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Investigating wastewater treatment plant impact on antibiotic resistance within UK river systems

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Submitted for the degree of Doctor of Philosophy

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Declaration

In accordance with the University of Warwick regulations for the degree of Doctor of Philosophy, I certify that this thesis has been written solely by me. The work contained in this thesis is my own unless otherwise stated. No aspect of this work has been submitted to any other institution for any other degree of award.

Abstract

Antimicrobial resistance (AMR) presents one of the most important threats to human health of the 21st century. The recent report on AMR predicted that by 2050 10 million deaths a year will be directly attributable to AMR bacterial infections. The dissemination of antibiotic resistance genes (ARG) in to the environment has previously been highlighted as an important route of transmission and was investigated in the current study.

Wastewater treatment plants (WWTP) have been highlighted to contribute to ARG pollution of rivers focusing on effluent impact on receiving water bodies. In this study the aim was to further investigate the effects of WWTP effluent on the receiving river, but also investigate the release of raw sewage resulting from combined sewer overflow (CSO) events on the receiving river. This study found that sediment samples carried a higher abundance of all ARG and therefore present a greater risk compared to water and that CSO spills are important in the spread of ARG likely contributing more substantially to the environmental spread of resistance than continuous release of treated wastewater.

In addition, the present study aimed to investigate the genetic potential of viable, potentially pathogenic *Escherichia coli* isolates from the river sediment to determine whether these human opportunistic pathogens carried the genetic capacity to spread resistance and cause disease. *E. coli* strains were shown to carry extensive resistance to many clinically relevant antibiotics, metals and biocides as well as carrying vast virulence-associated genes. This study identified ST940 as an important sequence type (ST) in the dissemination of the ESBL $bla_{CTX\text{-}M\text{-}15}$ gene and suggests further work to investigate the importance of this ST type in the transmission of this clinically important ARG.

The work presented here supports previous studies demonstrating extensive environmental ARG dissemination in rivers as a direct result of WWTP impacts and further highlights rivers as an important reservoir of ARG and antibiotic resistant bacteria (ARB). The discovery of clinically important viable *E. coli* isolates in sediment suggests more rigorous methods of wastewater treatment, specifically a reduction in the number of CSO release events, must be employed if further dissemination of ARB is to be prevented.

Abbreviations

L-Ara4N	4-amino-4-deoxy-L-arabinose
ANOSIM	Analysis of similarity
ABG	Antimicrobial resistance gene
AMR	Antimicrobial resistance
ARB	Antimicrobial resistant bacteria
ABC	ATP binding cassette
APEC	Avian pathogenic *Escherichia coli*
BOD	Biochemical oxygen demand
BRG	Biocide resistance gene
BSA	Bovine serum albumin
CR	Carbapenem resistant
CTX-M	cefotaxime - Munich
CTAB	Cetrimonium bromide
CRISPR	Clustered regularly interspaced short palindromic repeats
CMS	colistin methanesulphate, colistin sulphonyl methate, penta-sodium colistimethanesulphate
CSO	Combined sewer overflow
CA-MRSA	Community acquired MRSA
CARD	Comprehensive antibiotic resistance database
Cas	CRISPR associated
CGNF	Culturable Gram-negative fraction
DNA	Deoxyribonucleic acid
DAEC	Diffusely adherent *Escherichia coli*
DHPS	Dihyropeeroate synthase
NH4	Dissolved ammonium (NH_4)
Cl	Dissolved chloride (Cl)
F	Dissolved fluoride (F)
NO3	Dissolved nitrate (NO_3)
NO2	Dissolved nitrite (NO_2)
DOC	Dissolved organic carbon
SO4	Dissolved sulphate (SO_4)
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*

