Prevalence of plasmid genetic elements among ESBL-producing *E. coli* isolated from a UK river and the effects of waste water effluent release

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Abstract

The global antibiotic resistance crisis is a growing issue which poses a significant threat to human and animal health, and will limit the effectiveness of infection treatment in clinical settings. Traditionally research efforts have concentrated on discovering novel antibiotics, however an alternative approach is being utilised which focuses on the control and removal of antibiotic resistance from the environment. A major source of environmental antibiotic resistance originates from the release of treated waste water effluent (WWE) from local waste water treatment plants (WWTP) into natural aquatic environments, such as canals and rivers. Study has shown released WWE contains high numbers of antibiotic resistant bacteria and residual active antibiotics. In particular members of the *Enterobacteriaceae* family expressing extended spectrum beta-lactamases (ESBLs), such as the highly prevalent CTX-M group 1 genes, have been commonly identified in WWE and have been shown to be more prevalent in natural aquatic environments following the addition of WWE. These ESBL genes are commonly plasmid borne, and their prevalence has been attributed to their ability to be rapidly disseminated by horizontal gene transfer. Once spread through the bacterial population, antibiotic resistance plasmids are maintained by the selective pressure of high levels of antibiotics, introduced to the environment by WWE. This study aimed to identify the effect of WWE addition on the ESBL expressing *Escherichia coli* (ESBLEC) population of natural aquatic environments, and the genetic elements found on plasmids extracted from ESBLEC isolates. ESBLEC were isolated from the Sincil Dike, Lincoln, UK upstream, at the point of and downstream of WWE release. Plasmid DNA was then extracted from these isolates and the genetic elements they contained identified by PCR, CTX-M group 1, 3 addiction systems (Hok-Sok, PemK and ccdAB) and 3 incompatibility groups (IncF, IncN and IncP) were detected. This data was arranged into unique plasmid genotypes which were used to determine the effect of WWE addition on the individual genetic elements identified, and on the ESBLEC plasmid population as a whole. It was found that the number of ESBLEC isolated increased at sample sites that had received WWE and was attributed to the influx of ESBLEC producing organisms previously identified in WWE. Further genetic analysis revealed that the increase in the number of ESBLEC was accompanied by a shift from a few dominant plasmid types to a larger number of genetically diverse plasmids. This diversification event appeared to favour IncF plasmids containing alternate ESBL genes and multiple addiction systems, which allowed their persistence downstream while competing with naturally occurring genotypes. Overall it is clear that the addition of WWE to natural aquatic environments has a measurable impact on the number of ESBLEC and the plasmid genotype they contain. This may have a significant effect on the transfer and maintenance of antibiotic resistance in the environment. A larger study which detects a wider range of plasmid genetic elements across a larger number of sample sites would allow for a deeper insight into the effects of WWE release on plasmid containing ESBLEC in the environment.

Presentations of work

The work herein was present on two occasions:

The first a poster presentation at the 2016 Microbiology Society annual conference as part of the environmental microbiology forum, ACC Liverpool, 23rd March 2016 entitled:

Association of plasmid architecture with antibiotic resistance profiles reveals novel drug targets. Dinatale, A., Crewe, NJ., Goddard, A.

The second a poster presentation at the University of Lincoln postgraduate symposium, Riseholme Campus, Lincoln, 3rd June 2016 entitled:

Association of plasmid architecture with antibiotic resistance profiles reveals novel drug targets. Dinatale, A., Crewe, NJ., Goddard, A.

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Chapter 1 - Introduction

1.1 Antibiotics

1.1.1 The Antibiotic Revolution

Antibiotics can be broadly defined as chemical agents, often of natural origin, which possess the ability to destroy or inhibit the growth of a microorganism (Waksman, 1947). In 1909 the Nobel laureate Paul Ehrlich observed that synthetic dyes used at the time stained only specific organisms. This led to the idea of a "magic bullet", a compound which could exert its full antimicrobial action on the target with no effect to the host. Using this principle Ehrlich began a mass screening effort to discover an antimicrobial compound effective against the sexually transmitted infection syphilis, caused by the bacteria *Treponema pallidium*. This drive resulted in the discovery of a compound known as Salvarsan, an effective treatment for syphilis with far less associated side effects than the popular treatment of organic mercury (Fitzgerald, 1911; Aminov, 2010).

Following the success of Ehrlich, in 1929 Alexander Fleming made the next leap in antibiotic development by discovering the antimicrobial properties of the fungus *Penicillium chrysogenum,* from which the active ingredient of penicillin was purified. This new antibiotic was used to treat infections which previously would have been fatal, however the low activity and narrow spectrum of penicillin, then only available orally, meant it was not suitable to treat the majority of clinical infections. Research efforts turned to the modification of penicillin to create a more effective antibiotic active against a wider range of bacteria, kick starting the antibiotic revolution (Fleming, 1929; Acred *et al*. 1962).

During the mid-20th century research efforts were rewarded with the production of modified penicillins which became available for clinical use to treat previously fatal infections, such as pneumococcal pneumonia (Aldridge, 1999). These were widely used due to their low toxicity and high efficacy; the result of targeting only the components of prokaryotic cells, leaving host eukaryotic cells unaffected. Antibiotics were also easy to produce due to their natural fungal source which could be cultured effectively *in vitro* (Aminov, 2010). In the 20 years following the discovery of penicillin, prontosil, streptomycin, erythromycin and tetracycline were discovered and implemented for use as broad-spectrum antibiotics to treat a variety of infections, many of these are still used today (Lewis, 2013).

However, a multitude of factors has led to the abuse of virtually all of our antibiotics, complacency in prescribing combined with a lack of understanding by patients has led to their inappropriate use in millions of cases. A significant factor in the overuse of antibiotics came in America during the 1950s, when antibiotics were approved for the use as a growth promoter in cattle, a trend which quickly spread to commercial agriculture around the world (Gustafson and Bowen, 1997; Gross and Patel,

2007). This resulted in an inevitable decrease in their effectiveness due to overexposure and the eventual development of resistance by the target bacteria. In the same decade they were approved for agricultural use, tetracycline, methicillin, penicillin and erythromycin resistance was discovered in naturally occurring bacterial strains for the first time (Lewis, 2013).

In the years since antibiotic resistance first emerged, science has been engaged in a race to develop new antibiotics and slow the spread of resistance in an effort to prevent modern medicine returning to a pre-antibiotic era. Despite these efforts government bodies have been slow to react to this developing crisis, with the use of antibiotics as a growth promoter and their inappropriate use clinically still a major issue today.

1.1.2 Types of Antibiotic

As work on antibiotic modification progressed, many related antibiotics were produced for clinical use, which can be grouped by their method of bacterial inhibition or class. There are two main types of bacterial inhibition, bacteriostatic and bactericidal. Bacteriostatic antibiotics prevent cell replication and halt cell growth to lower the bacterial population within the host, allowing host defences to effectively mobilise and remove the infection. Bactericidal antibiotics act to induce rapid cell death to remove an infection without the use of host defences, typically by inhibiting ribosomal function (Weisblum and Davies, 1968; Pankey and Sabath, 2004; Kohanski *et al*. 2007). Table 1 shows the cellular targets and method of inhibition for some commonly used antibiotics.

Antibiotic(s)	Bacteriostatic/Bactericidal	Method of bacterial inhibition	Cellular Target
Tetracycline	Bacteriostatic	Halt cell metabolism	30s Ribosome (Kohanski *et al*. 2007)
Macrolide Family	Bacteriostatic	Halt cell metabolism	50s Ribosome (Kohanski *et al*. 2007)
Beta-Lactam	Bactericidal	Inhibit cell wall synthesis	DD-transpeptidase (Tomasz, 1979)
Quinolone Family	Bactericidal	Inhibit DNA synthesis	DNA Gyrase (Drlica and Zhao, 1997)

Table 1. Examples of commonly used antibiotics, their mode of action, method of inhibition and cellular target

As most newly implemented antibiotics are modifications of existing antibiotics they can also be grouped based on their common origin, known as a class. The main class of antibiotic used in this study are cephalosporins derived from the fungus *Acremonium spp.*, modification of which led to several new generations of antibiotic with slightly different properties to the previous generation. This structural change resulted in an increase in the spectrum of activity and reduction of resistance when used clinically (Jen *et al*. 1972; Perez-Inestrosa *et al*. 2005). Currently the third generation cephalosporins ceftriaxone, cefoperazone and cefotaxime are commonly used cephalosporins and are being utilised to treat a variety of infections, however resistance to these third generation antibiotics is rapidly growing (Davies, 2011; Bush, 2016; Goudanavar *et al*. 2016).

1.1.2.1 Beta-lactam Antibiotics

The cephalosporins belong to a further class of antibiotic with the penicillins, carbapenems and monobactams known as the beta-lactams, which all share a basic core structure of a nucleated beta lactam ring. Penicillin and all its derivatives contain a 6-aminopenicillanic acid (6-APA) side chain (Figure 1A) (Sheehan and Logan, 1959) while cephalosporin derivatives contain a 7- aminopenicillic acid (7-APA) side chain (Figure1B) (Nagarajan *et al*. 1971), this small change of nucleus location leads to a different class of antibiotic with a different spectrum of activity. Newer generations of beta-lactams have been developed by modifying this side chain to increase effectiveness or reduce resistance in the target organism.
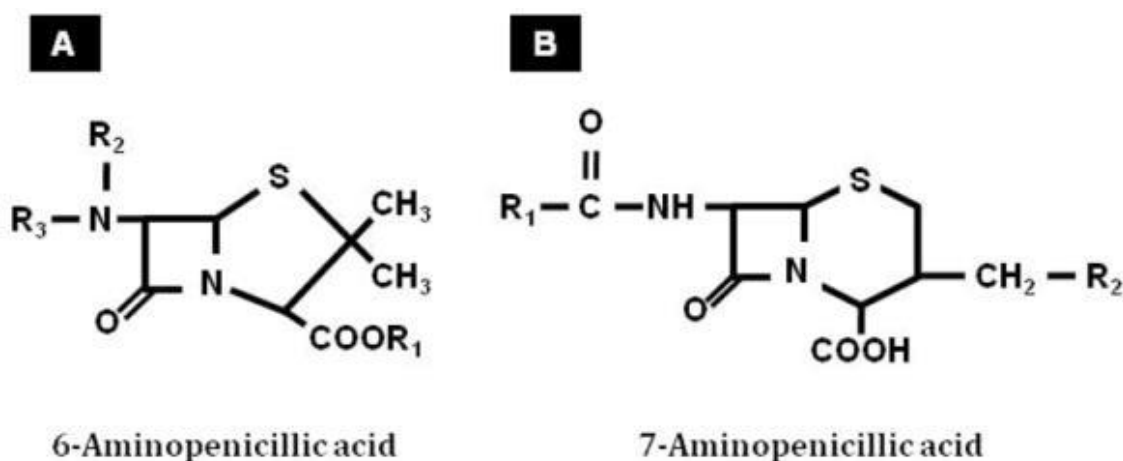
Figure 1. Structure of the lactam ring found at the centre of beta-lactam antibiotics, and the differing location of the aminopenicillic acid side chain (Kong *et al*. 2010)

Beta-lactams are bactericidal antibiotics that interfere with transpeptidase enzymes within the bacterial cell wall to prevent adequate linkage of the peptidoglycan subunits, inhibiting cell wall synthesis and inducing cell death (Wilke *et al*. 2005; Dorr *et al*. 2016). As human cells do not contain

an analogue of transpeptidase, beta-lactam antibiotics have a very low associated toxicity and a wide spectrum of activity. Their use is indicated against both Gram positive and Gram negative organisms, and in a range of infections such as urinary tract infection, soft tissue infection and as a prophylaxis to prevent infective endocarditis (Holten, 2000; Etebu and Arikekpar, 2016). The desirable properties of these antibiotics has led to an explosion in their use clinically, however their abundance has driven selective pressure for organisms which express resistance. The most significant mechanism of resistance to beta-lactams is the presence of the beta-lactamase enzyme which hydrolyses the beta lactam ring at the centre of beta-lactam antibiotics, rendering them inactive (Shaikh *et al*. 2015).

1.2 The Antibiotic Resistance Crisis

1.2.1 The Overuse of Antibiotics

The use of antibiotics has rapidly grown in the years since their introduction, forming a vital tool in the treatment of clinical infection. In theory, antibiotics should only be prescribed for bacterial infections which cannot be resolved by the host immune system, using an appropriate antibiotic at an appropriate therapeutic dose. However, in clinical practice antibiotics are used in bacterial infections that may have otherwise resolved naturally, or for infections that have not been confirmed as bacterial.

This has been supported by recommendations on antibiotic stewardship from the National Institute for Health and Care Excellence (NICE), which state that before prescribing antibiotic treatment, a physician should consider whether pharmaceutical intervention is necessary to improve the patients' prognosis, and prescribing antibiotics should be avoided where possible (NICE, 2015). This is in line with earlier recommendations made by the Infectious Disease Society of America (IDSA), which stated that antibiotic treatment is not appropriate in cases of asymptomatic bacteriuria, even when pyuria is observed (Nicolle *et al.* 2005). This is further supported by the American Academy of Otolaryngology who recommended antibiotic use in adult bacterial sinusitis only when complicating factors were present, such as upper respiratory infection, and when a bacterial cause can be identified in laboratory testing (Rosenfeld *et al.* 2007). The frequency of these recommendations suggest inappropriate antibiotic prescription is still a significant issue in the clinical setting.

Between 2000 and 2010 global antibiotic use grew by 30% increasing from 50 to 70 billion doses, 30 billion of which were prescribed in just 3 countries: India (13 Billion), China (10 Billion) and the United States (7 Billion) (CCDE, 2015). It is estimated that 60% of globally prescribed antibiotics were penicillin or carbapenem derivatives, with the number of prescribed last resort antibiotics, those used as a last line of defence such as colistin, increasing by 53% (Van Boeckel *et al*. 2014).

Many of these antibiotics were used appropriately however a large number were prescribed unnecessarily. It is estimated that antibiotics are misused in 30-50% of cases and a pathogen identified in only 7.6% of cases in the United States (Van Boeckel *et al*. 2014). Improper prescription could be due to the low toxicity and cost of most antibiotics making them practical to prescribe even when a firm diagnosis cannot be reached. A lack of patient understanding between bacterial and viral infection can also lead to pressure on physicians to prescribe antibiotics even when unnecessary, or the discontinuation of an antibiotic regime by the patient due to the alleviation of symptoms. These phenomena lead to an excess of antibiotics in the environment and their overexposure to clinically relevant organisms, placing selective pressure on resistant strains and allowing their proliferation (Ventola, 2015; Ashraf and Cook, 2016).

Furthermore, in some countries antibiotics are still used in large quantities in agriculture as a growth promoter. It has been long understood that by adding antibiotic supplement to animal feed it is possible to increase the meat yield and health of the animal, with 80% of antibiotics used in an agricultural application. This overexposure acts by the same mechanism as over prescription, however resistant organisms in animal microbiomes are able to enter the human food chain via detectable levels of resistant bacteria in meat and agricultural land. Waste from these sources can run off into aquatic or natural environments and result in exposure to other human and animal hosts (Bartlett *et al*. 2013; Frère and Rigali, 2016; Tasho and Cho, 2016).

Around the world there is a call for the restriction of antibiotic use, both in clinical and agricultural settings. However, statistics on global antibiotic use make it clear that even when governments have adopted policies on the restriction of these vital medicines, they have been too slow to act, while other nations are yet to recognise the threat of antibiotic overuse and continue to use antibiotics in an unsustainable way.

1.2.2 The Rising Incidence of Antibiotic Resistance

Since the recognition of growing antibiotic resistance, a global effort has been coordinated to prevent the end of the antibiotic era. Strategies focus on global monitoring schemes to measure and track the incidence of antibiotic resistance, and safeguarding vital last-resort antibiotics by regulating their use.

Methicillin resistant *Staphylococcus aureus* (MRSA) is one of the most well-known infection causing resistant bacteria in a clinical setting, and is often dubbed a 'Superbug' in the media due to its prolific spread and high associated mortality rate. Incidence of MRSA has been declining in many Western countries in the last decade due to better antibiotic regulation, in the UK incidence of MRSA fell by 84.7% between 2003 and 2011 and was only responsible for 1.6% of bacteraemias (Davies, 2011; Ventola, 2015). Other countries have responded less well to the call for antibiotic control and still have a significant MRSA issue. Sub-Saharan Africa, Latin America, India and Australia all have a rising incidence of MRSA infection with Latin America experiencing a resistance rate of up to 90% (CCDE, 2015).

