$-producing *E. coli* in low (100%), medium (99.99%) and high turbidity (99.97%) for seawater, and low (99.99%), medium (99.98%) and high turbidity (85.24%) for freshwater.

A. Seawater (Menai Strait)



B. Freshwater (river Conwy)

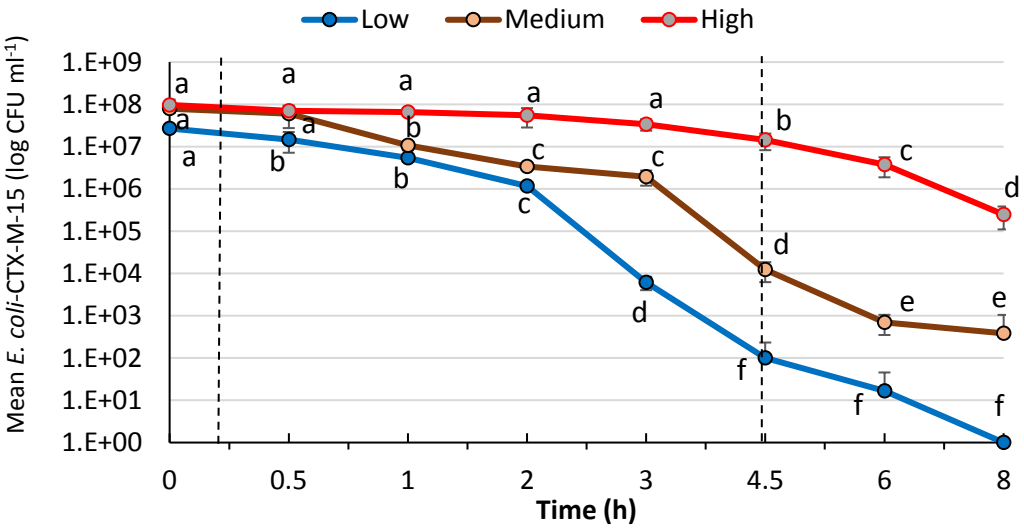


Figure 5.7 Mean (CFU ml^{-1}) (± SD; *n*=3) of *bla*$_{\text{CTX-M-15}}$-producing *E. coli* in (A) seawater and (B) freshwater recovered from the different levels of turbidity (Low, Medium and High turbidity) following different durations of exposure to UV radiation. The dashed lines represent the average of long time exposure in winter (14 min) and summer (4.5 h). Letters denote the significant differences (Mann-Whitney *U* test, *P* <0.05)

5.5 Discussion

Several published studies indicate that WWTPs play an important role in releasing ARB and their genes to the water environments (Wellington et al., 2013; Gao, Munir and Xagoraraki, 2012; Novo et al., 2013; Rizzo et al., 2013). A study by Amos et al. (2014) found that river sediments collected from downstream of WWTP were carrying ARB with *bla*$_{\text{CTX-M-15}}$ genes. A study in China found that river sediment was as rich reservoir of ESBL-producing *Enterobacteriaceae* (Lu et al., 2010). Karbasdehi et al. (2017) confirmed that seawater sediments were a reservior of pathogenic bacteria. Lawler et al. (2006b) and Pachepsky and Shelton (2011) confirmed that suspended sediments or turbidity is one of the most important factors in determining bacteria survival in the water environment, especially in high sediment concentrations.

Although numerous studies have examined sediments with ARB in environmental waters (Lu et al., 2010), it is not clear how sediment concentrations or turbidity affect survival (Pachepsky & Shelton, 2011). To the best of our knowledge, this study represents the first report of the effects of water turbidity levels in sea and freshwater on the survival of *bla*$_{\text{CTX-M-15}}$-producing *E. coli* under simulated winter and summer UV exposure.

Although the inclusion of no-sediment and no-sunlight controls (and the absence of both variables) would have added to the depth of understanding as to the relative importance of these variables on the survival of ESBL-producers in such waters, the aim was to imitate real conditions, where at least low levels of sediment would be expected, as would exposure to some sunlight (during daytime release of from a WWTP or STP). However, the limitations of this study were missing experimental controls within water samples such (i) without UV exposure, (ii) without sediments added, (iii) without sediments and without UV exposure under the dark condition. It would also have been of value to compare the survival of *E. coli* ESBL with non-ESBL producing *E. coli*, to determine whether non-ESBL producers can be used as a proxy for the survival of their ESBL-producing counterparts. We acknowledge that recovery may have been affected by the aggregation of cells after inoculation, and could test how desorption (e.g. low-energy sonication or applying a mild detergent to break down aggregates and flasks of cells) before plating or to apply direct microscopic methods (labelling, dead-alive staining, etc.) would have impacted results.

This study showed that suspended sediment concentrations influence the survival of *bla*$_{\text{CTX-M-15}}$-producing *E. coli* in both waters exposed to UV. In particular, survival was enhanced under

higher turbidity. These observations are concordant with those of Perkins et al. (2016), which demonstrated attachment of bacteria in highly turbid water can contribute to enhanced survival. Another study verified that differential turbidity levels in water are able to protect bacteria from sunlight (Lawler et al., 2006b). A study was conducted by Walters et al. (2014) to assess the impact of suspended sediment concentrations in river water and the UV inactivation of faecal coliforms. They found that there was relationship between sediment concentration and UV inactivation of faecal coliforms; i.e., increased sediment concentration reduced UV inactivation of faecal coliforms, concluding that UV exposure cannot reduce number of faecal coliforms in water environments when sediment concentration exceeded 100 mg l^{-1} (Walters et al., 2014). This finding is consistent with our study that survival of *bla*$_{CTX\text{-}M\text{-}15}$-producing *E*. *coli* in seawater and freshwater were increased with greater suspended sediment loads. At lower turbidity, *E. coli* were exposed to higher levels of UV radiation which probably led to damage in DNA by changing the position of the nucleotide acid on DNA stand (Ben Said et al., 2010). Thus, this bacteria cannot grow easily on usual media (Ding et al., 2016). In some cases, we did, however, find *E. coli* after enrichment of the water samples. This may be because when providing enrichment media, they can regain normal metabolic activity and have ability to become culturable once resuscitated (Pienaar et al., 2016). It must be noted that many pathogens can enter in environmental waters at levels below the detection limits of culturing. Ramamurthy et al. (2014) observed that the non-culturable pathogens have become a serious problem to public health due to their non-detectability via routine methods. Further work is needed to investigate non-culturable bacteria pathogens and activity over a longer period of UV exposure using advance detection techniques such as qPCR. Such a culture-independent method would also help ascertain whether *bla*$_{CTX\text{-}M\text{-}15}$ genes remain viable even if the *E. coli* have been eliminated.