ETEC	Enterotoxigenic *Escherichia coli*
EU	European Union
ESBL	Extended spectrum β-lactamases
ExPEC	Extraintestinal E. coli
GI	Gastrointestinal
GLM	Generalised linear model
HA-MRSA	Healthcare acquired MRSA
HiCA	HiCrome™ coliform agar
HipA	High persister protein A
HSD	Honest significant difference
HGT	Horizontal gene transfer
Inc	Incompatibility
KPC	*Klebsiella pneumoniae* carbapenemase
LPS	Lipopolysaccharide
LCB	Locally collinear blocks
MFS	Major facilitator superfamily
MNEC	Meningitis-associated *Escherichia coli*
MRG	Metal resistance gene
MRSA	Methicillin resistant Staphylococus aureus
MSSA	Methicillin susceptible Staphylococcus aureus
MGE	Mobile genetic element
MOB	Mobility
MDR	Multidrug resistance
MATE	Multidrug and toxic compound extrusion
NDM-1	New Delhi metallo-β-lactamase 1
OTU	Operational taxonomic units
OXA-48	Oxacillinase-48
PacBio	Pacific Biosciences
PEtN	Phosphoethanolamine
PCR	Polymerase chain reaction
PCE	Presumptive coliforms excluding E. coli
PCoA	Principal coordinates analysis
PMF	Proton motive force
qPCR	Quantitative polymerase chain reaction
QAC	Quaternary ammonium compound
Rep	Replication initiation
RND	Resistance nodulation division

RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
ST	Sequence type
STEC	Shiga-toxin producing E. coli
SPRI	Solid phase reversible immobilisation
SRP	Soluble reactive phosphorus
SCC	Staphylococcal cassette chromosome
SMR	Staphylococcal multiresistance
TDN	Total dissolved nitrogen
TDP	Total dissolved phosphorus
TP	Total phosphorus
TA	Toxin-antitoxin
TRACA	Transposon aided capture
TCS	Two component system
T4CP	Type IV coupling protein
T4SS	Type IV secretion system
UK	United Kindgom
UTI	Urinary tract infections
UPEC	Uropathogenic E. coli
WWTP	Wastewater treatment plant
WGS	Whole genome sequencing
WHO	World health organisation

Chapter 1

Introduction

1.1 Origins of antibiotic resistance

Antimicrobial resistance (AMR) no doubt constitutes one of the most important threats to human health of the 21st century. A post-antibiotic era whereby minor infections and common illness become fatal is predicted to occur within the century (O'Neill, 2016). The recent emergence of the mobilisable colistin conferring resistance genes *mcr-1* and *mcr-2* has highlighted the severity of the current situation and may result in untreatable bacterial infections (Liu, 2015, Xavier et al., 2016, McGann et al., 2016).

AMR is an ancient phenomenon (D'Costa et al., 2011), the study by D'Costa *et al.* investigated the Beringian permafrost to reveal AMR is at least 30000 years old. The first natural product antibiotics are estimated to have arisen two billion – 40 million year ago, which intuitively suggests that the first antibiotic resistance genes (ARG) must have arisen at a similar time point (Hall and Barlow, 2004, Baltz, 2005). The precise role of natural antibiotics is unknown and the clinical impacts of antibiotic production and ARG dissemination have far outweighed the call for studies to investigate the roles these products play in the natural environment. Not only has clinical importance outweighed the significance of these studies, the difficulties

associated with studying production and resistance in the natural environment are numerous. However, with the rapid dissemination of ARG throughout the clinic and the environment the call for studies investigating the natural roles these products play is becoming increasingly important. If the innate nature of antibiotics is elucidated, then it may be possible to find new mechanisms combating ARG evolution. Antibiotics are often found at sub-inhibitory levels in the environment (Michael et al., 2013, Kummerer, 2009, Bernier and Surette, 2013) and have been suggested to play a role in quorum sensing and biofilm formation. Selman Waksman, the microbiologist who coined the term "antibiotics" believed they played no significant role in "modifying or influencing living processes that occur in nature" (Waksman, 1961). Although there has been little evidence to disprove this, the complex nature of antibiotic production suggests, contrary to this, that antibiotics must play an important environmental role (Sengupta, 2013). In the natural environment survival is community driven with multi-cellular networks interacting making it difficult to determine the precise role antibiotics play (Bernier and Surette, 2013). Determining a clear-cut mechanism behind antibiotic action in the natural habitat is therefore incredibly intensive. The question posed by Julian Davis "are antibiotics naturally antibiotics?" is an important question that must be addressed in the future (Davies, 2006). Currently suggestions for natural product antibiotics include the obvious role of interspecies competition as well as signalling molecules, increased virulence, host-parasite interaction, SOS and DNA repair gene expression and quorum sensing (Sengupta, 2013).