Carbapenem-resistant *Enterobacteriaceae* (CRE) are defined by resistance to all last-resort carbapenems effective against Gram negative bacteria and are responsible for a significant number of infection associated mortalities. *Escherichia coli* and *Klebsiella pneumoniae* species are most likely to show resistance with an approximate 10-13% incidence in Europe, the United States and Asia, and a much higher incidence of 28% in parts of Latin America (CCDE, 2015). A systematic review of global CRE and carbapenem-susceptible *Enterobacteriaceae* (CSE) infection, comprising 20 studies reporting on 692 patients found that CRE was twice as likely to be fatal when compared to CSE. However, several methodology flaws were identified in these studies, including non-randomisation and a focus on critically ill ICU patients who would be less able to recover from any infection regardless of its resistance profile. It was also noted that combination antimicrobial therapy had a lower associated mortality rate than monotherapy, and that carbapenems prescribed with a last resort antibiotic colistin conferred a more favourable outcome (Falagas *et al*. 2014).

This increasing incidence of antibiotic resistant infection in the clinical setting not only has a human cost, but also a calculable economic cost. A review on the cost of antimicrobial resistance commissioned by the UK government in 2014 found that by 2050 antibiotic resistance will cause an estimated 10 million deaths per year with a 2-3.5% reduction in gross domestic product (GDP), costing $100 trillion globally (Oneill, 2014). To reverse the antibiotic resistance crisis, current efforts to regulate antibiotic use and promote research into novel antibiotics must be continued. However,

a focus on innovative solutions to remove antibiotic resistance from the environment and prevent its spread should also be implemented alongside current efforts.

1.2.3 Mechanisms of Antibiotic Resistance

In the 1940s antibiotics began to be utilised to treat serious infection among wounded World War II soldiers, resulting in the mass production of penicillin. Alexander Fleming himself identified the use of penicillin in the second Great War as a contributing factor to the increase in antibiotic research and development in his 1945 Nobel Prize speech. However, shortly after his discovery Fleming noted the emergence of penicillin resistance, and by the 1950s this had become a significant clinical issue which threatened to undo much of the progress made in the treatment of bacterial infection in the previous decade (Ventola, 2015; Fleming *et al*. 2016).

Overexposure of bacteria to antibiotics can lead to the development of protective systems in the target organism by natural selection. Bacterial strains able to survive in the presence of antibiotics are allowed to replicate and transfer these resistance mechanisms to the next generation of organism, while non-resistant organisms are eliminated from the environment. The three main mechanisms of antibiotic resistance are: alteration in cell wall permeability, degradation or modification of active antibiotics and alteration of target proteins (Hoffman, 2001; Munita and Arias, 2016).

In order to have an antimicrobial effect, all antibiotics regardless of mechanism of action must first cross the cell membrane in order to reach its cellular target. To prevent this, some bacteria are able to modify the cell wall membrane to prevent certain antibiotics from entering the cytoplasm (Frost, 2007; Blair *et al*. 2015). This can involve thickening of the cell wall to prevent diffusion into the cell cytoplasm, as observed in resistance of *Staphylococcus aureus* to vancomycin (Cui *et al*. 2003). Antibiotic that has crossed the cell membrane can also be removed back to the extracellular environment by the expression of efflux pumps, as observed in erythromycin resistance of *E. coli* (Li *et al*. 2015).

If an antibiotic is able to cross the cell membrane and enter the cytoplasm, it may still be subject to degradation or modification by a variety of enzymes (Debabov, 2013). Antibiotic degradation occurs in a number of bacterial strains and is typically mediated by hydrolysis of the ester or amide bonds which are central to the biological activity of most antibiotic compounds, rendering them inactive. Beta lactamase is the most prevalent degradative enzyme, which inactivates beta-lactam antibiotics such as penicillins and cephalosporins by cleavage of the beta-lactam ring central to the antibiotic structure. Antibiotics can also be modified by enzymes present in the cytoplasm, rendering them

inactive without breakdown of their original molecular structure. This process is usually mediated by transferase enzymes which attach a co-substrate such as ATP, acetyl-CoA or glutathione to the antibiotic structure, altering its ability to bind to the target site (Wright, 2005; Blair *et al*. 2015).

Some classes of antibiotic have protein targets necessary for cell growth and metabolism, such as ribosomal sub units or DNA replication machinery. By interfering with these vital proteins, cell growth can be slowed or even halted completely. Target organisms can spontaneously develop mutations in these proteins resulting in structural changes which allow the cell to carry out normal metabolic function, but can no longer be affected by the antibiotic. These organisms are positively selected over non-mutated organisms in antibiotic rich environments, facilitating their proliferation. Mutation of DNA gyrase can result in protection from quinolones, while mutation of RNA polymerase induces resistance to rifamycins (Lambert, 2005; Frost, 2007; Blair *et al*. 2015).

By further understanding the mechanisms responsible for antibiotic resistance, future research efforts can focus on the removal or interference of the biochemical processes responsible for the development of bacterial protective systems. This could result in the return of many antibiotics to therapeutic use which are currently ineffective due to widespread resistance.

1.2.3.1 Extended Spectrum Beta-Lactamases

Extended spectrum beta-lactamases (ESBLs) are a class of beta-lactamase enzyme responsible for the hydrolysis and inactivation of a wide range of beta-lactam antibiotics (Shaikh *et al*. 2015). ESBLs offer antibiotic resistance against all beta lactam antibiotics, except the broad spectrum carbapenems and the cephalosporin-like cephamycins, however resistance to these last line antibiotics is growing (Papp-Wallace *et al*. 2011; Korzeniewska and Harnisz, 2013).

ESBLs first emerged in Western Europe following inappropriate clinical use of extend spectrum beta-lactam antibiotics, but quickly spread to the United States and Asia. ESBL production is most commonly seen in clinical *E. coli* and *K. pneumoniae* isolates, where it is associated with an increased treatment failure rate (Pitout *et al.* 2005; Shaikh *et al.* 2015).

Beta-lactamase inhibitors such as clavulanic acid can be combined with beta-lactam antibiotics and effectively used on otherwise resistant ESBL producing organisms. These inhibitors share structural homology with penicillin antibiotics and contain a beta lactam ring, but show little or no antimicrobial properties alone. This similarity in structure allows beta lactamase inhibitors to reversibly bind to the ESBL enzyme, however upon cleavage of the beta lactam ring an acyl group

remains permanently covalently bonded within the enzyme active site, rendering it inactive (Buynak, 2006; Drawz and Bonomo, 2010).

ESBL genes were originally located on the bacterial chromosome, but have been recombined onto bacterial plasmids where they are easily spread through bacterial populations of different species. There are three main types of ESBL gene, SHV, TEM and CTX-M. TEM is the most prevalent ESBL type containing over 90 subtypes, with TEM-1 the most common in Gram negative bacteria, causing over 90% of ampicillin resistance in *E. coli.* TEM-1 is effective against penicillins, however selective pressure from the inappropriate use of beta-lactam antibiotics has resulted in the emergence of TEM types which are effective against extended spectrum beta lactam antibiotics, such as TEM-3 and TEM-12 (Livermore, 1995; Shaikh *et al.* 2015). There are few SHV subtypes with SHV-1 most prevalent, causing up to 20% of plasmid mediated resistance in *K. pneumoniae* isolates (Tzouvelekis and Bonomo, 1999). These enzymes show activity against broad spectrum penicillins but little activity against cephalosporins (Shaikh *et al.* 2015). CTX-M enzymes have emerged within the 21st century and show little structural or spectrum homology with other ESBL enzymes and contains several subtypes. CTX-M enzymes preferentially hydrolyse cefotaxime and are commonly found in *Enterobacteriaceae* such as *E. coli*. (Tzouvelekis *et al.* 2000).

1.2.3.1.1 CTX-M gene

The CTX-M genes encode a class of ESBL enzymes with action against ceftazidime and cefotaxime. The CTX-M family are the most widely disseminated ESBL enzyme type and are typically plasmid mediated and common in *Enterobacteriaceae* species, however recombination to the chromosome can occur.

The CTX-M family can be separated into different groups based on the similarity in amino acid sequence, genes containing over 94% similarity are classified in the same group while genes sharing less than 90% similarity are placed into distant groups. There are 5 distinct CTX-M groups when classified this way, containing 40 different enzymes in total, in particular the CTX-M group 1 enzyme CTX-M-15 is rapidly spreading and is often implicated in nosocomial infection (Table 2) (Boyd *et al.* 2004; Castanheira *et al. 2014*). Table 2 shows the 5 CTX-M groups and the common specific CTX-M types they contain (Bonnet, 2004; D'Andrea *et al*. 2013). A more recent classification system used the alignment of amino acid sequences to arrange CTX-M genes into 7 clusters of varying relatedness. Figure 2 shows these clusters and Table 3 shows the number of genes and percentage identity of each cluster (Zhao & Hu, 2013).

CTX-M group	Specific CTX-M types contained
CTX-M group1	CTX-M-1, -3, -10, -12, -15, -22, -23
CTX-M group 2	CTX-M-2, -4, -5, -6, -7, -20, -76, -77
CTX-M group 8	CTX-M-8, -40, -63
CTX-M group 9	CTX-M-9, -14, -15, -16, -17, -18, -19
CTX-M group 25	CTX-M-25, -26, -39, -41, -91

Table 2. The 5 CTX-M groups and the specific subtypes they contain (Bonnet, 2004).

CTX-M Cluster	Number of CTX-M genes contained	% identity of group members
CTX-M-3 Cluster	42	97.6-99.7%
CTX-M-14 Cluster	38	97.3-99.7%
CTX-M-2 Cluster	16	95.2-99.7%
CTX-M-25 Cluster	7	98.6-99.7%
CTX-M-8 Cluster	3	97.9-99.7%
CTX-M-64 Cluster	2	95.9%
CTX-M-45 Cluster	1	N/A

Table 3. The 7 CTX-M clusters, the number of genes they contain and their % identity (Zhao & Hu, 2013).
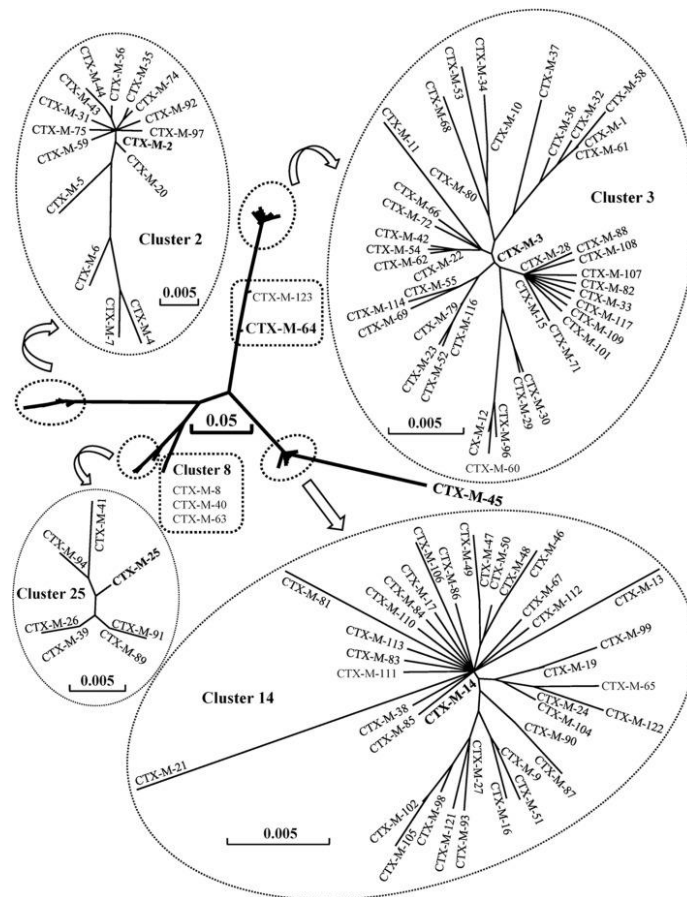
Figure 2. Phylogenic tree of the 7 CTX-M clusters (Zhao & Hu, 2013).

In Western Europe the CTX-M gene was first identified in Germany in 1989 but rapidly spread around the continent (Bauernfeind, A *et al.* 1990). In 2000 CTX-M was first detected in the UK and between July 2001 and February 2002 when an outbreak of CTX-M producing *K. pneumoniae* was identified(Alobwede *et al.* 2003; Brenwald *et al.* 2003). In addition to implications in clinical infection, CTX-M has also been detected in healthy human and animals which may serve as a reservoir, and study of UK waterways has revealed large reservoirs of CTX-M genes, including the first environmental instance of CTX-M-15 in the UK (Hawkey and Jones, 2009; Amos *et al.* 2014). The overuse of ceftriaxone and cefotaxime clinically may be the cause of CTX-M emergence and its maintenance in a widespread multi-locational reservoir (Alobwede *et al.* 2003).

Bacterial strains containing a member of the CTX-M family show a wide range of minimum inhibitory concentration (MIC) due to variation in CTX-M groups and other host genetics (Bonnet, 2004). In practice resistance to cefpodoxime is used as a phenotypic marker of CTX-M expression, however some CTX-M expressors are susceptible to cefpodoxime and therefore should be used in combination with an ESBL inhibitor such as clavulanic acid (EUCAST, 2015).

1.3 Mobile Genetic Elements

1.3.1 Overview of Bacterial Mobile Genetic Elements

Mobile genetic elements are sections of DNA that can move within a bacterial genome and be transferred between bacteria with genetic homology. Mobile genetic elements can contain a wide variety of genes, but most commonly contain virulence factors such as exotoxins or resistance genes which provide increased survivability and transferability in the expressing organism. There are many types of mobile genetic elements, but the most common are plasmids, transposons and gene cassettes.

Plasmids are a circular transferrable genetic elements that require a bacterial host for survival and contain a variety of genes, including resistance genes, addiction system genes, replication genes and genes responsible for segregationally stability. Typically, plasmids function to provide increased survivability or proliferation to their host organism, ensuring host survival and continued plasmid replication and dissemination. Resistance genes are commonly plasmid borne and are expressed by host machinery to provide protection from toxins within the environment, such as antibiotics or heavy metals. This protection results in the positive selection of bacterial cells containing expressible plasmids, resulting in a greater plasmid prevalence among the surviving bacterial population (Carattoli, 2009).

Transposons are single stranded sections of DNA that can mobilise themselves from bacterial chromosomal DNA into the cell cytoplasm. Once mobilised transposons can re-enter the chromosome at a different location causing mutation which can lead to the spontaneous generation of antibiotic resistance. Transposons, often coding antibiotic resistance genes, can also relocate to a plasmid within the cell cytoplasm which can then be transferred within the bacterial population and then recombine back to a bacterial chromosome, these are known as insertion sequences and require the host genome to translate the DNA sequence (Bennett, 2008; Siguier *et al*. 2014)

Much like a transposons, gene cassettes are mobile genetic elements which often contain antibiotic resistance genes which can relocate within a bacterial chromosome, or be transferred within the population by horizontal gene transfer. Gene cassettes also carry specific recombination sites, these are lengths of DNA responsible for the re-insertion of the gene cassette into a host chromosome. A gene cassette can only be inserted into a chromosome where its complimentary recombination site is present, this makes gene cassettes a useful tool in genetic engineering where a single gene needs to be inserted at a specific chromosomal location (Hall & Collis, 1995; Hall *et al.* 1996; Bennett 1999).

1.3.2 Plasmids

1.3.2.1 Definition, Function and Structure

As mentioned previously, plasmids are a circular transferrable genetic element that often contain antibiotic resistance genes and can disseminate throughout a bacterial population. In *Enterobacteriaceae,* plasmids carrying ESBL resistance genes are common, such as the *bla* gene family which encode SHV, TEM and CTX-M type enzymes (Carattoli, 2009).

As well as increased survival, increased dissemination can be provided by plasmids carrying virulence genes. Once expressed in an organism, these genes are able to induce a pathogenic phenotype in previously commensal bacterial strains, resulting in increased transmission and replication of the pathogen which in turn increases the number of plasmid copies present in the environment. In *E. coli,* virulence plasmids are responsible for the formation of several pathogenic strains, Table 4 shows the common intestinal pathogenic *E. coli* strains associated with virulence plasmids and their symptoms (Johnson and Nolan, 2009).