The UV exposure times tested were based on the findings of a previous study in north Wales by the Centre for Ecology & Hydrology. We found that the 14 min of UV exposure dose as simulated in winter time had little impact on the survival of *bla*$_{CTX\text{-}M\text{-}15}$-producing *E. coli* in all levels of water turbidity in both sea and freshwater. However, during simulated summer time, a significant reduction in survival of *bla*$_{CTX\text{-}M\text{-}15}$-producing *E. coli* was seen in all levels of water turbidity from both waters. This finding is consistent with the findings of others who have reviewed literature and report that the survival of faecal coliforms in sediment water was

higher in winter than summer (Pachepsky & Shelton, 2011). In our study, it was not possible to reduce the temperature of Suntest instrument below 15°C, because it depends on different ambient conditions. It is well-known that increased temperature affects bacterial survival. Nevertheless, more work is needed to investigate how mean seasonal temperature impact on survival in different water–sediment concentrations.

A study by Muela et al. (2000) measured the survival of *E. coli* in sterile river water during exposure to UV-A. They found that after the exposure to 14 kJ m^{-2}, a 10-fold decrease in *E. coli* was detected after only 24 hours. In contrast, our study found CFUs dropped by the factor of 10^8 within T_8 hours. It might be the stress, which *E. coli* is imposed to after growing in a very rich medium at 37°C and being transferred into nutrient-poor, colder environment, accelerates its death. This may mean the bacterium is at a competitive disadvantage to other microbes. Repeating the study using sterile, as well as unsterilised, samples would indicate the relative importance of microbial interactions compared to sediment and UV effects.

Although only one sample of each was used, this study showed that survival of *bla*$_{\text{CTX-M-15}}$-producing *E. coli* was longer in freshwater than in seawater. This is probably due to seawater is containing higher amounts of chemical elements and total dissolved solids as chlorine, sulphate, sodium, magnesium, calcium, and potassium (Talley et al., 2011); that could affect survival of bacteria (Pellerin et al., 2018; Tian et al., 2014). A review by Rozen and Belkin (2001) showed that antibiotics produced by microorganisms, parasitic predation and viral phage in marine environment can also cause a negative effect of survival. Other factors, e.g., levels of nutrient such as nitrate, carbon and nitrogen play a major part in survival of bacteria in water (Malham et al., 2014), and in this study, nutrient levels were higher in freshwater. Further study is needed to exanimate how chemical and biological factors impact on survival of *bla*$_{\text{CTX-M-15}}$-producing *E. coli* in freshwater and seawater derived from a WWTP.

5.6 Conclusions

The most important finding of this research was that sediment concentration might be a key issue for survival of ARB in environmental waters such sea and freshwater. This study found that higher sediment concentrations increased survival of *bla*$_{\text{CTX-M-15}}$-producing *E. coli*. Association with particle sediment might enhance the preservation of bacteria from UV exposure. Freshwater provided greater conditions for survival of *bla*$_{\text{CTX-M-15}}$-producing *E. coli*,

which might be due elevated nutrient concentrations or other biological or physiochemical factors. Looked at in isolation, typical UV exposure for wintertime did not eliminate *bla*$_{CTX\text{-}M\text{-}15}$-producing *E. coli*. Further investigation would help determine the relative importance (combined, and in isolation) of chemical and biological factors influence survival in environmental waters derived from wastewater.

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Chapter 6: General discussion

6.1 General discussion

As seen in Chapter 1, this thesis attempted to achieve a number of specific goals:

1. To perform a review of existing literature on antibiotic resistant bacteria (ARB), especially ESBL-producing *Enterobacteriaceae*, and their genes, prevalence and persistence within a wastewater treatment plant (WWTP) and following release in the water environment, and factors that influence survival and transport.
2. To further our knowledge and understanding of ARB in WWTP. For example, how demographic changes of human population may affect the numbers and diversity of ESBL-producing *Enterobacteriaceae* into and out of a WWTP. How factors such as sediment concentrations might affect survival in sea and freshwater after released by WWTP.
3. Finally, at the end of this chapter, to identify the areas related to ESBL-producing *Enterobacteriaceae* that warrant further research.

As is clear from Chapter 2, ARB as ESBL-producing *Enterobacteriaceae* have been increasing very rapidly in both clinical and environment settings over the last decade, and has been the focus of many studies. This has significantly improved our understanding of these *Enterobacteriaceae* and ESBL genes, such as $bla_{\text{CTX-M}}$ groups, bla_{SHV}, bla_{TEM} and bla_{OXA} across many countries. The work conducted within this PhD has shed light on the fate of these strains and their genes during treatment at a tertiary WWTP and following their release into fresh or marine water. By integrating the discoveries with those from existing literature, we have a clearer illustration of the role of WWTP in the spread of ESBL-producing *Enterobacteriaceae* in the environment.

The experimental work fulfilled our initial aims and objectives (Chapter 1). Here, we discuss the suggestions of our findings:

Previous researches have shown that WWTP are a main point for releasing ESBL-producing *Enterobacteriaceae* in the environment (Bréchet et al., 2014), and this study has further highlighted the potential for WWTP to lead to contamination of the wider environment with ESBL-producing *Enterobacteriaceae*. In Chapter 3 (experiment 1), we saw that mean presumptive *E. coli* cells were higher than other faecal coliforms (OFCs) (e.g. *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp. and *Citrobacter* spp.) at the four sites of the WWTP (effluent, primary sediment tank, aeration tank and effluent). This might be due to the prevalence of *E.*

coli within human faeces (Perkins et al., 2014). We found that Bangor's wastewater treatment plant eliminated about 99% of mean *E. coli* and OFCs, similar to that reported elsewhere (Mounaouer and Abdennaceur, 2015). We also interpreted the numbers of ESBL in OFCs was higher than in *E. coli*, which may reflect the wide range of species classed within OFCs as opposed to just *E. coli*. We found the most frequent ESBL gene among *E. coli* was $bla_{\text{CTX-M}}$ group 1, while ESBL-producing OFCs most frequently harboured bla_{SHV}. Importantly, it highlights that although treatment of wastewater significantly reduced counts of indicator bacteria from the influent to effluent stages, the process does not completely eradiate such bacteria and therefore we estimate that 300 billion each of both ESBL-producing *E. coli* and OFCs enter the water environment per day through the effluent released from this WWTP.