The mass production of penicillin in 1945 started the industrial era of antibiotic production which no doubt contributed to the rapid dissemination of ARG which Fleming had warned about in his Nobel lecture in 1945 (Aminov, 2010). Although ARG are a naturally occurring phenomenon, the rate at which these genes have evolved and disseminated is no doubt related to anthropogenic impact.

1.2 Antibiotics and resistance mechanisms

AMR is particularly problematic in Gram-negative bacteria with resistance typically emerging two-three years after introduction in to the clinic (Hall and Barlow, 2004).

There are multiple AMR mechanisms; target modification, reduced penetration, increased efflux, bypass mechanisms, target overproduction and enzymatic inactivation (Blair et al., 2015). Gram-negative bacteria are also characteristically less sensitive to antibiotics than Gram-positives due to their outer membrane which prevents some antibiotics e.g glycopeptide antibiotics permeating.

1.2.1 Beta-lactam antibiotics and resistance mechanisms

The beta-lactam antibiotics are the most important clinical antibiotic class. β-lactam activity is upon the murein layer of bacteria cell walls whereby interruption of cross-linking between murein strands through covalently binding the transpeptidase, responsible for cross-linking, and D-alanine D-alanine carboxypeptidase which removes the terminal D-alanine (Kong, 2010).

Despite the widespread resistance to β-lactams, they are routinely used and are often first-line response antibiotics due to their broad spectrum activity and low level toxicity, with third and fourth generation cephalosporins listed by the WHO advisory group as important antimicrobials in the treatment of human infections (WHO, 2013)(Livermore, 1996). The combined effect of the β-lactamase inhibitor clavulanic acid and β-lactam antibiotics is still one of the most successful drug combinations (Brown, 1976). In 2009, the international market for β-lactams was $15 billion per year and accounted for 65 % of the total antibiotics in the market (Thakuria and Lahon, 2013). Antibiotic resistance arose shortly after the discovery of penicillin in 1945 (Bellamy, 1948). Resistance to β-lactams arises through enzymatic cleavage of the β-lactam ring by β-lactamases. There are two classification schemes that have been used, one based on the amino acid sequence of β-lactamases and an updated system grouping based on molecular characterisation. There are ~900 unique protein sequences for β-lactamases which has made grouping difficult (Bush and Jacoby, 2010).

The first classification system consisted of four classes; A, B, C and D which are grouped according to sequence similarity (Ambler, 1980, Jaurin, 1981, Ouellette,

1987). Class A β-lactamases are primarily involved in the enzymatic cleavage of penicillin and ampicillin. They are also able to hydrolyse most cephalosporins but most carry out hydrolysis of a specific subset (Hall and Barlow, 2004). The class B β-lactamases, designated the metallo-β-lactamases due to the requirement of a bivalent metal ion (commonly Zn^{2+}) are structurally unrelated to the serine-β-lactamases (Classes A, C and D) and instead of hydrolysing substrates by forming an acyl enzyme through an active site serine, this utilise an active-site zinc ion to facilitate-β-lactam hydrolysis (Bush and Jacoby, 2010). Within this group there is much diversity, both by DNA and protein sequence. The complexity of this group has resulted in subgroups within the Class B enzymes: B1, B2 and B3 based on structural similarity (Galleni et al., 2001). Class C enzymes are generally referred to as the AmC β-lactamases and have a very broad spectrum of activity against cephalosporin's. Class D enzymes are, like Class A β-lactamases, primarily penicillinases, but are involved in hydrolysing oxacillin, cloxacillin, methicillin and carbenicillin (Naas, 1999). Similarly, Class D enzymes also specialise in the hydrolysis of specific subsets of cephalosporins (Naas, 1999).