Virulence plasmid associated *E.coli* Strain	Common associated symptoms
Enterotoxigenic *E. coli* (ETEC)	Watery diarrhoea
Enteroinvasive *E. coli* (EIEC)	Watery diarrhoea, Inflammatory colitis, Dysentery
Enteropathogenic *E. coli* (EPEC)	Non-bloody diarrhoea
Enterohemorrhagic *E. coli* (EHEC)	Non-bloody diarrhoea, Haemorrhagic colitis, Haemolytic-uremic syndrome
Enteroaggrevative *E. coli (EAEC)*	Persistent diarrhoea
Extraintestinal pathogenic E. coli (ExPEC)	UTI, Sepsis, Neonatal meningitis

Table 4. The 6 types of pathogenic *E. coli* caused by plasmid virulence genes and the commonly associated symptoms in human infection (Johnson and Nolan, 2009).

Structurally, plasmids consist of double stranded DNA in a circular structure which can exist in different conformations. *In vivo* plasmids are found in a supercoiled conformation with the circular structure twisted to introduce tension, decreasing its physical size. During replication of some plasmids a cut is made in a single strand of the closed circular DNA to release the tension from the supercoiled structure, known as nicked relaxed circular, while two cuts at the same point on both strands results in a linear plasmid. (Prazeres, 2011).

1.3.2.2 Methods of Transfer

Plasmids can be transferred vertically to daughter cells during replication or by horizontal gene transfer by three main methods: conjugation, transformation and transduction.

Conjugation is the exchange of genetic material between bacteria by direct cell to cell contact through attachment of a sex pilus. In *E. coli,* cells containing a plasmid that confers resistance, such as the broad-host- range RK2 plasmid, are known as R^+ cells, which express sex pilus on the cell surface and initially attach by cell-to-cell contact to a recipient cell. A nick is made in the donor plasmid allowing relaxation from its supercoiled conformation, and a single strand is transferred to the recipient R^- cell which does not express a sex pilus. Both cells synthesise a complementary strand to the single stranded DNA to form a double stranded circular plasmid, the R^- cell is now an R^+ cell and is capable of initiating conjugation. Through this method plasmid associated antibiotic resistance genes are readily spread throughout the environment. (Figurski *et al.* 1982; Griffiths *et al.* 2000).

Exogenous plasmid DNA can be found in bacterial environments following the lysis of plasmid containing cells, this DNA can be taken up by bacterial cells during transformation. Exogenous single stranded plasmid DNA first adheres to cell surface receptors of competent cells, those which express the required receptors and transport systems to internalise exogenous DNA. Single stranded plasmid DNA is then transported across the cell membrane by DNA translocase, where a complementary strand is synthesised. This plasmid DNA can then be recombined into the host chromosome or remain in the cell as a fully formed plasmid (Lorenz and Wackernagel, 1994).

During transduction bacterial viruses known as phage attach to the bacterial cell wall and establish metabolic control. Phage DNA is then injected into cell cytoplasm to hijack cellular machinery and create new virus DNA, which is packaged and assembled into a new virus particle and released upon cell lysis. During this process plasmid DNA can inadvertently be replicated and packaged with viral DNA and transferred to a secondary host contributing to the environmental spread of plasmids (Shrivastava, 2013).

1.3.2.3 Incompatibility Group

Once transferred to a host cell the new plasmid must undergo DNA replication to increase its copy number and be maintained within the bacterial cell. This is accomplished using host machinery to replicate the circular plasmid DNA by the same process as host chromosomal DNA replication (del Solar *et al.* 1998; Wang *et al.* 2009).

Plasmid replication begins at the origin of replication (ORI), a gene sequence which host replication machinery recognises and binds to initiate DNA replication. Typically, origins have a higher ratio of AT than GC base pairs, as AT base pairs are bound by 2 hydrogen bonds and are more readily denatured than the 3 hydrogen bonds found in GC base pairs. This lower melting point of origins allows for easier binding of replication machinery and a higher chance of plasmid retention within the bacterial cell (Thomas, 2014). There are many identified plasmid origins, including pSC101, R6K and 15A, which can be found in varying locations on the plasmid. Each origin produces a different copy number once replication has initiated, which is controlled by a negative feedback loop and has implications in protein expression and plasmid retention. Plasmids with a higher copy number are more likely to be expressed and retained within the host cell, compared to their low copy number counterparts (Nordstrom and Dasgupta, 2006; Carattoli, 2009).

Plasmids with origins of a similar sequence may be unable to co-exist within the same host without removal of one or both plasmids, this is known as plasmid incompatibility. A similar origin may result in plasmid removal due to competition for replication and regulation machinery, however sharing addiction and partition systems that require similar cellular elements may also lead to plasmid incompatibility. Incompatible plasmids are not always lost symmetrically from a host bacterial cell as the presence of secondary genetic features, such as a second ORI gene, may result in the retention of one plasmid and the removal of another. Several mathematical models have been created to simulate the inheritance of multiple plasmids which share an ORI gene (Carattoli, 2009; Thomas, 2014).

The Incompatibility (Inc) groups a plasmid belongs to can reveal vital information, such as host range and potential copy number, which are useful in understanding how the plasmid behaves in a bacterial population. Plasmids of the same Inc group cannot stably co-exist in a bacterial cell as they show homology in the ORI and partition genes necessary for replication and plasmid segregation. This homology results in competition for replication machinery within the cell, and the eventual removal of a plasmid. PCR techniques have been developed to quickly identify plasmid incompatibility groups which utilise primers designed to bind to any origin of the same Inc group. Currently Carattoli, A. *et al* have developed the most comprehensive PCR system for the detection of

plasmid Inc group, identifying 18 Inc groups including IncN, IncP and IncF. However, Inc group identification by PCR can be unreliable as some plasmids can exhibit multiple ORI genes of different Inc groups, a phenomenon particularly observed in IncF plasmids (Carattoli *et al.* 2005; Carattoli, 2011; Thomas, 2014).

1.3.2.4 Addiction Systems

Most plasmids have a symbiotic relationship with their bacterial host by providing resistance to antibiotics in the natural environment. Under these conditions antibiotic resistant plasmid-containing bacteria are naturally selected due to their increased survivability, encouraging plasmid maintenance in the bacterial population. However, if selective pressure is removed the plasmid no longer provides increased survivability and increases metabolic burden on host cells, making plasmid loss likely (Zielenkiewicz and Cegłowski, 2001). To prevent removal from the host cell many plasmids contain an addiction system to make plasmid removal detrimental to survivability even in the absence of environmental selective pressure. There are 2 main addiction systems observed in the natural environment, Antitoxin-Toxin (AT) systems and metabolism based (MB) systems (Kroll *et al.* 2010).

AT systems rely on the production of an unstable antitoxin and a more stable toxin encoded on the plasmid which bind to form a harmless dimer, these can be protein, antisense RNA or restriction modification mediated. During protein translation the more stable toxin is expressed at low levels while the unstable antitoxin is highly expressed. Cell survival continues while both toxin and antitoxin proteins are translated at the correct ratio, however should the plasmid be removed the antitoxin is degraded at a quicker rate than the toxin, leading to its accumulation within cell cytoplasm resulting in cell death and ensuring plasmid maintenance within the population (Kroll *et al.* 2010). Common AT protein systems include plasmid energy maintenance (PemKI), coupled cell division locus (ccdAB) and virulence associated protein (vagCD) (Mnif *et al.* 2010).

Antisense RNA systems use the same principle; however, an unstable RNA antisense molecule is produced which forms a dimer with a stable toxin mRNA molecule to prevent translation of toxin proteins. Following plasmid removal the stable toxin mRNA is not inactivated by missense RNA and is translated to a protein toxin which accumulates in cell cytoplasm causing cell death (Gerdes *et al.* 1997). Common AT RNA antisense systems include host killing (hok-sok) and promotion of nucleic acid degradation (pndAC) (Mnif *et al.* 2010).

The restriction modification model relies on two plasmid encoded antagonistic enzymes, a restriction endonuclease and a DNA modification enzyme. Upon expression of the modification enzyme the target sequence on the host chromosome is methylated rendering the host immune to

the expressed endonuclease while plasmid expression is maintained. Upon removal of the plasmid, methylation of the target sequence no longer occurs allowing restriction by endonuclease enzymes causing DNA damage and cell death (Handa and Kobayashi, 1999). The EcoRI sites are often targeted in plasmid containing *E. coli* (Kroll *et al.* 2010).

In MB systems a host gene vital for metabolism is inactivated and replaced by plasmid expression of a homologous gene, these can be divided into catabolism and anabolism based. In catabolism-based systems a gene encoding enzymes necessary for the degradation of carbon atoms in energy sources is inactivated, however metabolic activity is restored by plasmid expression of an enzyme with comparable activity. In anabolism-based systems an enzyme necessary for cell growth and repair is inactivated and again is replaced by plasmid expression of a similar gene. In both these systems cell metabolism is able to continue while the replacement homologous plasmid gene is transcribed, upon plasmid removal metabolism is interrupted resulting in cell death (Kroll *et al.* 2010).

These systems provide a novel target for the removal of resistance plasmids from environmental bacterial populations. Chemical agents which prevent the expression of antitoxin, antisense RNA or modification proteins could in theory be utilised to remove a cells protection to addiction mediated systems, lowering the number of plasmid containing bacteria and reducing plasmid mediated antibiotic resistance. This would reduce the overall levels of resistance expression in the environment and may lower the size of antibiotic resistance reservoirs, slowing the spread of resistance genes in both a clinical and environmental setting. This approach of combatting the source of antibiotic resistance as opposed to developing new antibiotics to fight already resistant organisms could allow the return of obsolete antibiotics to clinical use. Polymixin antibiotics were used approximately 30 years ago in the treatment of Gram-negative infection, however they were abandoned due to reports of decreasing efficacy and increasing toxicity. Following the emergence of multi drug resistant infection in recent years polymyxin E, also known as colistin, has been used as a last line antibiotic with a much higher efficacy and lower toxicity than previously reported, likely due to a lower prevalence of colistin resistance genes driven by the hiatus in polymyxin antibiotic use (Falagas *et al.* 2005).

1.4 Waste Water Effluent and the Natural Environment

1.4.1 The Waste Water Treatment Process

Waste water from residential, commercial and other sources from around the UK is directed towards waste water treatment (WWT) facilities. These facilities use a number of physical chemical and biological processes to remove harmful and pollutant elements from waste water before returning it back to the natural environment.

In the primary stage of WWT, solids, known as total suspended solids (TSS), are removed by filtration and sedimentation. Large particulate matter such as grit and sand are removed at this stage by allowing them to separate in large settling tanks, and the waste water transferred to the second stage of treatment. The second stage focuses on the removal of biological matter and aims to reduce the biological oxygen demand of the water. This is achieved by a variety of processes and varies around the country, but usually involves either the activated sludge process, membrane bioreactors or a rotating biological reactor. However, the most common form of sewage treatment is sand filtration, this uses layers of sand as filters to pass untreated water through and is often combined with chemicals, such as rapid and upward flow sand filters, however slow sand filters do not require the use of chemicals Trickle bed reactors are also common, and use a solid medium such as rocks, gravel or other sediment to establish an organic biofilm which absorbs or adsorbs pollutant such as nitrates and organic matter (Haig, S.J et al. 2011; Rittmann & Snoeyink 1984.) . Some WWT facilities utilise a final tertiary stage of treatment, however this varies depending on the treatment site. Disinfection is the most common tertiary process, with chlorination treatment the standard, however further treatment process is required to remove chlorine contamination from WWE before its release to prevent environmental pollution. Deep bed filtration can also be carried out to remove further solid waste following secondary treatment. Currently in the UK over 9,000 WWT facilities discharge WWE into natural in land waters and estuaries (DEFRA, 2002; DEFRA, 2012).

1.4.2 Pollution of Natural Aquatic Environments by Waste Water Effluent Release

Despite the stringent treatment processes in place for waste water in the UK, several studies have been undertaken investigating the chemical and biological pollution caused by the release of WWE into natural aquatic environments.

One source of chemical pollution is from incompletely metabolised antibiotics found in domestic, agricultural and hospital waste water. These residual antibiotics are not always effectively removed during treatment and are found at detectable levels in natural water systems receiving WWE. A Study of WWE in Wisconsin, USA detected 6 different antibiotic compounds: sulfamethazine, sulfamethoxalone, tetracycline, ciprofloxacin, erythromycin and trimethoprim, at concentrations less than 1.3 µg/L. Similar results have been obtained in European studies, however antibiotic concentrations were slightly lower (Karthikeyan and Meyer, 2006). The river of interest for this study, Sincil Dike, Lincoln, UK, was tested for concentrations of the antibiotic cefotaxime, with results suggesting the antibiotic was not present. However, the study identified several issues with the detection method used, including improper sample preparation and lack of standard spike introduction during HPLC. Therefore, it is still possible that Sincil Dike, Lincoln UK contains antibiotic

contamination similar to that found globally, but more sensitive and refined analytical techniques are required for its detection (Cheswick, 2014).

As well as chemical pollution, WWE release also introduces biological pollutants to the natural environment. A study has shown that large numbers of bacteria are able to survive the water treatment process, and are released into the natural environment with WWE. A high proportion of these organisms have been shown to be ESBL producing *E. coli* (ESBLEC), with up to 600 million released by WWE into the environment daily (Brechet *et al.* 2014). It has been identified that these large numbers of organisms are able to persist through the WWT process due to inadequate removal of organic matter during disinfection, with the CFU/ml of antibiotic resistant organisms unaffected after chlorine disinfectant treatment (Munir *et al.* 2011)

From the studies performed on the purity of WWE released into natural environments, it is apparent that the current WWT process is not stringent enough to ensure the removal of all harmful contaminants before its release into the environment. Therefore, a review of the current treatment technologies and investigation into how they can be refined would improve the health of our natural waterways and prevent the introduction of resistant bacteria to the environment.

1.4.3 The Effects of Waste Water Effluent Release on Antibiotic Resistant Organisms

It has been previously shown that release of WWE into natural aquatic environments introduces a variety of chemical and biological pollutants, however, a little study has been conducted on how the introduction of these pollutants effects the native bacterial population and the levels of resistance within this population.

A study of river sediment samples collected between 2009 and 2011 from the midlands, UK, both upstream and downstream of WWE release points, showed a significant increase in third generation cephalosporin resistance in isolates exposed to WWE. It was also shown that this increase in resistance was caused by dissemination of the group 1 CTX-M gene CTX-M-15, which is often associated with resistant nosocomial infection. However, it was not clear if CTX-M-15 bearing plasmids were introduced by WWE or already existed in the environment and were allowed to proliferate. It was hypothesised that increased proliferation could be due to favourable conditions caused by the introduction of WWE, such as the presence of antibiotic residues which drove natural selection (Amos *et al.* 2014).

A 2014 study of the Sincil Dike, Lincoln, UK found similar results to that obtained by Amos, *et al.* 2014. Water samples were collected upstream at the point of and downstream of WWE release and

colony counts of ESBLEC performed, these isolates were then screened for phenotypic and genotypic antibiotic resistance. It was determined that ESBLEC counts were significantly higher at the point of and downstream of WWE release. Comparison to a nearby river not receiving WWE showed a 10-fold increase in the number of ESBLEC isolated from sites exposed to WWE. It was hypothesised that this dramatic increase can be attributed to the introduction of resistant organisms by WWE, and rapid horizontal gene transfer of plasmid associated antibiotic resistance genes (Cheswick, 2014).

The small body of evidence currently available suggests that the introduction of contaminates by WWE has a significant and measurable effect on the prevalence of antibiotic resistant organisms, particularly those carrying ESBL genes such as the CTX-M family. To fully understand the effect of WWE, larger scale study is needed which focuses on the origin of the increase in resistance, and the specific genetic and chemical mechanisms responsible.

1.5 Aims and Objectives

The aim of this study was to perform a series of genetic analysis on plasmids extracted from ESBLEC isolated from aquatic environments receiving WWE. This analysis revealed the genetic elements present on circulating plasmids, which were then used to develop a plasmid architecture of commonly associated genetic elements to characterise, and track the environmental spread of antibiotic resistance carrying plasmids through the addition of WWE.

Initially water samples from a river receiving WWE were collected at three points, 500m up stream of WWE release, at the point of WWE release and 500m downstream of WWE release. This provided a natural control of samples that have not been in contact with environments receiving WWE. Samples were filtered onto selective media for ESBLEC, however subsequent biochemical testing was used to confirm bacterial species and ESBL production independently.