The findings of Chapter 4 (experiment 2) highlight how large demographic changes (in this case, the arrival of overseas students) can influence the inputs and outputs of WWTP and the presence of ESBL-producing bacteria. These arrive in a short space of time and from across the world, therefore the local WWTP is likely to be subject to sudden and considerable changes in microbial inputs. Bangor is a relatively small city, of which a significant proportion of the population are students. However, what is less clear is which demographic changes factor might be the key rising of ARB in the environment. The findings of this study indicated the effect of a dilution factor by heavy rainfall reducing counts of such bacteria found per ml sampled, corroborating the results of others (Lucas et al., 2014). A number of *E. coli* before welcome week were ESBL-producers, and a similar number were found after students had arrived. However, in OFCs, the number of ESBLs increased after the arrival of students. However, the diversity of *bla* resistance genes was increased genes (in both *E.coli* and OFCs) after the arrival of students. We also found the new genes were detected after arrival of students; with $bla_{\text{CTX-M-14,}}$ ($bla_{\text{CTX-M-15}}$ + $bla_{\text{OXA-1}}$) and ($bla_{\text{CTX-M-27}}$ + $bla_{\text{OXA-1}}$) found in *E. coli* and as ($bla_{\text{TEM-1}}$ + $bla_{\text{SHV-2}}$), ($bla_{\text{TEM-19}}$ + $bla_{\text{SHV-12}}$ + $bla_{\text{OXA-1}}$), ($bla_{\text{TEM-120}}$ + $bla_{\text{SHV-12}}$ + $bla_{\text{OXA-1}}$) and $bla_{\text{SHV-12}}$ found in OFCs, corroborating similar results found by others that movement people around the world may increase ARB and their genes in the environment (Barlam & Gupta, 2015). The $bla_{\text{CTX-M-15}}$ gene was predominate in *E. coli*, while $bla_{\text{SHV-12}}$ and $bla_{\text{TEM-1}}$ were more frequently genes in OFCs.

However, it is not clear what variables influence the survival of these strains in the water environment, post release from the WWTP. In Chapter 5 (experiment 3), we identified that

particle sediment concentrations may aid their survival, such as what would be encountered following storm events. The ability of ESBL-producing *E. coli* to attach with sediments in water is well established (Karbasdehi et al., 2017; Lawler et al., 2006; Malham et al., 2014). However, what is ambiguous is how sediment concentration might regulate ARB survival. The work sheds light on this as it showed the notable link in survival of *bla*$_{CTX\text{-}M\text{-}15}$-producing *E. coli* and concentrations of sediment in both fresh and sea water. We also illustrated the increase survival of *bla*$_{CTX\text{-}M\text{-}15}$-producing *E. coli* in water of greater sediment loads might be due to greater protection from UV radiation (Lawler et al., 2006). Furthermore, the characterisations of freshwater were of greater importance in long survival of *bla*$_{CTX\text{-}M\text{-}15}$-producing *E. coli* periods because of available nutrients as nitrate, carbon and nitrogen. We showed that the average time of UV exposure in winter in north Wales might be insufficient to ensure complete disinfection of this strain at three levels of water turbidity. Under simulated summer conditions, *bla*$_{CTX\text{-}M\text{-}15}$-producing *E. coli* numbers were significantly reduced at all levels of water turbidity in both waters, except in freshwater of high turbidity, where low numbers were still recovered after 4.5 h of UV exposure.

6.2 Overall conclusion

The experimental work in this thesis demonstrate that demographic changes might increase the diversity of ESBL-producing *Enterobacteriaceae* in Bangor's tertiary WWTP. Furthermore, a small number of these strains are likely to be released to the environment through treated wastewater. Reviewing the regulation or procedures for bacterial removal from wastewater is needed to reduce the survival of these microorganisms in the wider environment. It is obvious that ESBL-producing *E. coli* can become associate with sediments, especially during storm events (high turbidity), and that this can contribute to such stains surviving a long period into water environment. The existence of ESBL-producing *Enterobacteriaceae* in the environment might pose risks to human and animal health.

6.3 Further work

Further research and recommendation are required as follows:

- There is a need to develop ways to eradicate ESBL-producing *Enterobacteriaceae* in effluent by stricter regulations and higher levels of treatment.
- There is a need to better understand how factors such as turbidity and rainstorm event influences the effectiveness of treatment.
- Using a molecular approach, it would also be of value to better understand how UV irradiation impacts on the genetic material of these microorganisms, whether there is any repair of damaged DNA, and regrowth
- Research into how ESBL-producing *Enterobacteriaceae* transfer resistance genes to other bacteria through horizontal gene transfer or genetic mutation is needed, and how this might be influenced by WWTP operating conditions and degree of treatment.
- Further studies are needed to understand how the diversity of resistance genes entering and exiting a WWTP can change with demographic changes and how sources such as hospital wastewater might increase ARB and their genes.
- The impact of the size of sediment on the spread ARB in water requires clarifying. Similarly, how other environmental variables (e.g. temperature and nutrient levels) affect the survival and transport of ESBL-producing *Enterobacteriaceae* would be of value.
- Further study is required to better understand the survival and decay of $bla_{CTX\text{-}M\text{-}15}$-producing *E. coli* following release from wastewater treatment plants. Such work could study the influence of wave effects on dispersal and seasonal effects (e.g. weaker UV during winter) (Hassard et al., 2016), depth, dilution effects during storm events and the impacts of salinity on survival.