The updated classification of β-lactamases groups enzymes in to 3 groups, group 1 (which contains all Class C) cephalosporins, group 2 (Classes A and D) broad-spectrum, inhibitor resistant and extended-spectrum β-lactamases (ESBL) serine carbapenemases and group 3 which consists of the metallo-β-lactamases. Group 1 cephalosporinases are chromosomally encoded β-lactamases which are encoded by many *Enterobacteriaceae* (Jacoby, 2009). They are generally not inhibited by clavulanic acid and display resistance to cefotaxime but not ceftazidime (Yu, 2008). AmpC expression is low but inducible on exposure and when induced in large amounts group 1 enzymes are able to confer resistance to carbapenems, particularly ertapenem (Bush, 1982, Jacoby, 2009, Livermore, 1987, Weber, 1990, Bradford, 1997, Jacoby, 2004, Quale et al., 2006). The most common plasmid-mediated subgroup of the group 1 β-lactamases are bla_{CMY}, bla_{ACT}, bla_{DHA},bla_{FOX} and bla_{MIR} (Jacoby, 2009).

Group 2 serine β-lactamases (classes A and D) is the largest group of β-lactamases. They contain the ESBL resistance genes which are currently the most frequently found plasmid-mediated β-lactamases (Jacoby, 2009). This group also represents the penicillinases which are the most common β-lactamases within Gram-positive cocci (Kernodle, 1989). Group 2 is sub grouped in to 12 groups; 2a, 2b, 2be, 2br, 2ber,2c, 2ce, 2d, 2de, 2df, 2de and 2df. Group 2a consists of penicillinases, 2b contains β-lactamases which hydrolyse penicillins and early cephalosporins but are strongly inhibited by clavulanic acid and tazobactem, examples include bla_{TEM} and bla_{SHV}. Group 2be is the ESBLs consisting of $bla_{CTX\text{-}M}$ enzymes, also sensitive to clavulanic acid. Group 2br comprised broad-spectrum β-lactamases which are resistance to the inhibitor clavulanic acid, examples include $bla_{TEM\text{-}30}$, and 5 of the 72 bla_{SHV} enzymes (Jacoby and Munoz-Price, 2005, Bush and Jacoby, 2010). Group 2ber consists of bla_{TEM} enzymes that are relatively resistant to clavulanic acid and have been termed complex mutant TEM (CMT). Group 2c are penicillinases which are effectively inhibited by clavulanic acid, Group 2ce have expanded activity against cefepime and cefpirome (Potron, 2009). The subgroup 2d consists of β-lactamases able to hydrolyse cloxacilin/oxacillin more effectively than benzylpenicillin and consist of the bla_{OXA} enzymes. 2de comprises cloxacillin/oxacillin hydrolysing enzymes which have an extended spectrum of activity to include the oxyimino-β-lactams. The subgroup 2df enzymes have carbapenem hydrolysing activities and are found primarily in *Acinetobacter baumannii* on the chromosome (Walther-Rasmussen and Hoiby, 2006). Subgroup 2e β-lactamases can hydrolyse extended-spectrum cephalosporins and are inhibited by clavulanic acid or tazobactam. The final subgroup of the Group 2 serine β-lactamases is subgroup 2f which consist of carbapenemases which are inhibited more efficiently by tazobactam than clavulanic acid (Bush and Jacoby, 2010).