Extracted plasmid from these samples was used in PCR for the detection of ESBL, incompatibility group and addiction system genes. The prevalence of each gene was assessed and compared to current published data, and the effects of WWE on the incidence of each gene studied. Finally, the genetic data for each sample was arranged into the common genotypes observed and analysed to determine how these genotypes changed with the addition of WWE.

This information allowed for a better understanding of the genetic elements currently present on plasmids isolated from ESBLEC and how addition of WWE effects the genetics of ESBL producing organisms.

Chapter 2 - Methods and Materials

2.1 Media and Supplements

2.1.1 Brilliance ESBL Chromogenic Agar

Brilliance ESBL chromogenic agar plates (Oxoid, PO5302) were used to isolate presumptive ESBLEC*.*
Plates were purchased prepared, and under optimum conditions are able to presumptively identify
E. coli, KESC (*Klebsiella, Enterobacter, Serratia and Citrobacter*), *Proteus, Morganella and providencia*
species (Figure 3). Plates were stored at 4°c until the stated use by date.

Blue or Pink
E. coli

Green
Klebsiella, Enterobacter, Serratia
and *Citrobacter*

Brown halo
Proteus, Morganella, Providencia

Colourless
Salmonella, Acinetobacter or other*
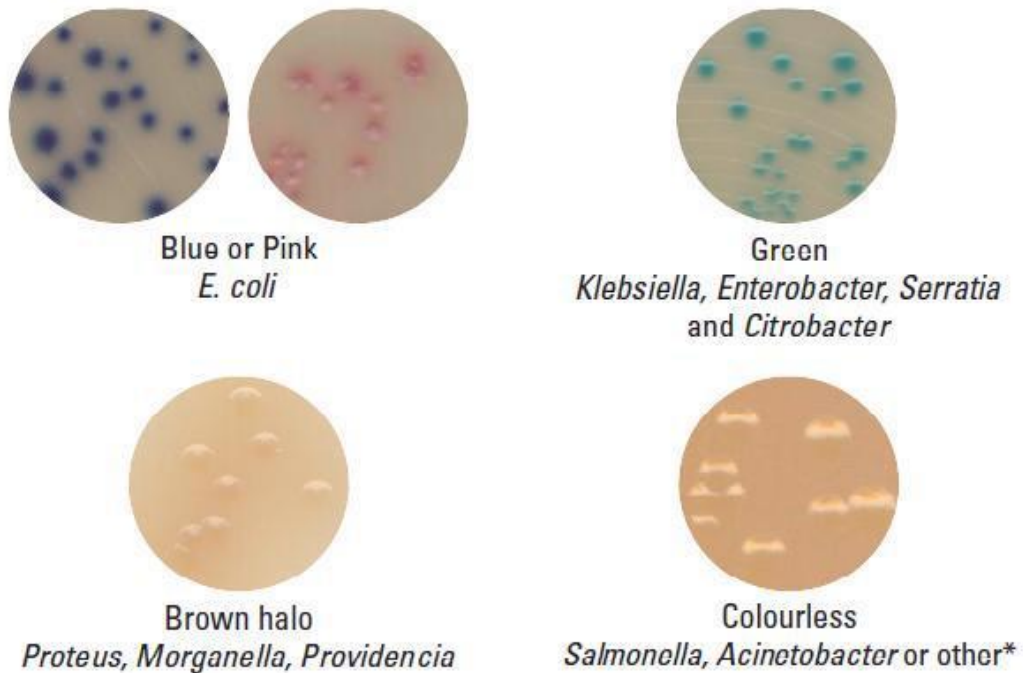
Figure 3. Chromogenic change of ESBL brilliance agar when cultured with identifiable species
(Oxoid, 2010)

Typical Formula	g/L
Sodium Chloride	5.0
Peptones	12.0
Phosphate Buffers	4.0
Chromogenic Mix	4.0
Antibiotic Mix	0.28
Agar	15.0

2.1.2 Agar Technical No.3

1.2% w/v agar technical no.3 (Oxoid, LP0013) was added to broth media to create a solid media.
Agar powder was added to the broth solution and autoclaved according to broth media instructions.

2.1.3 Membrane Lauryl Sulphate Agar (MLSA)

MLSA was used for confirmation of *E. coli* after presumptive identification from *Brilliance* ESBL chromogenic agar (2.1.1). 76.2 g of membrane lauryl sulphate broth (Oxoid, MM0615) powder was dissolved in 1 L of distilled water with 1.2 g of agar technical no.3 (2.1.2), and autoclaved at 121°c for 15 minutes. After autoclaving, molten agar was poured into polystyrene Petri dishes (Fisher Scientific, AS4052) and allowed to cool until set, plates were stored at 4°c for up to 3 weeks.

Typical Formula	g/L
Peptone	39.0
Yeast Extract	6.0
Lactose	30.0
Phenol Red	0.2
Sodium Lauryl Sulphate	1.0
Agar Technical no.3	12.0

2.1.4 Nutrient Broth

Nutrient Broth (Oxoid, CM0001) was used as a general liquid growth medium. 13 g of nutrient broth powder was dissolved in 1 L of distilled water and transferred to glass universals in 10 ml aliquots. The universals were autoclaved at 121°c for 15 minutes and stored at room temperature for up to 3 months.

Typical Formula	g/L
'Lab-Lemco' Powder	1.0
Yeast Extract	2.0
Peptone	5.0
Sodium Chloride	5.0

2.1.5 Nutrient Agar

Nutrient Agar (Oxoid, CM0003) was used as a general solid growth medium. 28 g of nutrient agar powder was dissolved in 1L of distilled water and autoclaved at 121°c for 15 minutes. After autoclaving molten agar was poured into polystyrene petri dishes (Fisher Scientific, AS4052) and allowed to cool until set, plates were stored at 4°c for up to 3 weeks.

Typical Formula	g/L
'Lab-Lemco' Powder	1.0
Yeast Extract	2.0
Peptone	5.0
Sodium Chloride	5.0
Agar	15.0

2.1.6 Cefotaxime Stock Solution

Cefotaxime was used as an additive to solid and liquid media for the selection and maintenance of ESBLEC. 0.1 g of cefotaxime sodium salt (Sigma-Aldrich, C7039) was dissolved in 50 ml of distilled water to create a 1000x stock (2 g/L). The stock solution was passed through a 0.45µm nylon filter (Fisher Scientific, 09-719D) to sterilise and stored in 0.5ml aliquots at -20°c.

2.1.7 Cefotaxime Supplemented Nutrient Agar

Cefotaxime supplemented agar was used to maintain cefotaxime resistant ESBLEC. Nutrient agar was prepared as described above (2.1.5) but autoclaving the media was allowed to cool to 55°c and 1 ml of cefotaxime stock solution (2.1.6) was added to 999 ml of media and thoroughly mixed. The supplemented molten agar was poured into polystyrene petri dishes (Fisher Scientific, AS4052) and allowed to cool until set, plates were used immediately. This gave a final cefotaxime concentration of 2mg/ml as described previously (Doumith *et al.* 2012).

2.1.8 Mueller-Hinton Agar

Mueller-Hinton agar (Oxoid, CM0337) was used during combination antibiotic susceptibility disc testing. 38 g of Mueller-Hinton agar powder was added to 1 L of distilled water and autoclaved at 121°c for 15 minutes. After autoclaving molten agar was poured into polystyrene petri dishes (Fisher Scientific, AS4052) and allowed to cool until set, plates were stored at 4°c for up to 3 weeks.

Typical Formula	g/L
Beef, Dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0

2.1.9 Tryptone Broth

Tryptone broth was used as a growth media during the indole test (2.2.3). 10g of tryptone (Oxoid, LP0042) and 5g of sodium chloride (Oxoid, LP0005) was dissolved in 1L of distilled water and transferred to test tubes in 5ml aliquots. The test tubes were autoclaved at 121°c for 15 minutes and stored with caps at 4°c for up to 1 month.

Typical Formula	g/L
Tryptone	10.0
Sodium Chloride	5.0

2.1.10 LB Broth

LB broth (Fisher Scientific, BP9723) was used as a culture medium for *E. coli* prior to plasmid extraction (2.4.1.2). 37 g of LB broth granules was dissolved in 1 L of distilled water and transferred to glass universals in 10 ml aliquots. The universals were autoclaved at 121°c for 15 minutes to sterilise.

Typical Formula	g/L
Casein peptone	10.0
Yeast Extract	5.0
Sodium Chloride	10.0

2.2 Isolation of *ESBL* Producing *E.coli*

2.2.1 Sample Collection Method

Samples were taken at 3 different sites from the Sincil Dike, Lincoln, UK. Samples were taken 500m upstream of the wastewater effluent release point, at the release point and 500m downstream of the release point (Figure 4). Samples were collected approximately 30 cm below the surface in a 500 ml screw-top Duran bottle sterilised by autoclaving. Samples were filtered on the day of collection (2.2.2.2).

Figure 4. Map of WWT facility and sample points S1 (500m upstream of WWE release point), S2 (At WWE release point) and S3 (500m downstream of WWE release point).

2.2.2 Sample Filtration and Bacterial Isolation

2.2.2.1 Filter Membranes

Gridded cellulose nitrate membrane filter discs of pore size 0.45 µm (Nalgene, DS0205) were used during water sample filtration.

2.2.2.2 Differentiation of ESBL Producing Organisms

A vacuum pump (Liquiport, NF 100) was connected to filtration manifolds (Sartorius, Combisart) and sterilised by Bunsen burner with all valves fully open, manifold cups were sterilised separately using a clamp stand and Bunsen burner. Flat forceps were ethanol sterilised and used to place a sterile 0.45µm cellulose nitrate filter (2.2.2.1) onto the filtration pedestal. A manifold cup was placed over the filter and clamped in place with the valve closed. 100ml of water sample was transferred to the manifold cup and the valve opened until all sample had been drawn through the filter. The manifold cup was removed and the filter transferred using ethanol sterilised forceps to the surface of *Brilliance* ESBL agar (2.1.1) ensuring the filter remained face up with no trapped air between the filter and agar surface.

Brilliance ESBL agar plates (2.1.1) were then incubated at 37°c for 24 hours, presumptive ESBLEC colonies were selected by a chromogenic change to blue or pink, and sub cultured by streak plate onto MLSA (2.1.3) to confirm presence of *E. coli*. MLSA plates (2.1.3) were incubated at 30°c for 4 hours followed by 44°c for 14 hours, yellow colonies indicated the presence of *E. coli* and were retained for further testing.

E. coli ATCC 25922 was used as a negative control strain and *K. pneumoniae* ATCC 700603 used as a positive control strain as recommended by the *Brilliance* ESBL agar data sheet. These were cultured from glycerol stocks created by Cheswick, 2014.

2.2.3 Indole Test

All presumptive *E.coli* isolates from MLSA testing were confirmed as *E.coli* by the indole test. Tryptone broth (2.1.9) was inoculated with a single colony of each isolate and incubated at 37°c overnight. Isolates were confirmed as *E.* coli if a cherry red layer was seen on the media surface immediately after 5-7 drops of Kovacs reagent (Biorad, 55313) was added.

2.2.4 Combination Antibiotic Susceptibility Disc Testing

2.2.4.1 Cefpodoxime Combination Disc Kit

Cefpodoxime combination disk kit (Oxoid, DD0029) was used to confirm the ESBL status of *E. coli* isolates following positive *E. coli* identification from indole testing (2.2.3). Two types of disc were included in the kit, cefpodoxime 10 µg and cefpodoxime + clavulanic acid 10µg/1µg. Discs were dispensed onto the surface of Mueller Hinton agar using a disc dispenser (Oxoid, ST6090).

2.2.4.2 Combination Antibiotic Disc Testing Method

All presumptive *E. coli* isolates from MLSA testing were confirmed as ESBL producers by the combination antibiotic disk method (Carter *et al.* 2000). Nutrient broth (2.1.4) was inoculated with a single colony of each isolate and incubated at 37°c overnight. Cultures were diluted to a 0.5 mcfarland standard and 0.1ml spread onto Mueller-Hinton agar (2.1.8) as described by British standards (EUCAST, 2015).

A cefpodoxime and a cefpodoxime+clavulanic acid combination disk (2.2.4.1) were placed on the agar surface and incubated at 37°c for 24 hours. Following incubation, zone of inhibition for each disk was measured in mm. Organisms were confirmed as ESBL producers if the zone of inhibition around the combination disk was ≥5mm larger than that of the cefpodoxime disk alone (Carter *et al.* 2000). In this study *E. coli* NCTC 13353 was used as a positive control and *Klebsiella pneumoniae* ATCC 700603 was used as a negative control as recommended by the product manufacturer and were cultured from glycerol stocks created by Cheswick, 2014.

2.3 Long Term Storage of *E. coli* Strains

2.3.1 50% Glycerol Solution

Sterile 50% glycerol solution was mixed with bacterial culture prior to flash freezing and long term glycerol storage. 50g of glycerol (Sigma-Aldrich, G5516) was dissolved in 100ml of distilled water and autoclaved at 121°c for 15 minutes.

2.3.2 Glycerol Stock Method

For long term storage glycerol stocks of the isolates were created. 0.5ml of overnight nutrient broth culture was mixed with 0.5ml of 50% glycerol solution (2.3.1) in a screw-top cryovial (Sigma-Aldrich, BR114840) and flash frozen in liquid nitrogen and stored at -80°c.

2.4 DNA Extraction

2.4.1 Plasmid Extraction

2.4.1.1 Monarch Plasmid Mini Prep Kit

For the extraction of pure plasmid DNA from *E. coli* isolates a Monarch plasmid mini prep kit (New England BioLabs, T1010) was used. The kit contains resuspension buffer (B1), plasmid lysis buffer (B2), plasmid neutralisation buffer (B3) wash buffers 1 and 2 and elution buffer. The kit also contains spin columns used to purify plasmid DNA from other substances following extraction.

2.4.1.2 Plasmid Extraction Method

1.5ml of overnight nutrient broth culture was added to a sterile 1.5ml Eppendorf and centrifuged (ThermoFisher, 75002430) at 16,000 x g for 30 seconds and supernatant discarded from the bacterial pellet which was resuspended in 200µl of resuspension buffer (B1). 200µl of plasmid lysis buffer (B2) was added and gently inverted 6 times until the solution became dark pink and viscous to ensure cell lysis had occurred. The solution was then neutralised with 400µl neutralisation buffer (B3), inverted until uniformly yellow with a white precipitate and incubated for 2 minutes at room temperature. Samples were then spun for 5 minutes at 16,000 x g and the supernatant was transferred to a silica spin column and centrifuged at 16,000 x g for 1 minute. Flow through was discarded and 200µl of wash buffer 1 added to the spin column before centrifugation at 16,000 x g for 30 seconds, this was repeated with 400µl of wash buffer 2 and all flow through discarded. Finally the spin column was transferred to a clean 1.5ml Eppendorf tube and 50µl of elution buffer added and left to incubate for 1 minute before centrifugation at 16,000 x g for 1 minute to elute bound plasmid DNA from the spin column (New England BioLabs, 2016). Plasmid samples were stored at 4°c for the duration of the project.

2.4.2 Control DNA Extraction Method

Control strains for CTX-M group 1 required extraction of both plasmid and chromosomal DNA as the CTX-M group 1 gene could be found on both. Control strains were streak plated onto nutrient agar and incubated overnight at 37°c. 3 colonies of each control was suspended in 50µl of PCR grade water in a sterile 1.5ml Eppendorf tube and heated to 100°c for 10 minutes in a heat block Samples were centrifuged at 17,000 x g for 10 minutes to separate cellular debris and 1µl of the supernatant used as template DNA during PCR.

2.5 Visualisation of DNA Fragments

2.5.1 TAE Buffer

TAE buffer (Fisher, BP1332) was used to make agarose gels and as a running buffer during electrophoresis. A purchased 50x stock solution was diluted to a 1x working concentration using distilled water.

Typical Formula	%
Tris	24.0
Acetic acid	5.0
EDTA	<2.0

2.5.2 Agarose Gels

Agarose gels were used during visualisation of PCR products and restriction enzyme fragments. 0.8-1.5% w/v agarose powder (Fisher, BP160), depending on the application, was added to 1 x TAE buffer (2.5.1) in a clean conical flask and microwaved in 1 minute bursts for 3 minutes, or until completely melted. Molten agarose was allowed to cool to 50°c then poured into a casting tray with the necessary combs and allowed to set.