6.4 References

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Appendix

Influent (raw wastewater)

Primary Sediment Tank

Aeration Tank

Effluent (post UV radiation)

Figure I Sample sites of Treborth wastewater treatment plant were collected

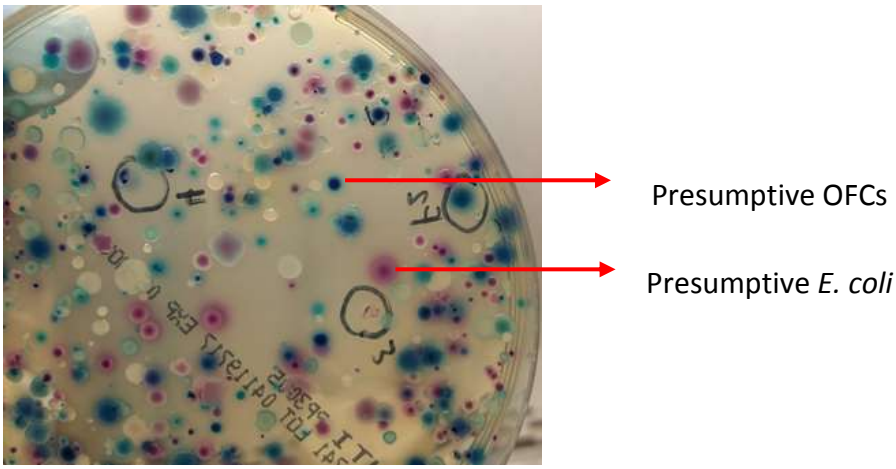
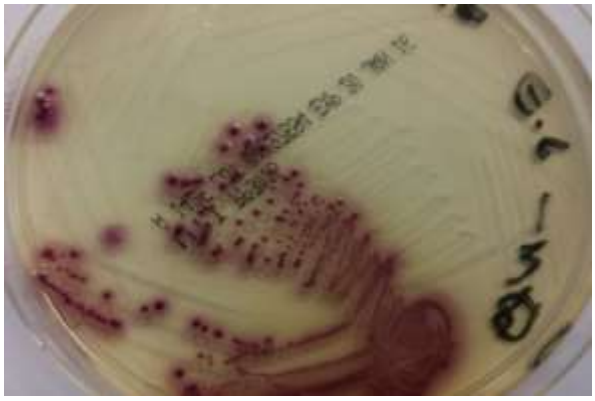


Figure II Presumptive other faecal coliforms (OFCs) and *E. coli* cultured over Chromogenic Primary UTI agar medium (PP3005; E&O Laboratories Ltd.)



E. coli – CTX-M- 15 (13353 NCTC)

Enterobacter aerogenes (10006 NCTC)

K. pneumoniae -SHV-18 (13368 NCTC)

Figure III Control strains of *E. coli* and OFCs (e.g. *Klebsiella pneumonia* and *Enterobacter aerogenes*) obtained from the National Collection of Type Cultures (NCTC), cultured over Chromogenic Primary UTI agar medium

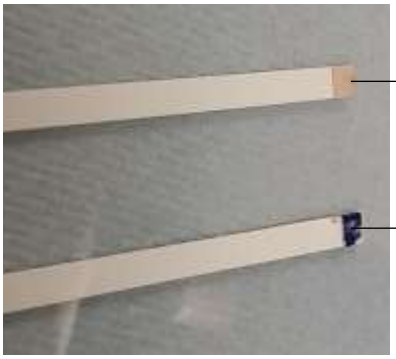


Figure IV Detection of the cytochrome oxidase enzyme activity (oxidase-positive showed dark purple blue and oxidase-negative showed no colour change)

A Water samples

A Sediment sample

B Water samples

B Sediment sample

Figure V Water and sediment sampling sites were collected from near WWTP at (A) Menai Strait and (B) river Conwy

A B

Figure VI (A) Experimental box containing the sediment sample and (B) Oven dry sample of the sediment at 105°C