2.5.3 Preparation of DNA Ladders

DNA ladders were used during gel electrophoresis to quantify fragment BP size by comparing to reference bands of known size in the ladder. Ladders were prepared as shown in Table 5 and loaded into agarose gels next to DNA samples. 100bp, 1kb and Lambda DNA-HindIII digest ladders (New England BioLabs, N3231, N3232 & N3012) were used in this study, as shown in Figure 5.

	Volume or concentration
DNA Ladder, 500µg/ml (See below)	0.125µg
Water	4.75µl
6x Purple Stain	1µl
Final Volume	25µl

Table 5. Components and volumes used in preparation of DNA ladders
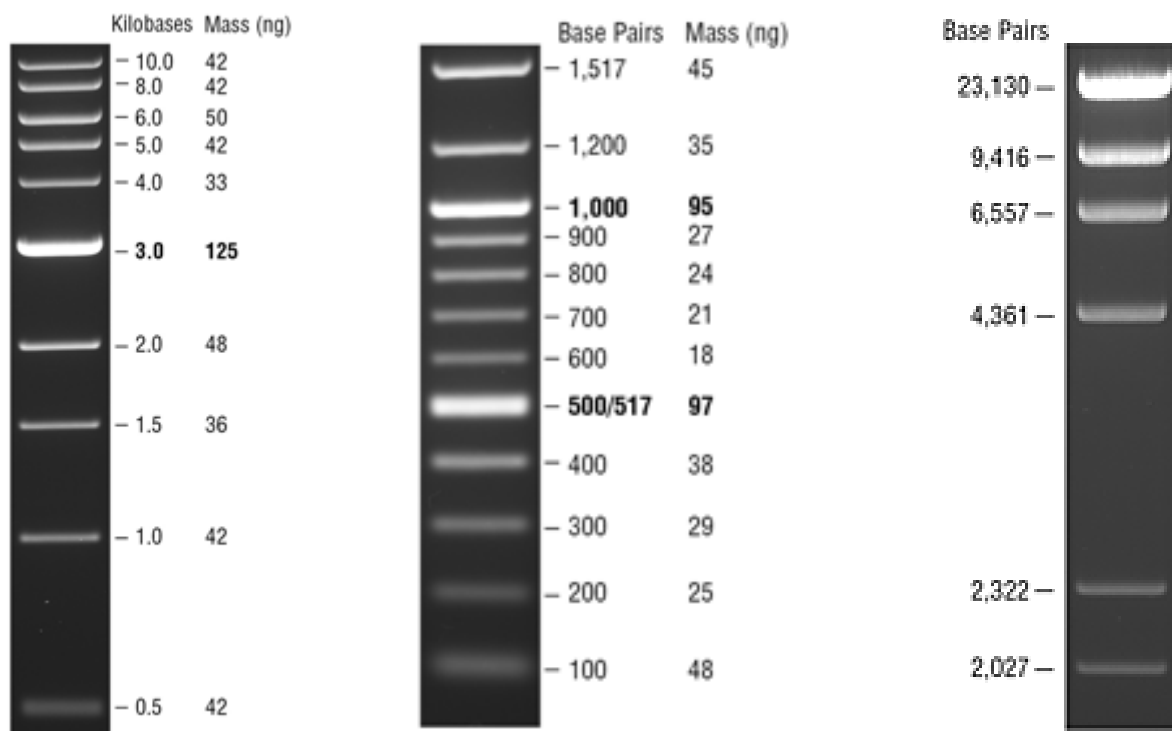
Figure 5. (Left to Right) 100bp Molecular ladder, 1kb molecular ladder and Lamda DNA-HindII digest molecular ladder (New England BioLabs, N3231, N3232 & N3012).

2.5.4 Gel Electrophoresis Method

Gel electrophoresis was used to separate DNA fragments based on their base pair size and quantify them using a DNA ladder (2.5.3). Prepared agarose gels (2.5.2) were placed in an electrophoresis tank and filled to approximately 5mm above the agarose gel with 1 x TAE buffer. Samples were loaded into individual wells will DNA ladders at the ends of each gel. The gel tank, anode and cathode were assembled and connected to a power supply (Biorad, PowerPac) and set at a constant of 100V, running time depended on percentage of agarose used and size of the gel, and stopped when the loading dye reached approximately 3/4 of the way down the gel.

2.5.5 Ethidium Bromide Staining and Visualisation

Ethidium bromide (Thermo Scientific, 15585011) was used as a DNA stain following agarose gel electrophoresis. Ethidium bromide was purchased as a 10 mg/ml solution and 50μl was added to 1L of water to give a final concentration of 5μg/ml. Gels were submerged in the ethidium bromide solution for 15 minutes following electrophoresis and destained for 10 minutes in distilled water to reduce background staining. Gels were then imaged on a ChemiDoc gel imaging system (Biorad, 1708280) and analysed using ImageLab software suite.

2.6 Detection of Plasmid Genetic Elements

2.6.1 *Taq* 5x Master Mix

T*aq* 5x master mix (New England BioLabs, M0285) was used during PCR as a source of DNA polymerase and dNTPs. *Taq* 5x master mix was used in PCR reaction mixtures to a final 1x working concentration and stored at -20°C.

2.6.2 Primer Stock Preparation

Custom primer sequences were used during PCR to bind to specific sequence sites in the target gene. First dehydrated primers were briefly centrifuged at full speed to prevent loss and rehydrated using the amount of TE buffer recommended by the manufacturer (Sigma-Aldrich) to give a concentration of 100μM. Primers were further diluted to 10 μM in TE buffer before being added to PCR reaction mixtures to give a final concentration of 1 μM. Table 6 shows primer sequences used in this study.

Gene Detected	Primer	Sequence (5'to 3')	Fragment Yielded (bp)	Annealing temperature (°c)	Source
CTX-M group 1	Forward	AAAAATCACTGCGCCAGTTC	415	52	(Woodford *et al.* 2006)
	Reverse	AGCTTATTCATCGCCACGTT			
IncF	repB Fw	TGATCGTTTAAGGAATTTTGA	270	52	(Carattoli *et al.* 2005)
	repB Rv	GAAGATCAGTCACACCATCC			
IncN	Rep 1	AGTTCACCACCTACTCGCTCCG	164	55	(Gotz *et al.* 1996)
	Rep 2	CAAGTTCTTCTGTTGGGATTCCG			
IncP	oriT1	CAG CCT CGC AGA GCA GGA T	110	57	(Gotz *et al.* 1996)
	oriT2	CAGCCGGGCAGGATAGGTGAAGT			
Hok-Sok	Hok up	AGATAGCCCCGTAGTAAGTT	203	54	(Mnif *et al.* 2010)
	Sok low	GATTTTCGTGTCAGATAAGTG			
PemK	PemK up	AACGAGAATGGCTGGATGC	232	54	(Mnif *et al.* 2010)
	PemK low	CCAACGACACCGCAAAGC			
ccdAB	ccdB low	AGGAAGGGATGGCTGAGGT	230	54	(Mnif *et al.* 2010)
	ccdA up	GGTAAAGTTCACGGGAGAC			

Table 6. Sequence and yielded fragment sizes of primers used in this study.

2.6.3 PCR Reaction Mix

PCR reactions were setup in 0.2ml PCR tubes as described in Table 7. Template plasmid DNA was quantified using a Nanodrop 2000 (ThermoFisher, ND-2000) and diluted to 1ng/µl in PCR grade water.

	Volume	Final Concentration
Taq 5x Master Mix	5µl	1x
Diluted Primer	2µl	1µM
Diluted Template DNA	1µl	1.0 ng
Water	17µl	-

Table 7. Components, volumes and final concentrations used in assembly of PCR reaction mix

2.6.4 PCR Cycling Conditions

PCR reaction mixtures were placed in a thermocycler (Biorad, T100) for amplification of the target gene. Tables 8-11 show the cycling conditions for each gene detected. Following thermos cycling 5µl of PCR product was mixed with 1µl of 6x purple stain (New England Biolabs, B7024S) for agarose gel loading.

PCR Step	Temperature and Duration
Initial Denaturation	94°c for 5 minutes
35 Cycles	94°c for 40 seconds
	X°c for 40 seconds
	72°c for 50 seconds
Final Elongation	72°c for 6 minutes

Table 8. PCR cycling conditions for CTX-M group 1 (X represents the annealing temperature used. See Table 5).

PCR Step	Temperature and Duration
Initial Denaturation	94°c for 5 minutes
35 Cycles	94°c for 1 minute
	X°c for 30 seconds
	72°c for 5 minute
Final Elongation	72°c for 10 minutes

Table 9. PCR cycling conditions for IncF (X represents the annealing temperature used. See Table 5).

PCR Step	Temperature and Duration
Initial Denaturation	94°c for 5 minutes
35 Cycles	94°c for 1 minute
	X°c for 1 minute
	72°c for 1 minute
Final Elongation	72°c for 5 minutes

Table 10. PCR cycling conditions for IncN and IncP (X represents the annealing temperature used. See Table 5).

PCR Step	Temperature and Duration
Initial Denaturation	94°c for 5 minutes
35 Cycles	94°c for 40 seconds
	X°c for 1 minute
	72°c for 1 minute
Final Elongation	72°c for 5 minutes

Table 11. PCR cycling conditions for Hok-Sok, Pemk and ccdAB (X represents the annealing temperature used. See Table 5).

2.6.5 Preparation of sample for gel electrophoresis

6 x Loading dye was mixed with PCR grade water in a 2:5 dye:water ratio. 7μl of PCR product was mixed with 5μl of 2:5 dye:water mix and loaded into a 1.5% agarose gel and ran at 100V for approximately 90 minutes followed by ethidium bromide staining and visualisation.

2.7 Full procedure workflow

Water samples were collected from the 3 sites of the Sincil Dike shown in Figure 3, 500m upstream of the WWE release (S1), at the point of WWE release (S2) and 500m downstream of WWE release (S3) (2.2.1). Samples were collected twice during the project, then filtered and cultured on *Brilliance* ESBL agar under specific conditions to presumptively identify ESBLEC (2.1.1). Colonies suspected as the organism of interest by *Brilliance* ESBL agar were sub cultured onto MLSA agar (2.1.3) to identify *E. coli* (2.2.2.2), which were sub-cultured in tryptone broth (2.1.9) for confirmation as *E. coli* by the indole test (2.2.3). Once confirmed as *E. coli* all samples were cultured in nutrient broth (2.1.4) and prepared for antibiotic susceptibility testing to confirm ESBL production by the combination antibiotic disk method (2.2.4.2). Samples confirmed as *E.* coli by indole testing and ESBL producing by combination antibiotic disk testing were cultured in nutrient broth (2.1.4). Plasmid DNA of each sample was extracted using a plasmid mini prep kit for future genetic analysis (2.4.1.2) and bacterial isolates were prepared as glycerol stocks for long term storage (2.3.2).

Plasmid extracts were used as template DNA in PCR reaction mixtures (2.6.3) containing a single primer pair specific to the gene detected (2.6.2). PCR reaction mixtures were cycled in a thermocycler (2.6.4) and visualised by gel electrophoresis on a 1.5% agarose gel (2.6.5) followed by ethidium bromide post stain and imaging (2.6.5) to determine the presence of DNA fragments of the expected size (2.6.2). A negative (*E. coli* ATCC 25922) and positive (*E. coli* NCTC 13353) control were used to ensure the validity of CTX-M group 1 PCR results. DNA from these strains was extracted separately to yield both chromosomal and plasmid DNA as CTX-M Group 1 can be both chromosomal and plasmid borne. Control strains for the other genetic elements detected could not be obtained.

Chapter 3 – Isolation of ESBLEC and Characterisation of Circulating Plasmid Genotype

3.1 Introduction

Research has previously shown that WWE contains high levels of ESBL producing *Enterobacteriaceae* despite its stringent cleaning process, and its release into river environments causes an increase in the total numbers of ESBL producing bacteria observed. It is understood that this increase in ESBL production is associated with the introduction of highly prevalent ESBL genes such as the CTX-M family, which are frequently disseminated by horizontal genetic transfer of plasmid DNA (Amos *et al.* 2014). However, less is known about how the addition of WWE affects other secondary genetic factors of bacterial plasmids such as incompatibility group or addiction systems. In the first section of this study ESBLEC were isolated pre, during and post WWE addition to compare the prevalence of ESBLEC and determine the plasmid genetic elements they contain.

ESBLEC were isolated from 3 sites of the Sincil Dike, Lincoln, UK to assess the effect of WWE introduction on numbers of circulating ESBL producing bacteria. Plasmid DNA was extracted from each isolate and characterised by PCR for 3 incompatibility groups, 3 addiction systems and the CTX-M group 1 ESBL genes. These characterised plasmids were then arranged into groups of the common genetic elements they contained, and the occurrence and persistence of these groups was studied before, during and after the addition of WWE. This revealed the effect of WWE on the plasmid genotypes present in isolated ESBLEC and the prevalence of each element detected.

3.2 Results

In total 88 ESBLEC were isolated from all sample sites, 13 from S1 containing 6 unique plasmid genotypes, 40 from S2 containing 15 unique plasmid genotypes and 35 from S3 containing 14 unique plasmid genotypes. Table 12 shows each genotype with the genetic elements it contained, with the number of organisms it was detected in by sample site. Figure 6 shows a bar chart of the number of ESBLEC isolated by sample site.

In text genotype designator	Addiction systems	Incompatibility group	CTX-M group 1 status	Number isolated from S1	Number isolated from S2	Number isolated from S3	
α	3	IncF	+	5	8	5	
β	3	IncF	-	0	5	4	
γ	3	IncF, IncN	+	0	1	1	
δ	3	IncF, IncN	-	0	1	0	
ε	3	IncF, Inc P	-	0	1	0	
ζ	3	None Detected	+	3	0	1	
η	3	None Detected	-	0	0	1	
θ	2	IncF	+	0	2	7	
ι	2	Inc F	-	1	2	2	
κ	2	IncF, IncN	-	0	0	1	
λ	2	IncF, IncP	+	0	1	0	
μ	2	IncF, IncP	-	0	2	0	
ν	2	None Detected	+	2	4	3	
ξ	2	None Detected	-	1	0	0	
o	1	IncF	+	1	2	0	
π	1	IncF	-	0	3	2	
ρ	1	IncF, IncN	-	0	0	1	
σ	1	None Detected	+	0	4	3	
τ	1	None Detected	-	0	2	3	
υ	None Detected	None Detected	+	0	2	1	
			Total	**13**	**40**	**35**	**88**

Table 12. Table of ESBLEC isolated and the plasmids genotype contained by sample site.
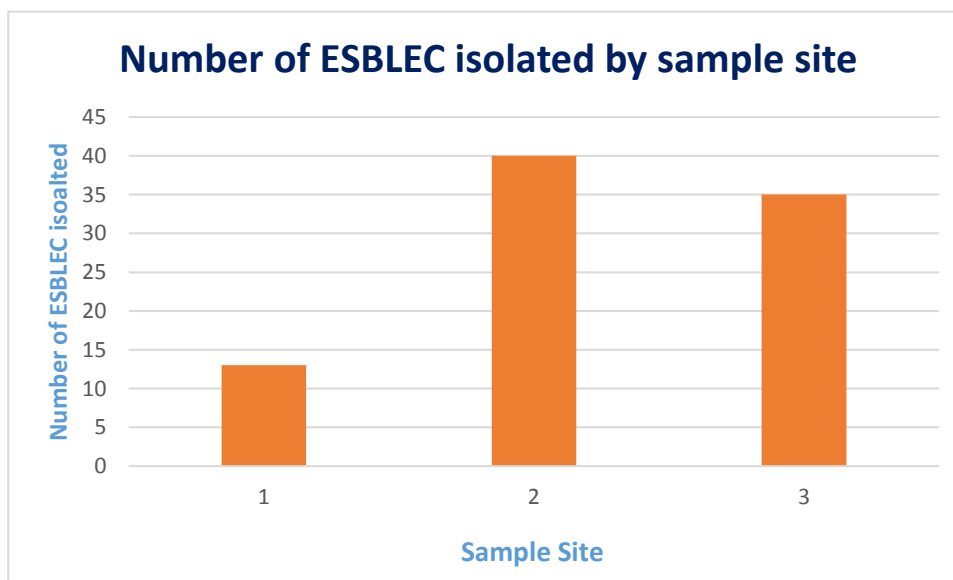

Figure 6. A bar chart to show the number of ESBLEC by sample site

3.3 Discussion

3.3.1 Effect of WWE on Number of ESBLEC Isolated

At the point of WWE release the number of ESBLEC isolated increased from 13 to 40 (Figure 5). An increase in the number of resistant and non-resistant organisms was expected at this site as the result of an in influx of ESBLEC that survived the WWT process, as WWE has been shown in previous study to release over 600 million ESBLEC into a river environment daily (Brechet *et al.* 2014). Amos *et al.* 2014 measured the number of isolated third generation cephalosporin (3GC)-resistant *E. coli* found in river sediment samples upstream and downstream of a WWE release point, and reported similar results of a 7-fold increase in the number of 3GC-resistant bacteria. This suggests the increase in the number of resistant organisms found both at the point of WWE release and further downstream was due to the influx of ESBL producing bacteria shown to be present in WWE.