A) Hanna pH 209 Bench Top Meter **B**) JENWAY 4520 electrical conductivity Meter

C) Spectrophotometer measured NO_3 and PO_4^{3-} **D**) TOC and TOC instrument

Figure VII Instruments measured physio chemical elements

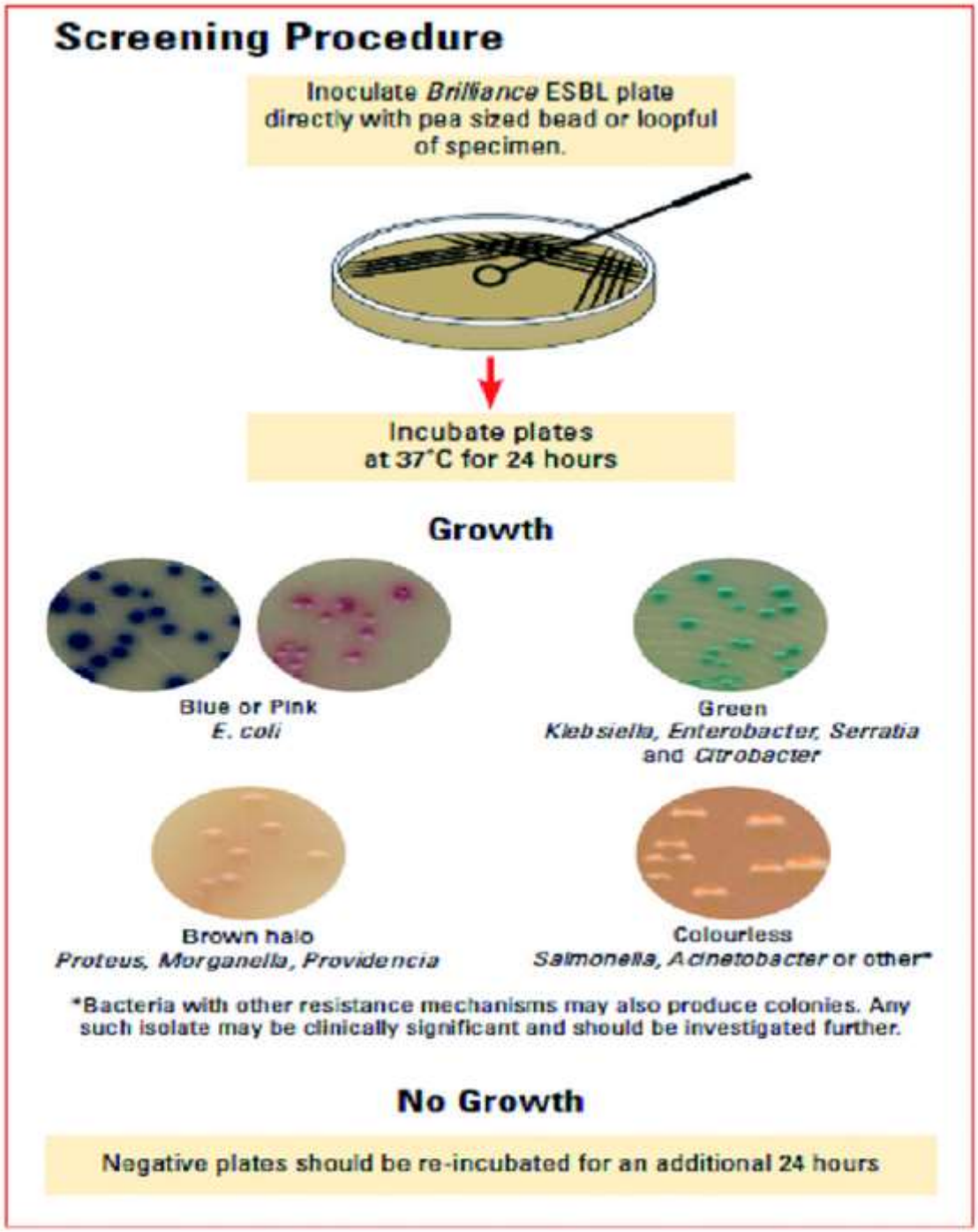


Figure VIII Procedure of Brilliance™ ESBL agar plate for detecting ESBL-producing strains (http://www.oxoid.com/pdf/oxoid-Brilliance-ESBL.pdf)

Table I Mean and Standard Error of Mean presumptive counts of *E. coli* and other faecal coliform (OFCs) at each stage of the WWTP. Percentage value refers to the total platable counts of oranisms at each WWTP site

Location	Organisms	Mean (CFU ml^{-1})	% of organisms
Influent	*E. coli*	276,727	100
	OFCs	199,727	100
Primary Sediment Tank	*E. coli*	163,333	59.0
	OFCs	76,061	38.1
Aeration Tank	*E. coli*	178,576	64.5
	OFCs	82,394	41.6
Effluent	*E. coli*	232	0.08
	OFCs	132	0.07

Table II Results of bacterial isolates over seven times collecting samples and confirmed by oxidase and PCR (*uidA* & *lacZ* genes) tests

Sample site	Organism	Number isolated per sampling event							Total No. isolated
		1st	2nd	3rd	4th	5th	6th	11th	
Influent	*E. coli*	2	8	4	5	6	7	9	41
	OFCs	2	4	6	6	4	5	12	39
Primary Sediment Tank	*E. coli*	2	3	6	8	5	6	10	40
	OFCs	2	3	4	9	5	6	10	39
Aeration Tank	*E. coli*	3	5	4	7	5	6	8	38
	OFCs	3	3	4	5	6	6	14	41
Effluent	*E. coli*	2	2	6	3	7	6	10	36
	OFCs	1	0	6	4	5	6	11	33
Total	*E. coli*	9	18	20	23	23	25	37	155
	OFCs	8	10	20	24	20	23	47	152

Table III Total numbers of international students at Bangor University during 2015/16

Country	Number	Country	Number	Country	**Number**
Afghanistan	2	India	78	Philippines	2
Albania	1	Indonesia	3	Qatar	5
Antigua and Barbuda	3	Iran	9	Russia	5
Argentina	1	Iraq	27	Saudi Arabia	88
Australia	6	Israel	3	Seychelles	2
Azerbaijan	9	Jamaica	14	Sierra Leone	1
Bahamas	27	Japan	10	Singapore	16
Bahrain	57	Jordan	5	South Africa	6
Bangladesh	30	Kazakhstan	1	Sri-Lanka	2
Barbados	2	Kenya	10	St Kitts Nevis	1
Belize	2	Korea Rep	8	St Lucia	2
Bermuda	2	Kuwait	61	St Vincent	1
Bosnia and Herzegovina	1	Lebanon	1	Sudan	11
Botswana	7	Lesotho	2	Swaziland	1
Brazil	10	Libyan AR	10	Switzerland	10
Brunei	11	Liechtenstein	1	Taiwan	13
Burundi	2	Macao	1	Tanzania	6
Cameroon	16	Malagasy Rep	2	Thailand	10
Canada	29	Malawi	13	Trinidad and Tobago	1
Cayman Islands	2	Malaysia	26	Turkey	6
Chile	3	Mauritius	6	UAR	4
China	563	Mexico	1	USA	112
Colombia	10	Mongolia	1	Uganda	10
Congo Peoples Rep	1	Morocco	1	Ukraine	1
Croatia	1	Myanmar (Burma)	1	United Arab Emirates	9
Cuba	1	Namibia	1	Unknown (OS)	1
Dominica	1	Nepal	2	Uzbekistan	4
Ecuador	1	New Zealand	1	Vietnam	18
Ghana	16	Niger	1	Virgin Is Brit	1
Grenada	2	Nigeria	151	Yemen AR	1
Guam	1	Norway	3	Zambia	10
Guyana	5	Oman	3	Zimbabwe	3
Honduras	1	Pakistan	34	Total	**1737**
Hong Kong	52	Papua New Guinea	1		
Iceland	1	Peru	3		

Table IV Results of bacterial isolates collected over eight times at WWTP sites and confirmed by oxidase and PCR (*uidA* & *lacZ* genes), and number of ESBL-producing bacterial isolates detected

Collecting time	Location	Influent		Sediment Primary Tank		Aeration Tank		Effluent		Total	
	Organism	*E. coli*	OFCs	*E. coli*	OFCs	*E. coli*	OFCs	*E. coli*	OFCs	*E. coli*	OFCs
1	Isolates	20	8	18	9	17	7	18	3	73	27
	Confirmed by oxidase and PCR	11	8	13	9	6	7	6	3	36	27
	ESBLs	0	2	1	5	0	1	0	0	1	8
2	Isolates	17	14	14	10	12	16	15	9	58	49
	Confirmed by oxidase and PCR	17	6	14	6	7	7	15	6	53	25
	ESBLs	2	0	3	0	0	1	0	0	5	1
3	Isolates	15	15	15	15	16	18	16	8	62	56
	Confirmed by oxidase and PCR	12	11	13	15	7	17	10	6	42	49
	ESBLs	1	1	0	0	0	0	0	2	1	3
4	Isolates	25	16	24	15	23	16	28	16	100	63
	Confirmed by oxidase and PCR	22	12	22	12	11	15	22	15	77	54
	ESBLs	0	4	0	0	0	0	0	2	0	6
Total Before	Isolates	77	53	71	49	68	57	77	36	293	195
	Confirmed by oxidase and PCR	62	37	62	42	31	46	53	30	208	155
	ESBLs	3	7	4	5	0	2	0	4	7	18
5	Isolates	18	19	19	11	25	17	28	14	90	61
	Confirmed by oxidase and PCR	15	18	8	11	9	17	24	14	56	60
	ESBLs	0	1	0	0	0	1	2	1	2	3
6	Isolates	29	16	27	13	29	13	28	15	113	57
	Confirmed by oxidase and PCR	21	16	19	8	7	10	13	10	60	44
	ESBLs	0	9	0	3	0	0	1	2	1	14
7	Isolates	22	18	29	13	24	16	27	16	102	63
	Confirmed by oxidase and PCR	16	17	25	11	11	15	22	16	74	59
	ESBLs	0	1	0	0	0	2	1	2	1	5
8	Isolates	21	16	24	16	28	13	29	16	102	61
	Confirmed by oxidase and PCR	15	16	11	13	6	12	16	16	48	57
	ESBLs	0	0	0	0	1	4	1	0	2	4
Total After	Isolates	90	69	99	53	106	59	112	61	407	242
	Confirmed by oxidase and PCR	67	67	63	43	33	54	75	56	238	220
	ESBLs	0	11	0	3	1	7	5	5	6	26
Overall	Isolates	167	122	170	102	174	116	189	97	700	437
	Confirmed by oxidase and PCR	129	104	125	85	64	100	128	86	446	375
	ESBLs	3	18	4	8	1	9	5	9	13	44

Table V Mean counts and standard error mean (SEM) of presumptive *E. coli* and OFCs before and after the arrival of students at WWTP sampling sites (based on percentage of confirmatory test)

Organism	Site	Welcome week	Mean (CFU ml^{-1})	Mean (63.7%)	SEM
E. coli	Influent	Before	337,917	215,301	75551
		After	124,778	79,501	24898
	Primary Sediment Tank	Before	273,750	174,418	52460
		After	96,639	61,573	16621
	Aeration Tank	Before	307,917	196,187	36221
		After	230,694	146,985	46700
	Effluent	Before	1,770	1,127	570
		After	3,013	1,920	684
Organism	Site	Welcome week	Mean	Mean (85.8%)	SEM
OFCs	Influent	Before	176,667	151,602	32950
		After	103,778	89,054	31262
	Primary Sediment Tank	Before	100,417	86,170	18040
		After	47,556	40,809	13214
	Aeration Tank	Before	134,167	115,132	25217
		After	85,583	73,441	26278
	Effluent	Before	938	804	479
		After	1,264	1,085	338

Table VI Mean turbidity levels (NTU) of low turbidity (LT), medium turbidity (MT) and high turbidity (HT) of (A) seawater and (B) freshwater before and after adding sufficient amount of sediments to 190 ml of water sample. For seawater LT, 0.0191 g was added, 0.1770 g for MT, and 0.5358 g for HT. For freshwater LT, 0.0091 g was added, 0.0876 g for MT, and 0.2679 g for HT

A. Menai Strait (seawater)

Turbidity	Original turbidity reading			Mean	Overall mean	Post-adjustment of turbidity			Mean	Overall mean
Low	1	2	3			1	2	3		
1-LT	3.72	2.85	3.09	3.22	3.33	11.54	10.77	11.35	11.22	10.97
2-LT	3.78	3.4	3.01	3.40		11.16	10.74	11.27	11.06	
3-LT	3.21	3.46	3.45	3.37		9.35	10.83	11.73	10.64	
Medium										
1-MT	2.26	2.24	1.19	1.90	2.28	143	153	174	156.67	154.33
2-MT	2.16	2.13	2.38	2.22		160	141	180	160.33	
3-MT	3.22	2.03	2.95	2.73		137	152	149	146	
High										
1-HT	2.63	2.17	2.13	2.31	1.79	411	398	406	405.00	409
2-HT	1.73	1.28	1.39	1.47		399	418	431	416.00	
3-HT	1.7	1.47	1.6	1.59		408	406	404	406.00	

B. The river Conwy (freshwater)

Turbidity	Original turbidity reading			Mean	Overall mean	Post-adjustment of turbidity			Mean	Overall mean
Low	1	2	3			1	2	3		
A-LT	0.6	0.77	0.54	0.64	0.65	12.08	12.92	10.1	11.70	12.44
B-LT	0.56	0.82	0.58	0.65		11.81	11.1	13.69	12.20	
C-LT	0.54	0.62	0.78	0.65		10.55	11.91	17.76	13.41	
Medium										
A-MT	0.68	0.89	0.47	0.68	0.58	148	166	153	155.67	155.56
B-MT	0.5	0.39	0.62	0.50		153	146	164	154.33	
C-MT	0.52	0.72	0.43	0.56		146	160	164	156.67	
High										
A-HT	0.55	0.52	0.58	0.55	0.53	409	415	407	410.33	415.22
B-HT	0.43	0.49	0.42	0.45		417	437	386	413.33	
C-HT	0.56	0.7	0.55	0.60		423	417	426	422.00	

Table VII Average time of UV light exposure in winter and summer as measured by the Centre for Econology and Hydrology at Hiraethlyn, north Wales