Further downstream the number of ESBLEC isolated remained higher than in the natural environment but decreased compared to levels observed at the site of WWE release to 35 (Figure 5). This could demonstrate that ESBLEC introduced by WWE were able to persist through the natural environment, however the number begins to decrease as the environment regains its natural stasis.

This stabilisation could be caused by increased competition between newly introduced ESBLEC that have lower survivability in the new environment compared to native organisms.
The number of ESBLEC isolated may also have been affected by the time of year sampling was carried out. A study has shown that a higher rate of faecal *E. coli* shedding occurs in cattle in England and Wales during the summer months, where heavy rainfall can then cause these organisms to be washed into natural aquatic environments where they cause an increase in the number of human *E. coli* infections (Money P *et al.* 2010*)*. This is supported by figures released by the CDC in 2015 which observed an increased infection rate of *E. coli* O157 in the months of July-September, in particular it noted an increase in Waterborne transmission in June and leafy vegetable associated transmission in September (Heiman KE *et al*. 2015). As the Sincil dike is surrounded by agricultural land and sampling carried out in September 2015 and June 2016, there may have been an unusually high number of ELBEC sampled, compared to cooler winter months.

As significantly less ESBLEC were isolated from S1 than S2 or S3, the number of each plasmid type detected at each site was used to compare the prevalence of each plasmid type at a sample site, as the number of each plasmid type detected represents a proportion of the bacterial population at that site. When comparing the prevalence of plasmid types between sample sites a percentage was used due to the significantly different population sizes collected from S1, S2 and S3. This allows us to

determine how the proportion of each plasmid type changes by sample site despite the varying sample size, however this is still a significant limiting factor of this study.

3.3.2 Persistence of Native Plasmid Genotypes

The 13 isolates collected from S1 were arranged into 6 unique genotypes based on the common genetic data collected (Table 12). Plasmid types ι, ξ and o were found in 1 organism each at S1 and showed large variation in the genetic elements they contained. This could be due to the random transformation of genetic material onto host plasmids from the environment, as exogenous DNA has been show to survive in aquatic environments especially when adhered to sand or clay particles, the random nature of genetic transformation also explains the low frequency of these plasmids (Davison, 1999). Plasmid types α, ζ and v were present in 5, 3 and 2 isolates respectively at S1 and represent part of the dominant microbiome in this environment. These dominant species contained 2 or more addiction systems and CTX-M group 1 genes on plasmids of IncF or an undetermined incompatibility group. These types are likely to be more dominant in the natural unaltered environment due to the conferred survivability of CTX-M expression which provides resistance to cefotaxime antibiotics commonly found in UK rivers (Singer *et al.* 2014), while the presence of multiple addiction systems ensures plasmid retention and continued expression of resistance genes. The high frequency of these plasmids is more likely as a result of clonal expansion and conjugation between ESBLEC cells.

At S2 the percentage of isolates containing the dominant plasmid type α and v increased from 38.5% to 20% and from 15.4% to 10% respectively. As the number of these plasmid types appear more frequently at S2 they could be carried on ESBLEC shown to be introduced to the environment by WWE in section 3.4.1, however they represent a smaller proportion of the population at S2 and are no longer the majority type. This also suggests alternate ELBLEC plasmid types were also introduced, however conjugation and clonal expansion of both native and introduced plasmid types may also be responsible if the introduced types have an improved survivability in the new environment. The previously dominant type v plasmid was eliminated from the environment at S2 and did not belong to the highly transferrable IncF incompatibility group observed in other dominant plasmid types. This could cause a decreased rate of conjugation, explaining its removal from the environment. It also suggests conjugation plays an important role in plasmid incidence in this environment.

Further downstream at S3 incidence of type α plasmids fell from 20% to 14.3% of isolates returning to levels observed before the addition of WWE, while incidence of type v plasmids fell from 10% to 3 8.6% of isolates, lower than at the site of WWE release, but still more prevalent than at S1. This shows that native plasmid types were able to persist throughout the river environment and survive

the return to a more stable environment despite a decrease in their overall numbers. The proportion of these plasmid types once again decreased at S3, suggesting that while they can survive in the new environment, the addition of WWE continues to diversify the plasmid population further downstream. These plasmids may contain genetic elements which provide a selective advantage over other organisms in the S2 environment, such as the presence of CTX-M group 1 or other resistance genes, due to the introduction of antibiotic pollutants by WWE (Brown *et al.* 2006). Further downstream these pollutants may be at a lower concentration, therefore presence of resistance genes no longer provides a selective advantage, accounting for the decrease in numbers and proportion of these plasmids observed at S3.

3.3.3 Introduction of Novel Plasmid Genotypes by WWE

With the addition of WWE, the number of unique plasmid types increased from 6 at S1 to 15 at S2 and remained elevated further downstream at S3 with 14 unique plasmid types detected.

At the site of WWE release several new dominant plasmid types appeared, most notably type β plasmids which contained 3 addiction systems on an IncF plasmid, similar to the dominant type α plasmid observed at S1, however CTX-M group 1 was not detected, indicating the presence of an alternate ESBL producing gene. This plasmid type was first observed at the point of WWE release in 12.5% of isolates at S2 and persisted downstream in 10% of isolates at S3, showing that this new dominant genotype is able to persist downstream at a similar level to type α plasmids and its prevalence in the population remains steady. This indicates the diversification of ESBL genes present on dominant plasmid types with the addition of WWE, while the presence of multiple addiction systems and IncF incompatibility group still conferred high survivability and transferability in the new environment.

The significant plasmid types σ and π were also introduced with WWE, observed in 10% and 8.6% of isolates at S2 and survived downstream in 7.5% and 5.7% of isolates at S3 respectively. These plasmid types contained only 1 addiction system and showed variability in the incompatibility group and ESBL gene detected. This shows that while the prolific IncF plasmids containing 3 addiction systems are able to prosper in the environment, other plasmids containing a variety of genes are also conferred increased survivability by the disruption of environment caused by WWE addition. As type τ plasmids contained an unidentified incompatibility group and type ρ contained an unidentified ESBL gene it is impossible to determine why these particular plasmids were able to survive as well as previously established dominant genotypes. This also suggests the genes present on these plasmids confer a similar survivability to the addiction systems and incompatibility groups in previously dominant plasmid types.

Finally, the type θ plasmid containing 2 addiction systems and CTX-M group 1 genes on an IncF plasmid was not detected at S1 but appeared with the introduction of WWE in 5% of isolates at S2, with its incidence increasing downstream detected in 20% of isolates at S3. Type θ plasmids differ from the previously dominant type α plasmid detected at S1 by loss of a single addiction system, therefore It would be expected that these plasmid types would occur at a similar rate throughout the sampled river due to genetic homology, however plasmid type θ becomes the most prevalent plasmid observed at S3. This strongly suggests that extra genetic factors present on the plasmid or chromosome have an important role in the selection and persistence of ESBLEC downstream of WWE release, and may play a more significant role in the maintenance of plasmid borne ESBL genes than the genetic elements detected in this study.

Overall an increase in the number of unique plasmid genotypes and a decrease in the prevalence of the previously most dominant plasmid types was observed with the addition of WWE indicating a significant diversifying event, with these plasmids moving from the previously dominant type A to several new types with a lower incidence, but more likely to contain fewer addiction systems, unknown incompatibility groups and unknown ESBL genes. This could be a result of the introduction of ESBLEC carrying novel plasmid types by the addition of WWE, which proliferate by conjugation and clonal expansion, or the transformation of genetic material to plasmids which did not previously contain ESBL genes. These novel plasmids were also able to persist downstream, though the number and occurrence of each type does not appear to depend on the genetic elements they contained.

3.3.4 Role of Pollutants in Natural Selection of Alternate Plasmid Genotypes

A previous study has shown that WWE introduced to the environment contains a significant number of resistant organisms including the ESBLEC isolated in this study, however it has also been proven that high levels of pollutants such as active antibiotics, heavy metals and disinfectants are also carried by WWE. Addition of these pollutants alters the natural environment in which the studied bacteria exist and shifts the selective pressures placed on these bacteria (Baquero *et al.* 2008; Singer *et al.* 2014).

While this study showed that plasmid addiction and transferability play a significant role in the persistence of native and novel ESBLEC downstream of WWE release, natural selection of organisms expressing resistance genes to pollutants in the environment may also contribute to their persistence. Organisms that do not possess resistance genes to the pollutants added at S2 by WWE release will be removed from the population allowing more resources for those organisms possessing the necessary resistance genes. This natural selection ensures the bacterial population

expresses a high level of resistance as bacterial cells incorporate genes which confer resistance to the variety of pollutants now present in the environment. Ultimately, the persistence of plasmid types throughout the river environment depends on its ability to confer resistance to pollutants present in the new environment to its bacterial host.

3.3.5 Issues with Sampling and Isolation

The large variation in the number of isolated ESBLEC between S1 and S2 may affect the comparison of the incidence of plasmid genetic elements. 100ml of water sample from each site was filtered onto *Brilliance* ESBL plates in an equal number of replicates with all identifiable ESBLEC sub-cultured for further testing. Therefore, discrepancy in the number of isolates was due to a lower CFU/ml of those organisms in each water sample as opposed to inconsistent sample collection and isolation. This change in sample size could lead to a lower detection of genes studied later at S1 when compared to S2 which may be falsely attributed to WWE release. As this study focuses on the presence of selected genes on ESBLEC rather than their overall occurrence in the environment, genetic data will be presented as the increase in raw numbers of isolates positive for the gene of interest at each sample site, allowing for direct comparison despite variation in sample number.

The location for sample collection (Sincil Dike, Lincoln, UK) could not be reached during winter which limited sampling opportunities to once in September and once in June. This sample can only be representative of the environment at the time of collection as other factors may affect the studied parameters. As mentioned previously the prevalence of *E. coli* infection is higher during June and September (Department of Health, 2001), in humans a higher rate of water borne and food borne by leafy greens transmission rate is observed (Heiman *et al.* 2015). This higher incidence of infection would cause an increase in the number of ESBLEC detected in WWE.

3.3.6 Evaluation of *Brilliance* ESBL Agar for the Isolation of ESBLEC

Brilliance ESBL agar plates were used for the presumptive identification of ESBL producing bacteria, and in this study used to identify ESBLEC. 26/114 (22.8%) isolates shown to be ESBLEC by *Brilliance ESBL* agar were shown by further testing to be incorrectly identified, giving *Brilliance* ESBL agar an overall specificity of 77.2% for the identification of ESBLEC. However the *Brilliance* ESBL agar data sheet states a specificity of 94%, this could lead to the inclusion of non-ESBLEC if supporting biochemical testing is not used due to the increased false positive rate. Distinguishing between blue or green colonies on *brilliance* ESBL agar plates was difficult due to similarity in appearance and may have resulted in the incorrect selection of *K. pneumoniae* (Green) instead of *E. coli* (Blue), responsible some of the observed low specificity.

The sensitivity of *Brilliance* ESBL agar could not be calculated as colonies indicated as non-ESBLEC were not isolated or verified by further testing, therefore the number of false negatives is unknown. The agar data sheet states sensitivity as 95%, if this figure is also inaccurate many ESBLEC colonies may not have been isolated. This could mean that the number of ESBLEC isolated from each sample site is not representative of the bacterial population.

3.4 Conclusion

Water samples were collected from Sincil dike, Lincoln, UK upstream, at the point and downstream of WWE release and filtered onto *Brilliance* ESBL agar for the isolation of ESBLEC. Numbers of ESBLEC isolated increased following the introduction of WWE at S2 consistent with current research, which was attributed to the introduction of WWE shown to carry high levels of resistant bacteria. Plasmid was extracted from each ESBLEC isolated and characterised for a range of addiction systems, incompatibility groups and CTX-M group 1 family of ESBL genes. This genetic data was then used to arrange isolates into common plasmid types. It was discovered that before the introduction of WWE there were few unique plasmid types, of which type A F and M formed part of the dominant microbiome at this site, some of which were able to persist downstream of WWE and throughout the river environment. With WWE addition there was an increase in the number of unique plasmid types observed, with a transition from few common type to many less frequent type, many of which contained fewer addiction systems, fewer IncF and fewer CTX-M group 1 genes than previously observed, these again were able to persist downstream but which plasmids were more prevalent at each site appeared to depend on the transferability of the plasmid with possible influence from other genetic elements not detected. Overall the introduction WWE to the environment caused a significant increase in the number and diversity of circulating plasmid genotypes of ESBLEC.

Chapter 4 – Effect of WWE on Prevalence of CTX-M Group 1

4.1 Introduction

In the previous chapter 88 ESBLEC were isolated from 3 sites of Sincil Dike, Lincoln, UK and it was found that introduction of WWE increased the number of ESBLEC isolated, and increased the genetic diversity of the plasmid types present by the introduction of resistant organisms with WWE. The organisms were able to persist throughout the 100m river environment tested, though survivability was dependant on the genetic elements the plasmid contained. In this section the prevalence of CTX-M group 1 amongst the ESBLEC isolated in Chapter 3 was determined and the effect of WWE addition om incidence of CTX-M group 1 presence amongst ESBLEC plasmids was studied.

4.2 Results

Sample Site	Number of isolates	Number of CTX-M group 1 positive isolates	% CTX-M group 1 positive isolates
S1	13	11	84.6
S2	40	25	62.5
S3	35	21	60.0
Total	88	57	64.8

Table 13. Number and percentage of isolates positive for CTX-M group 1 by sample site
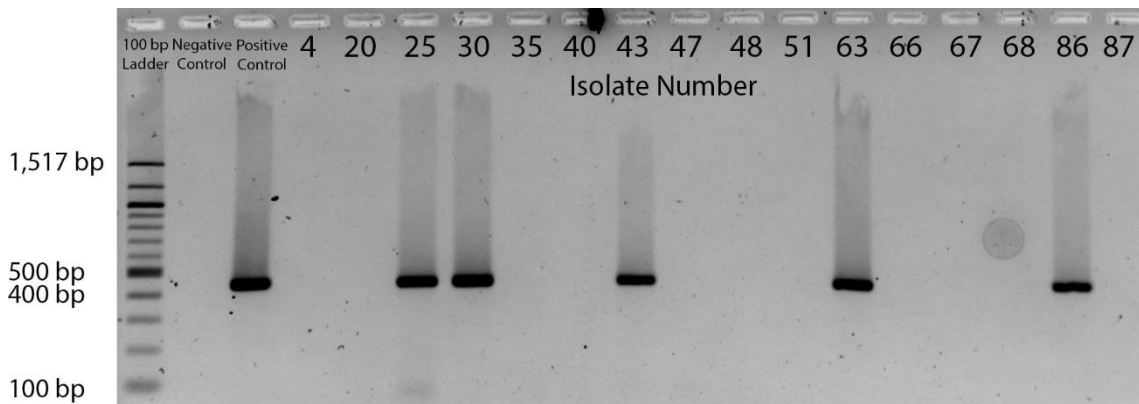
Figure 7. An example electrophoresis agarose gel for PCR products obtained by CTX-M group 1 amplification. Lane 1 shows a 100bp DNA ladder, Lane 2 contained the negative control (*E.coli* ATCC 25922) and showed no amplification while lane 3 contained the positive control (*E.coli* NCTC 23352) and yielded a DNA fragment at the expected size of 415 bp. Lanes 6, 7, 10, 14 and 18 contain samples 25, 30, 43, 63 and 86 and were positive for CTX-M group 1 showing a 415 bp band. Lanes 4, 5, 8, 9, 11, 12, 13, 15, 16, 17 and 19 contain samples 4, 20, 35, 40, 47, 48, 51, 66, 67, 68 and 87 which were negative for CTX-M group 1 as they showed no PCR product.