Mimicking an average winter day at 300-400 nm	
Average in January 2017	38.17 W m^{-2} = J s^{-1} m^{-2} = 38.17 x 3600 h x 4.534 h = 623 kJ h^{-1} = 623 ÷ 18.7 (295-3000nm = 18.7x based on solar radiance in Central Europe) = 33.3 kJ h^{-1} m^{-2}
Average hours of sun per day	4.534 h
Time in Suntest at 40 W m^{-2}	= 40 x 3600 J h^{-1} m^{-2} = 144,000 J h^{-1} m^{-2} = 144 kJ h^{-1} m^{-2} (33.3 kJ /144) = 0.23 h = 14 min
Mimicking an average summer day at 300-400 nm	
Average in June 2017	261.11 W m^{-2} = J s^{-1} m^{-2} = 261.11 x 3600 h x 12.88 h = 12,107,148.48 kJ h^{-1} = 12,107,148.48 ÷ 18.7 (295-3000nm = 18.7x based on solar radiance in Central Europe) = 647 kJ h^{-1} m^{-2}
Average hours of sun per day	12.88 h
Time in Suntest at 40 W m^{-2}	= 40 x 3600 J h^{-1} m^{-2} = 144,00 J hour m^{-2} = 144 KJ h^{-1} m^{-2} (674 kJ /144) = 4.5 h

Physiochemical procedures

1. pH measurement Hanna pH 209 Bench Top Meter

Calibrating the pH meter

The pH meter is calibrated by a two-point method using appropriate buffer standards. If low/acidic values are expected, buffer standards with a pH of 4 and 7 are used; if high/alkaline values are expected, buffer standards with a pH of 7 and 10 are used.

Procedure

Insert the pH electrode into the supernatant and record the pH after 30 seconds.

2. Electrical conductivity (EC) measurement

Calibrating the EC meter

The EC meter is calibrated by standard value1413 µS cm^{-1}.

Procedure

Immerse the probe into the prepared standard. Wait a few minutes for the reading to stabilise display on the screen.

3. Nitrate concentration (NO_3^-) measurement by (Biotek PowerWave XS; microplate spectrophotometer).

Reagent preparation

(1) Dissolve 0.16 g Vanadium Chloride (VCl_3) in 20 ml of 1M HCl. Vortex/shake until solution turns clear blue.
(2) Dissolve 0.02 g N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD) in 20 ml DI H_2O.
(3) Dissolve 0.4g Sulfanilamide in 20 ml 1M hydrochloric acid (HCl).
(4) Store all reagents in the dark at 4°C.

Standard preparation

The Nitrate standard NO_3 (Fluka, 1000 mg l^{-1}) prepare a set of at least six standards ranging from 0 – 10 mg NO_3^--N l^{-1}.

$$\frac{\text{Desired Concentration (mg } l^{-1}\text{) x Flask Volume (ml) Vol}}{\text{Stock Concentration (mg } l^{-1}\text{)}} = \text{ml of stock solution to use}$$

Procedure

(1) Add 100 µl of each standard and sample to a well in the microplate.
(2) Use the multi-channel pipette to add 100 µl of VCl_3.
(3) Add 50 µl of NEDD.
(4) Add 50 µl of Sulfanilamide and mix.
(5) Allow pink colour to develop for 20 min at room temperature.
(6) Read absorbance at 540 nm on the microplate-reader.

Calculations

In Excel, plot a calibration curve with NO^{3-} standard concentrations (x-axis) and absorbance values (y-axis). Apply the equation of sample absorbance values to calculate mg NO_3^--N l^{-1}.

4. Phosphate (PO_4^{3-}) measurement

Reagent preparation

(1) 10% (w/v) ascorbic acid solution: add 1 g of ascorbic acid to 10 ml of distilled water and shake thoroughly until dissolved.
(2) AMES reagent: this makes 500 ml of 0.42 % Ammonium Molybdate (NH4Mo) in 0.5M Sulphuric Acid (H_2SO_4), in a fume hood, carefully and slowly add 14 ml of concentrated H_2SO_4 to ~400 ml of distilled water and make up to 500 ml to make a 0.5 M H_2SO_4 solution. Add 2.1 g NH4Mo and shake vigorously until dissolved, and store at 4°C.

Standard preparation

Prepare a range of standards PO_4^{3-} stock solution (1000 mg l^{-1}) prepare a set of at least six standards ranging from 0 – 16 mg using the following equation:

$$\frac{\text{Desired Concentration (mg } l^{-1}\text{) x Flask Volume (ml) Vol}}{\text{Stock Concentration (mg } l^{-1}\text{)}} = \text{ml of stock solution to use}$$

Procedure

(1) Add 80 µl of standards and samples to each well.
(2) Add 180 µl of AMES reagent to each well, using a multi-channel pipette.
(3) Add 30 µl of 10% ascorbic acid and mix.
(4) Allow the blue colour to develop for 30 min at room temperature.
(5) Read absorbance at 820 nm on the plate-reader.

Calculations

In Excel, plot a calibration curve with PO_4^{3-} standard concentrations (x-axis) and absorbance values (y-axis). Apply the equation of sample absorbance values to calculate mg PO_4^{3-} m l^{-1}.

5. Total organic carbon (TOC) and of total organic nitrogen (TON) measurements by Analytik Jena Multi NC 21005 TOC TN Analyser

Standard preparation

TOC

Prepare a set of at least six standards ranging 0 – 50 mg and a stock solution (1000 mg l^{-1}).

TON

Prepare a set of at least six standards ranging from 0 – 1 mg and a Nitrate Nitrogen stock solution (1000 mg l^{-1}).

	TOC standards				**TN standards**			
	Standard conc mg l^{-1}	Flask Vol (ml)	Stock solution mg l^{-1}	µl of stock solution to add to flask	Standard conc mg l^{-1}	Flask Vol (ml)	Stock solution mg l^{-1}	µl of stock solution to add to flask
Std 1	0.5	20	1000	10	0.25	20	1000	5
Std 2	1	20	1000	20	0.5	20	1000	10
Std 3	5	20	1000	100	1	20	1000	20
Std 4	10	20	1000	200				
Std 5	25	20	1000	500				
Std 6	50	20	1000	1000				

Procedure

(1) Add 1000 µl of standards and samples to each glass vial.
(2) Add 10 µl of of 2M HCl to standards and samples to each glass vial.
(3) Start to run standards and samples according to manufacturer's structures.