4.3 Discussion

4.3.1 Effect of WWE on Prevalence of CTX-M Group 1

Overall, CTX-M group 1 was detected in 57 (64.8%) samples and explained the majority of ESBL phenotypes at all sample sites. This is consistent with studies which have reported that CTX-M type genes are the most prevalent ESBL producing genes detected in natural aquatic environments similar to that sampled in this study, with CTX-M group 1 the most prevalent CTX-M group detected in municipal waste water. This high incidence of CTX-M genes in the environment is due to horizontal transfer of plasmids between bacteria (Brechet *et al.* 2014; Borgogna *et al.* 2016).

A study mentioned in the previous chapter by Amos *et al.* 2014 demonstrated that WWE increases the numbers of 3GC-resistant organisms along with showing that the presence of CTX-M genes increased with WWE addition, and attributed this to the introduction of CTX-M carrying bacteria by WWE that was able to undergo horizontal gene transfer. In this study the number of CTX-M group 1 positive samples increasing from 11 (84.6%) before the introduction of WWE at S1 to 25 (62.5%) at the site of WWE release, S2, while the percentage that were CTX-M group 1 positive decreased. Between S2 and S3 the number and percentage of CTX-M group 1 positive organisms decreased to 21 (60%), consistent with the decrease in ESBLEC isolated at S3.

This increase in the numbers with decreasing percentage of the population positive for CTX-M group 1 shows that while WWE addition increases the instance of CTX-M group 1 amongst ESBLEC plasmids, its representation in the population decreases. This can be explained by the introduction of alternate ESBL producing genes carried by ESBLEC at the site of WWE release which were not detected in this study such as SHV, TEM or other CTX-M groups alongside the introduction of CTX-M group 1 carrying ESBLEC, resulting in the diversification of circulating ESBL genotypes. Amos *et al* 2014 found high incidence of the ESBL producing gene TEM alongside CTX-M upstream and downstream of WWE release, if similar TEM expression is present in the river sampled in the current study it is possible that plasmids containing CTX-M group 1 undergo less clonal expansion and conjugation due to competition from TEM expressing organisms. Downstream of WWE release percentage of CTX-M group 1 decreases slightly and remains lower than observed before the addition of WWE indicating that while ESBLEC carrying novel ESBL genes are able to persist downstream, they may begin to diminish due to competition from CTX-M group 1 carrying ESBLEC which show resistance to cefotaxime antibiotics present in the environment.

4.3.2 Potential issues with Chromosomal Contamination

Plasmid DNA was extracted from ESBLEC isolates using NEB plasmid mini prep kits. These kits required a detergent to initiate cell lysis and release chromosomal and plasmid DNA, followed by a sodium hydroxide solution to increase pH and cause denaturation of DNA from a double stranded to a single stranded conformation. Potassium acetate was then used to acidify the solution and cause the renaturing of plasmid DNA while chromosomal DNA remained single stranded and precipitated out of solution. This precipitate was centrifuged into a pellet to leave pure plasmid DNA in solution, however carryover of some chromosomal DNA into the plasmid sample was possible. As CTX-M group 1 is commonly found both on plasmid and chromosomal DNA it is possible that chromosomal contamination could lead to false identification of CTX-M group 1 genes on plasmid DNA. In future to prevent chromosomal carryover affecting PCR results plasmid extracts will be transformed into an attenuated *E. coli* laboratory strain such as DH5α to allow greater efficiency during extraction. Chromosomal carryover from DH5α is still possible but would not contain any gene detected in this study and therefore would not yield a false positive result.

4.4 Conclusion

PCR amplification of CTX-M group 1 genes was performed on 88 ESBLEC isolates to determine the prevalence and effect of WWE on CTX-M group 1 genes amongst circulating ESBLEC. CTX-M group 1 was found in the majority of samples at all sites and explained the ESBL phenotype in most isolates. Following the addition of WWE, the number of CTX-M group 1 positive organisms increased noticeably and began to return to levels observed before WWE release further downstream, consistent with the change in the number ESBLEC isolated previously. It is suspected that introduction of other ESBL genes such as SHV and TEM, which were found at high incidence with CTX-M in other studies, were introduced by WWE and diversified the genetic source of ESBL production by horizontal gene transfer and clonal expansion of novel ESBLEC. These results demonstrate the ability of introduced ESBLEC by WWE to persist downstream of WWE release, however it appears that native ESBLEC species are able to out compete novel ESBLEC possibly due to increased resistance to cefotaxime antibiotics commonly found in this type of environment.

Chapter 5 – Effect of WWE on Prevalence of Incompatibility Group

5.1 Introduction

In the previous chapter the prevalence of CTX-M group 1 genes was studied, and the effect of WWE release on incidence of CTX-M group 1 carried on ESBLEC plasmids determined. CTX-M group 1 expression explained the ESBL phenotype in the majority of samples, with the number of CTX-M positive samples increasing with WWE introduction, consistent with the previously established rise in ESBL producing organisms. This was attributed to the introduction of novel ESBL genes by WWE which were allowed to proliferate, resulting in the diversification in observed ESBL genotypes.

Plasmid associated ESBL genes such as CTX-M are easily disseminated by conjugation in which a plasmid is transferred to a recipient cell and undergoes successful replication. To prevent competition between plasmids of similar origin, each contains a collection of genes known as an incompatibility group, members of each group cannot successfully replicate in the same cell leading to removal of one or both plasmids.

In this section the same PCR techniques from chapter 3 were used to establish the presence of 3 incompatibility groups, Inc F, N and P in each plasmid sample. These groups were selected based on their previous association with ESBLEC and availability of singleplex PCR methodology. Prevalence of each group and association with CTX-M group 1 genes before and after the introduction of WWE was studied to assess its effects on numbers of circulating plasmid incompatibility groups

5.2 Results

5.2.1 IncF

Sample Site	Number of ESBLEC isolates	Number of IncF positive samples	% IncF positive samples	Number of IncF plasmids positive for CTX-M group 1	% IncF plasmids positive for CTX-M group 1 positive
S1	13	7	53.8	6	85.7
S2	40	28	70.0	14	51.9
S3	35	23	65.7	13	56.5
Total	88	58	65.9	33	57.9

Table 14. Number and percentage of IncN positive samples with number and percentage of those positive for CTX-M group 1
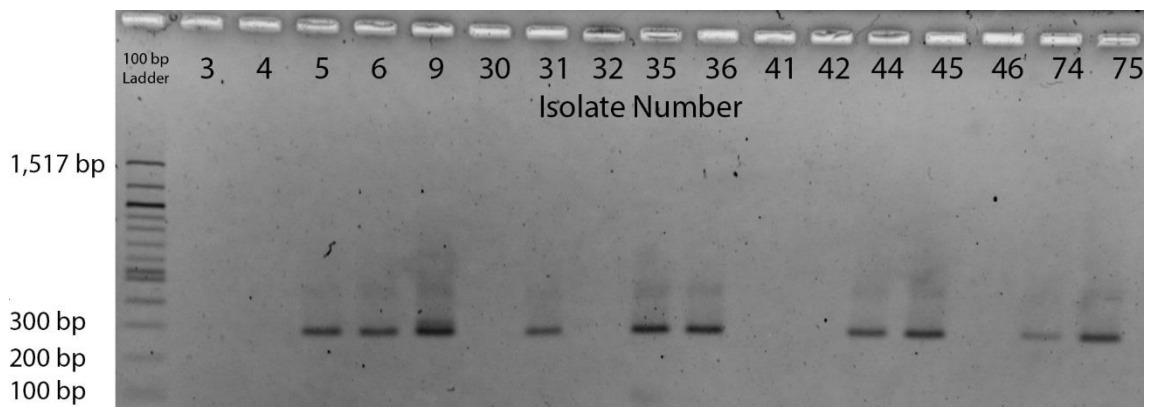
Figure 8. An example gel for PCR products obtained from Inc F amplification. Lane 1 shows a 100bp DNA ladder, lanes 4, 5, 6, 8, 10, 11, 14, 15 and 18 contain samples 5, 6, 9, 31, 35, 36, 44, 45, 74 and 75 and were positive for Inc F showing an approximately 270 bp band. Lanes 2, 3, 7, 9, 12, 13, 16 and 75 contain samples 3, 4, 30, 32, 41, 42 and 46 which were negative for Inc F as they showed no PCR product. Positive and negative controls could not be obtained for this PCR.

5.2.2 IncN

Sample Site	Number of ESLBEC isolates	Number IncN positive samples	% IncN positive	Number of IncN and CTX-M group 1 positive samples	% IncN plasmids positive for CTX-M group 1 positive
S1	13	0	0.0	0	0.0
S2	40	2	5.0	1	50.0
S3	35	2	5.7	1	50.0
Total	88	4	4.5	2	50.0

Table 15. Number and percentage of IncN positive samples with number and percentage of those positive for CTX-M group 1
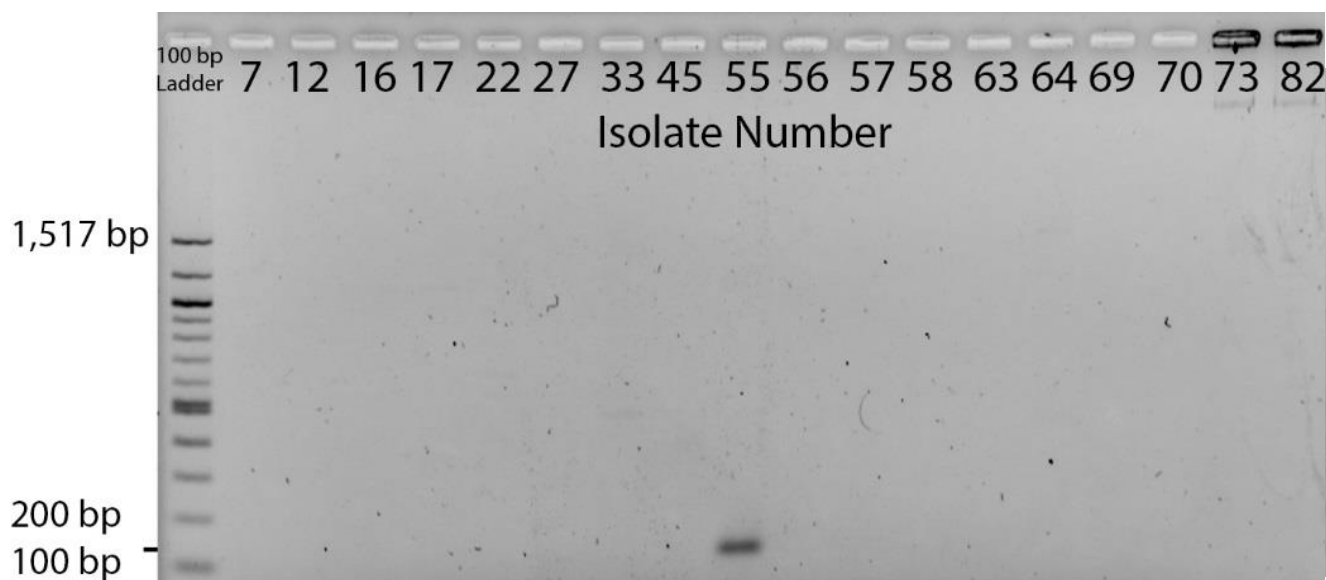
Figure 9. Example electrophoresis agarose gel of PCR products obtained by IncN amplification. Lane 1 shows a 100bp DNA ladder, lanes 6, 8, 12 and 16 contain samples 18, 23, 66 and 75 and were positive for IncN showing an approximately 164 bp band. Lanes 2, 3, 4, 5, 7, 9, 10, 11, 13, 14, 15, 17, 18 and 19 contain samples 5, 7, 8, 10, 14, 20, 33, 45, 54, 68, 69, 70, 80 and 82 which were negative for IncN as they showed no PCR product. Positive and negative controls could not be obtained for this PCR.

5.2.3 IncP

Sample Site	Number of ESBLEC isoaltes	Number IncP positive samples	% IncP positive	Number of IncP and CTX-M group 1 positive samples	% IncP and CTX-M group 1 positive
S1	13	0	0.0	0	0.0
S2	40	3	7.5	0	0.0
S3	35	0	0.0	0	0.0
Total	88	3	3.4	0	0.0

Table 16. Number and percentage of IncP positive samples with number and percentage of those positive for CTX-M group 1

Figure 10. Example electrophoresis agarose gel of PCR products obtained by Inc P amplification. Lane 1 shows a 100bp DNA ladder, lane 10 contains sample 55 which was positive for Inc P showing an approximately 110 bp band. Lanes 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 17, 18 and 19 contain samples 7, 12, 16, 17, 22, 27, 33, 45, 46, 58, 63, 64, 69, 70, 73 and 82 which were negative for Inc P as they showed no PCR product. Positive and negative controls could not be obtained for this PCR.

5.3 Discussion

5.3.1 Effect of WWE on Prevalence of IncF Plasmids & Association with CTX-M Group 1

Overall, IncF was observed in 58 (65.9%) isolates and was the most represented incompatibility group tested. A study has shown IncF to be the most prevalent incompatibility group amongst plasmids harbouring ESBL genes in *E. coli* and is most often associated with CTX-M ESBL genes, particularly CTX-M group 1 members CTX-M-1, and -15 (Marcade *et al.* 2009). The percentage of IncF plasmids detected increased at the point of WWE from 53.8% at S1 to 70.0% at S2. This shows that WWE introduced ESBLEC containing IncF plasmids at a higher rate than that already seen in the environment, increasing the dominance of this incompatibility group. Further downstream the number of ESBLEC carrying IncF plasmids decreased to 65.7%, this subsequent decrease following WWE addition could represent the stabilisation of the ESBLEC community as numbers of IncF plasmids return to pre-WWE levels suggesting introduced IncF plasmids are unable to persist as well as native IncF plasmids.

IncF plasmids were found to contain CTX-M group 1 genes in 57.9% of cases, comparable with results obtained by Wang *et al.* 2009 which found IncF CTX-M positive plasmids in 64.3% of clinical isolates, while Amos *et al.* 2014 commented that IncF was the most associated incompatibility group with CTX-M positive samples in river sediment receiving WWE. At the site of WWE release the number of IncF plasmids containing CTX-M group 1 increased from 6 to 14 and remained stable at 13 at S3, consistent with the increase seen in the number of CTX-M group 1 positive isolates previously. This indicates that IncF plasmids carrying CTX-M group 1 genes contribute significantly to the increase in CTX-M group 1 observed in chapter 4. However, the percentage of IncF plasmids carrying CTX-M group 1 genes decreased between S1 and S2 from 85.7% to 51.9% and increasing to 56.5% at S3. This suggest that while IncF plasmids carrying CTX-M group 1 genes are present in WWE, IncF plasmids carrying alternate ESBL genes also comprises a significant number of ESBL carrying plasmids in WWE.

Marcade *et al.* 2009 found that IncF plasmids were commonly associated with the CTX-M group 1 gene CTX-M-15 and the CTX-M group 9 gene CTX-M-14, while Wang, *et al* 2009 found SHV genes the second most commonly IncF associated ESBL gene behind CTX-M, with no instance of TEM type genes associated with IncF plasmids. If WWE in this study contains a similar distribution of ESBL genes as observed in other studies, it is likely that the increase in IncF plasmids with decreasing association with CTX-M group 1 at the point of WWE release is due to the introduction of IncF plasmids carrying SHV and CTX-M-14 genes which were not detected in this study. These plasmids are able to proliferate easily among ESBLEC by conjugation due to the transformability of IncF plasmids leading to an overall diversification of available ESBL genotypes.

5.3.2 Effect of WWE on prevalence of IncN plasmids & association with CTX-M group 1

IncN was identified in 4 (4.5%) samples across all sites and was only identified after the addition of WWE, in 2 (5%) samples from S2 and 2 (5.7%) samples from S3. Inc N has been detected in clinical ESBLEC isolates at low levels consistent with IncN prevalence found in this study (Carattoli, 2011). As WWE introduced to the Sincil Dike may contain waste of a clinical origin from the nearby hospital it is possible that IncN plasmids were introduced to the environment through WWE. However, IncN plasmids carrying ESBL genes are more commonly associated with ESBLEC in animal infection. A significant reservoir of IncN plasmids carrying ESBL genes can be found in commercial agricultural settings such as poultry, pig, cow and dairy farms (Dolejska *et al.* 2011), which can be found surrounding the Sincil Dike as Lincolnshire contains a large area of farmland. Heavy rainfall can cause introduction of agricultural waste to aquatic environments such as rivers and could be responsible for the introduction of IncN plasmids at S2 and S3 (Bortolaia *et al.* 2011; Hammerum *et al.* 2014). IncN plasmids of an animal origin are commonly associated with CTX-M type ESBL genes from several groups, a study of CTX-M positive ESBLEC on commercial duck farms revealed presence of several CTX-M genes including CTX-M group 1 genes CTX-M-14, -24 and -27, while a study of a dairy farm found 39% of ESBLEC samples were positive for CTX-M group 1 enzyme CTX-M-1, the majority of which were carried on IncN plasmids, consistent with the 50.0% of the IncN samples detected in this study which were positive for CTX-M group 1.