Calculations

Make calibration curve by plotting average peak area against known standard concentrations.

Physiochemical results

Table VIII Mean pH of seawater (Menai Strait) and freshwater (river Conwy) samples (A, B and C), and standard error mean (SEM)

Water sample	pH	Mean	SEM
Sea_A	7.93	7.92	0.04
Sea_B	7.85		
Sea_C	7.97		
Freshwater_A	7.58	7.41	0.09
Freshwater_B	7.32		
Freshwater_C	7.33		

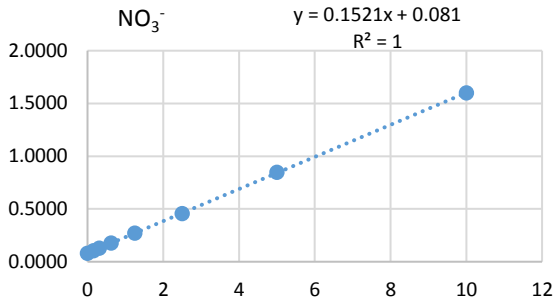
Table IX Mean EC of seawater (Menai Strait) and freshwater (river Conwy) samples (A, B and C), and standard error mean (SEM)

Water sample	EC (mS cm^{-1})	Mean (mS cm^{-1})	SEM
Sea_A	40.6	40.43	0.09
Sea_B	40.3		
Sea_C	40.4		
Freshwater_A	0.0917	0.10	0.01
Freshwater_B	0.1065		
Freshwater_C	0.089.8		

A

Standard concentration		Value
Blank	0	0.0810
Std2	0.15625	0.103
Std3	0.3125	0.128
Std4	0.625	0.177
Std5	1.25	0.27
Std6	2.5	0.455
Std7	5	0.848
Std8	10	1.6

B



C

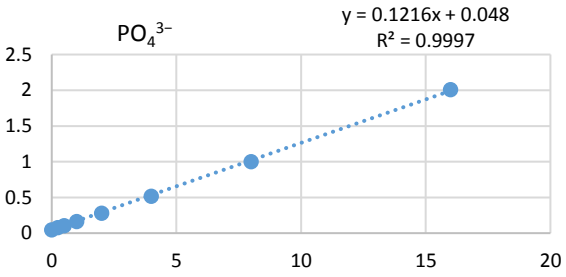
Water sample	Value	NO_3^- (mg l^{-1})	Mean (mg l^{-1})	SEM
Sea_A	0.124	0.28	0.20	0.04
Sea_B	0.101	0.13		
Sea_C	0.109	0.18		
Freshwater_A	0.216	0.89	0.89	0.01
Freshwater_B	0.219	0.91		
Freshwater_C	0.216	0.89		

Figure IX (A) standard concentrations (B) calibration curve and (C) mean and standard error mean (SEM) of NO_3^- in seawater and freshwater samples

A

Standard concentration		Value
Blank	0	0.048
Std1	0.25	0.076
Std2	0.5	0.105
Std3	1	0.161
Std4	2	0.279
Std5	4	0.519
Std6	8	1.002
Std7	16	2.009

B



C

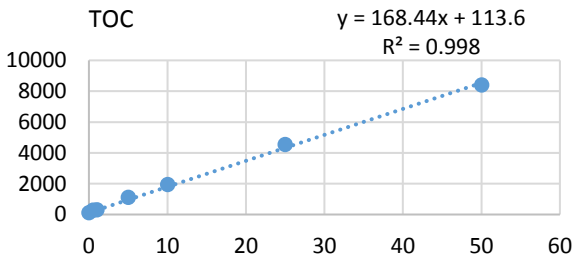
Water sample	Value	PO_4^{3-} (mg l^{-1})	Mean (mg l^{-1})	SEM
Sea_A	0.059	0.09	0.04	0.03
Sea_B	0.049	0.01		
Sea_C	0.051	0.02		
Freshwater_A	0.049	0.01	0.02	0.01
Freshwater_B	0.052	0.03		
Freshwater_C	0.051	0.02		

Figure X (A) standard concentrations (B) calibration curve and (C) mean and standard error mean (SEM) of PO_4^{3-} in seawater and freshwater samples

A

Standard concentration		Value
Blank	0	113.6
Std1	0.5	274.32
Std2	1	309.34
Std3	5	1107.26
Std4	10	1937.57
Std5	25	4527.97
Std6	50	8389.44

B



C

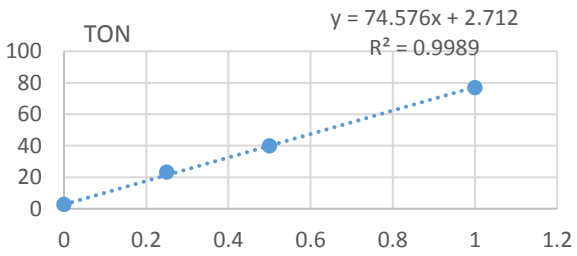
Water sample	Value	TOC (mg l^{-1})	Mean (mg l^{-1})	SEM
Sea_1	184.62	4.22	4.47	0.60
Sea_2	208.165	5.61		
Sea_3	173.96	3.58		
Freshwater_1	189.815	4.52	5.92	1.10
Freshwater_2	250	8.10		
Freshwater_3	199.98	5.13		

Figure XI (A) the standard concentrations (B) calibration curve and (C) mean and standard error mean (SEM) of TOC in seawater and freshwater samples

A

Standard concentration		Value
Blank	0	2.71
Std1	0.25	23.13
Std2	0.5	39.93
Std3	1	76.88

B



C

Water sample	Value	TON (mg l^{-1})	Mean (mg l^{-1})	SEM
Sea_A	9.551	0.92	0.57	0.19
Sea_B	6.6095	0.52		
Sea_C	4.7825	0.28		
Freshwater_A	11.462	1.17	1.42	0.19
Freshwater_B	16.0015	1.78		
Freshwater_C	12.358	1.29		

Figure XII (A) the standard concentrations (B) calibration curve and (C) mean and standard error mean (SEM) of TON in seawater and freshwater samples