5.3.3 Effect of WWE on Prevalence of IncP Plasmids & Association with CTX-M Group 1

Inc P was identified in 3 (7.5%) samples and was the least represented incompatibility group tested. IncP is less commonly found in the natural environment but has been identified in ESBLEC isolated from WWE at similar levels to those identified in this study. No IncP positive samples were found to contain CTX-M group 1 genes which is expected as OXA genes, a Class D ESBL enzyme which shows hydrolytic activity against oxacillin antibiotics and are commonly found in *E. coli*, are most commonly associated with IncP plasmids (Tennstedt *et al.* 2003; Schluter *et al.* 2007). IncP was only detected at the site of WWE release suggesting it was introduced by WWE and not a naturally occurring incompatibility group in this environment. It was also unable to persist downstream suggesting the IncP plasmid was quickly eliminated from its host despite containing 2-3 addiction systems, which should ensure its maintenance in host species, however elimination from the small sample may also be due to the relatively low number of IncP plasmids present in the environment.

IncP occurred at low frequency, detected in approximately 1 in every 13 samples and therefore may have been present upstream at S1 where sample size was significantly smaller. If IncP was in fact present upstream and at the point of WWE release but not detected further downstream, it could

indicate elimination of the bacterial host by natural selection as they did not contain CTX-M group 1 genes which appear to provide a selective advantage in this environment.

5.3.4 Occurrence of Multiple Incompatibility Systems

IncP and IncN groups were only detected in samples also positive for IncF in all cases. Data from previous study has demonstrated that IncP and IncN can exist alone and in the presence of IncF (Carattoli *et al.* 2005).Two scenarios exist for the possible appearance of multiple incompatibility groups in one sample, either a single plasmid belongs to multiple incompatibility groups simultaneously, or 2 unique plasmids of different incompatibility groups are able to replicate successfully in a single bacterial cell. Plasmids that contain multiple incompatibility groups show an increased ability to transfer genetic material by conjugation due to an increased host range, however both IncP and IncN were found in relatively low proportions suggesting several unique plasmids are responsible. If this is the case, genetic data collected from these samples would be representative of several plasmids working in unison as opposed to a single unique plasmid, however as they express their genotype together the genetic data collected is still relevant for the individual bacterial cell, but may affect results for grouping of plasmid genetic elements.

5.4 Conclusion

PCR amplification of 3 incompatibility groups was performed to understand the effect of WWE on prevalence of circulating incompatibility groups amongst plasmids of ESBLEC and to determine their association with CTX-M group 1. IncF was the most common group detected occurring in approximately half of all samples and increased with the introduction of WWE due to an influx of ESBLEC containing IncF plasmids, however the percentage of IncF plasmids positive for CTX-M group 1 decreased. This is potentially a result of the same diversifying effect of WWE addition mentioned in chapters 3 and 4, caused by the introduction of ESBLEC carrying IncF plasmids bearing novel ESBL genes which proliferate by conjugation and clonal expansion throughout the ESBLEC population.

IncN and IncP were detected infrequently only after the addition of WWE. IncN is commonly associated with CTX-M group 1 expression in commercial farm animals but has also been identified in some clinical isolates. IncP has been previously been detected in WWE and contained the ESBL genes OXA responsible for resistance to oxacillin. It is possible that both IncP and IncN were introduced to the environment at S2 by WWE, however IncN plasmids were more likely introduced by waste run off from neighbouring farms. This demonstrates the WWE is able to introduce novel genetic elements to natural environments to increase genetic diversity, however they may not be able to persist further downstream due to lower survivability in the environment compared to native organisms. As InP and IncN were detected in less than 1 in every 13 samples at S2 and S3, they may

both have been present at S1 but the sample number was too low to be detected. As such IncP and IncN may be present throughout the river environment, and were not introduced by WWE. IncN and IncP were only detected in samples positive for IncF, similar results have been reported for the co-existence of IncP, N and IncF groups in a single plasmid. This can also be explained by the presence of multiple unique plasmids in a single isolate which would mean genotypic elements detected may be describing several plasmids working in unison. The diversification of incompatibility groups and CTX-M group 1 genes seen with the addition of WWE in this section supports the idea that WWE introduces plasmids with a variety of genetic elements which are able to disseminate by conjugation and clonal expansion to diversify ESBLEC genotypes.

Chapter 6 – Overall Conclusion

It has been long understood that the global antibiotic resistance crisis is a significant threat to human health with the majority of available antibiotics predicted to be redundant in a few decades. Research has mainly focused on the occurrence of resistant organisms in a clinical setting but recently has turned to the occurrence and monitoring of antibiotic resistance in the environment. A major source of this resistance has been found to originate from treated waste water that contains significant levels of resistant organisms following inadequate water treatment which is then released back into natural aquatic environments shared by humans and animals alike. In particular, the extended spectrum beta-lactamase expressing *Enterobacteriaceae* family has been implicated in clinical infection and found commonly in natural aquatic environments, with the CTX-M gene family implicated as the major genetic source of ESBL expression in a number of species. The CTX-M gene has rapidly spread globally due to its frequent association with mobile plasmids that are easily disseminated through bacterial populations by horizontal gene transfer. WWE release sites have also been identified as a major site of plasmid transfer and is speculated to significantly contribute to environmental antibiotic resistance. This study confirmed results found in comparative research that the addition of WWE to natural aquatic environments measurably increases the number of ESBLEC present when compared to the same environment that has not received WWE. Further genetic analysis of the plasmids contained within ESBLEC isolated from these environments indicated that the increase in resistance is as a result of an increase in the diversity of plasmid genetics as opposed to the amplification of plasmid genotypes naturally present in the environment. The introduction of WWE saw a shift from few dominant plasmid types to a large number of genetically diverse plasmids less likely to contain CTX-M group 1 genes in favour of other undetected ESBL genes, there was also a marked decrease in the number of plasmid addiction systems present. The organisms introduced by WWE were shown to frequently contain plasmids of an IncF incompatibility group and were less commonly associated with CTX-M group 1 genes than their natural counterparts, they were also shown to persist further downstream of WWE release and were able to outcompete naturally occurring plasmid types in some cases while others were eliminated shortly after their introduction. Which plasmids were able to persist and which were eliminated appeared to depend on the plasmids ability to transfer horizontally through the population and be maintained with the host cell. However, some of the most dominant plasmid types downstream of WWE release contained few of the genetic elements associated with stable inheritance and maintenance in favour of CTX-M group 1 genes which provided increased survivability, suggesting plasmid prevalence is also related to the clonal expansion of host cells. It is clear that WWE release has a dramatic effect on the levels of resistance observed in natural aquatic environments which is accompanied by a diversification of plasmid genetic elements responsible for expression of this resistance, however it was difficult to determine which plasmid genetic elements were the primary cause of plasmid dissemination due to

the small nature of this study. In reality it is probably a combination of genetic factors which results in plasmid dissemination and spread of antibiotic resistance genes in the environment. A larger scale study has been proposed to better understand which factors should be monitored more closely to prevent further dissemination.

The diversification of antibiotic genes in the environment is a bad omen for the occurrence of antibiotic resistant organisms in clinical practice as these large natural aquatic environments can act as reservoirs for ESBLEC, resulting in their return back to clinical environments where they are able to cause infection with a poor outcome for the patient.

Chapter 7 – Further Study

This study revealed the significant role WWE addition plays in diversification of ESBLEC plasmid genetic elements and the increase in ESBL producing organisms in a single UK river in a small time frame. While these results are important in understanding exactly how WWE addition effects our natural waterways, the focused nature of the study means the same conclusions may not be relevant in all situations where WWE is released into the natural environment. Future study would use more effective methods of gene detection and arrangement into genetic plasmid types and use a wider data set to truly understand the dynamic relationship between antibiotic resistance in the environment and the large scale release on improperly treated WWE. A larger scale study could collect a larger number of water samples from more sites up and downstream of WWE at regular intervals throughout the year to gain an accurate insight into the changing dynamics of this complex environment and the bacteria it contains.

A wider range of genetic elements would also be detected, including more addiction systems, incompatibility groups and ESBL genes as well as gene families not detected in this study such as metal resistance genes. More advanced gene methods would be used, such as sanger sequencing and next generation sequencing platforms which allow an entire plasmid genome to be sequenced quickly and accurately. This data could then be compared to a database of known plasmid genetic elements, revealing not only the specific elements each plasmid contains, but also the genetic context in which they are found. A better understanding of the genetic elements and their context contained on these plasmids could potentially reveal which elements are responsible for the persistence or elimination of specific plasmid types with the introduction of WWE. Alongside this wealth of genetic data, study of antibiotic, heavy metal and disinfectant content of the water samples may also reveal the selective pressures placed on organisms at each sample site and aid in the understanding of which genes confer increased survivability.

This large scale study performed over several years would provide a much deeper insight into the microbiome of natural aquatic environments and allow us to ascertain exactly how bacteria introduced by WWE interact with native bacteria. This study would be repeated in multiple rivers receiving WWE around the country, with the collaboration of other higher education institutes located near river environments also receiving WWE, to compare the effect of WWE originating from different treatment facilities. Comparison of the treatment methods used in each facility could reveal which methods produce cleaner, safer WWE which would be used to reduce the disruption to natural microbiomes and prevent dissemination and diversification of ESBLEC plasmid genotypes. This could be achieved by collaboration with industry figures such as Anglian water and other local water authorities who could provide water samples and details of water treatments process, these companies may be willing to help as they have a vested interest in the safety of WWE released into

the environment. This would aid in the reform of waste water treatment processes and ensure safety of natural aquatic environments around the country. To establish which genetic elements are most important in dissemination and persistence of introduced EBLEC plasmid types an *in vitro* model of a river environment would be created with use of a chemostat to maintain a stable chemical environment similar to that which occurs naturally. This model could be used to assess transfer of a variety of naturally occurring and introduced ESBLEC plasmid types to ascertain which are more likely to be transferred horizontally throughout the bacterial population and develop strategies to interfere with this transfer.

Due to the rural nature of the sampling area (Lincoln, UK), some regions in the local area utilise a private water treatment facility shared by few households, and in some cases a single family. This arrangement can be used as a model to monitor how diet, medical history or current antibiotic treatment effects the number and type of ESBLEC plasmid released. This data could then be used to determine likely sources of ESBLEC within the population, and develop strategies for minimising their accumulation.

By collecting this information from a wide range of ESBLEC plasmids we can begin to minimise the effect of WWE release on the natural environments shared by both humans and animals and decrease levels of environmental resistance. Elimination of this environmental resistance reservoir could aid in the prevention of clinically significant ESBLEC infection and contribute to the reduction of antibiotic resistance globally.

Appendix A – Full Table of Results

New Systematic Label	Location	CTX-M Group 1 Status	Incompatibility Group					Addiction System		
			IncF	Inc N	Inc P	Inc W	Inc Q	HokSok	ccAB	PemK
1	S1	+	-	-	-	-	-	+	+	+
2	S1	+	-	-	-	-	-	+	+	-
3	S1	+	-	-	-	-	-	+	+	+
4	S1	-	-	-	-	-	-	+	-	+
5	S1	+	+	-	-	-	-	+	+	+
6	S1	+	+	-	-	-	-	+	+	+
7	S1	+	-	-	-	-	-	+	+	-
8	S1	+	-	-	-	-	-	+	+	+
9	S1	+	+	-	-	-	-	+	+	+
10	S1	+	+	-	-	-	-	+	+	+
11	S1	+	+	-	-	-	-	+	+	+
12	S2	+	+	-	-	-	-	-	+	-
13	S2	+	-	-	-	-	-	-	+	-
14	S2	+	+	-	-	-	-	-	+	+
15	S2	+	+	-	-	-	-	-	+	+
16	S2	+	-	-	-	-	-	-	-	-
17	S2	+	+	-	-	-	-	-	+	-
18	S2	+	+	+	-	-	-	+	+	+
19	S3	-	-	-	-	-	-	-	+	-
20	S3	-	+	-	-	-	-	+	-	-
21	S3	+	+	-	-	-	-	+	+	+
22	S3	+	-	-	-	-	-	+	+	+
23	S3	-	+	+	-	-	-	-	+	-
24	S3	+	+	-	-	-	-	+	+	+
25	S3	+	+	-	-	-	-	+	-	+
26	S3	-	+	-	-	-	-	-	+	-
27	S3	+	-	-	-	-	-	-	-	-
28	S3	-	+	-	-	-	-	+	-	+
29	S3	-	+	-	-	-	-	+	+	+
30	S3	+	-	-	-	-	-	-	+	-
31	S3	-	+	-	-	-	-	-	+	+
32	S3	+	-	-	-	-	-	-	+	+
33	S3	+	-	-	-	-	-	-	+	+
34	S3	+	+	-	-	-	-	-	+	+
35	S1	-	+	-	-	-	-	-	+	+
36	S1	+	+	-	-	-	-	-	+	-
37	S2	+	-	-	-	-	-	-	+	-
38	S2	+	+	-	-	-	-	+	+	+
39	S2	+	-	-	-	-	-	-	+	+
40	S2	-	+	-	+	-	-	-	+	+
41	S2	+	+	-	-	-	-	+	+	+

42	S2	-	-	-	-	-	-	-	+	-
43	S2	+	-	-	-	-	-	-	+	+
44	S2	+	+	-	-	-	-	+	+	+
45	S2	+	+	-	-	-	-	-	+	+
46	S2	+	-	-	-	-	-	-	+	+
47	S2	-	+	-	-	-	-	+	+	+
48	S2	-	+	-	-	-	-	-	+	-
49	S2	+	+	-	-	-	-	+	+	+
50	S2	+	-	-	-	-	-	-	+	-
51	S2	-	+	-	+	-	-	-	+	+
52	S2	-	+	-	-	-	-	+	+	+
53	S2	+	-	-	-	-	-	-	+	+
54	S2	-	+	-	-	-	-	+	+	+
55	S2	-	+	-	+	-	-	+	+	+
56	S2	-	+	-	-	-	-	-	+	+
57	S2	+	-	-	-	-	-	-	+	-
58	S2	-	-	-	-	-	-	-	+	-
59	S2	-	+	-	-	-	-	+	+	+
60	S2	-	+	-	-	-	-	-	+	-
61	S2	+	+	-	-	-	-	+	+	+
62	S2	+	+	-	-	-	-	+	+	+
63	S2	+	-	-	-	-	-	-	+	-
64	S2	+	+	-	-	-	-	+	+	+
65	S2	+	+	-	-	-	-	+	+	+
66	S2	-	+	+	-	-	-	+	+	+
67	S2	-	+	-	-	-	-	+	+	+
68	S2	-	+	-	-	-	-	+	+	-
69	S2	+	-	-	-	-	-	-	-	-
70	S3	+	-	-	-	-	-	-	+	-
71	S3	+	+	-	-	-	-	+	+	+
72	S3	+	+	-	-	-	-	-	+	+
73	S3	+	+	-	-	-	-	+	+	+
74	S3	+	+	-	-	-	-	+	+	-
75	S3	+	+	+	-	-	-	+	+	+
76	S3	+	-	-	-	-	-	-	+	-
77	S3	+	+	-	-	-	-	-	+	+
78	S3	+	+	-	-	-	-	-	+	+
79	S3	-	+	-	-	-	-	+	+	+
80	S3	-	-	-	-	-	-	+	+	+
81	S3	-	-	-	-	-	-	-	+	-
82	S3	-	+	-	-	-	-	+	+	+
83	S3	-	+	-	-	-	-	-	+	+
84	S3	+	+	-	-	-	-	-	+	+
85	S3	-	+	-	-	-	-	+	+	+
86	S3	+	+	-	-	-	-	+	+	+
87	S3	-	-	-	-	-	-	-	+	-
88	S3	+	-	-	-	-	-	-	+	+

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