The Group 3 metallo-β-lactamases are structurally and functionally separate from Group 1 and Group 2 β-lactamases. The capacity to hydrolyse carbapenems was originally the defining feature of this group, however there are now some serine β-lactamases also able to hydrolyse these antibiotics. Group 3 metallo-β-lactamases

are not able to effectively hydrolyse monobactams and are not inhibited by clavulanic acid or tazobactam but can be inhibited by metal chelators such as EDTA however are able to hydrolyse virtually all other-β-lactams (Marchiaro et al., 2008). The original discovery of metallo-β-lactamases did not prompt concern because they were found in non-pathogenic organisms and were encoded on the chromosome but the mobilisation of bla_{VIM} and bla_{IMP} in the 1990s on plasmids and transposons was the first indication of the clinical relevance these resistance genes might have (Lim, 1988, Walsh, 1994, Cornaglia, 2011). The most concerning enzyme within this group is the bla_{NDM-1-} which was first detected in 2008 (Yong et al., 2009). Within this group, there are 3 subgroups designated B1, B2 and B3 based on structure and 3a. 3b and 3c when sub grouped based on function (Frere et al., 2005, Galleni et al., 2001, Garau et al., 2004, Bush and Jacoby, 2010). Subgroup 3a consists of the major plasmid-encoded metallo-β-lactamases bla_{VIM} and bla_{IMP}. Subgroup 3b is a smaller group that will preferentially hydrolyse carbapenems compared with penicillin's and cephalosporins. The bla_{NDM-1} bla_{VIM} and bla_{IMP} are all subgroup B1.

The first bla_{CTX-M} gene was identified from an *E. coli* isolate from a cancer patient in 1989 and the first publication was from an *E. coli* isolate from a 4-month old child suffering with otitis media in Munich hence the name CTX-M; CefoTaXime and the –m from Munich (Bernard, 1992, Schwaiger et al., 2014). Since the discovery of these ESBLs the presence of these enzymes has nearly displaced all other ESBL genes in *Enterobacteriaceae* and they represent some of the most clinically important resistance genes are present (Hawkey and Jones, 2009, Canton, 2012). Dissemination is likely due to the cumulative effects of insertion on to highly mobilisable genetic elements and the location of these genes within particularly successful clones as well as co-selective effects of the mobile genetic elements (MGEs) particularly with respect to carriage of aminoglycoside and fluoroquinolone genes (Woodford et al., 2009, Dhanji, 2011, Woodford, 2008, Dhanji et al., 2011, Canton and Ruiz-Garbajosa, 2011). The most widespread bla_{CTX-M} are the $bla_{CTX-M-3,}$ $bla_{CTX-M-14,}$ $bla_{CTX-M-15,}$ and $bla_{CTX-M-9}$. There are five main clusters of bla_{CTX-M} genes. The chromosomal *bla* gene *kluC* from *Kluyvera cryocrescens* is considered the ancestor of the $bla_{CTX-M-1}$ cluster of genes (Decousser, 2001), *kluA* from *K. ascorbata* is considered the ancestor of the

bla$_{CTX\text{-}M\text{-}2}$ gene cluster and the gene clusters *bla*$_{CTX\text{-}M\text{-}8}$, *bla*$_{CTX\text{-}M\text{-}9}$, *bla*$_{CTX\text{-}M\text{-}25}$ are believed to have originated from *K. georgiana kluG, kluY and bla*$_{CTX\text{-}M\text{-}78}$ respectively (Humeniuk et al., 2002, Poirel, 2002 , Olson, 2005, Rodriguez et al., 2010). Although it is widely accepted that *Kluyvera* spp. are responsible for the evolution of the mobilisable *bla*$_{CTX\text{-}M}$ genes there has been some speculation due to evidence suggesting that the evolutionary relationships of *bla*$_{CTX\text{-}M}$ and *Kluyvera* 16S do not completely correlate (Canton, 2012). Another concern over the suggestion that *Kluyvera* is the ancestral origin of *bla*$_{CTX\text{-}M}$ is that three suggested ancestral chromosomal genes originate from one species each forming separate clusters (Canton, 2012). And thirdly, the *bla*$_{KLU}$ gene upstream region is identical to the genetic organisation in *Enterobacter aerogenes* which suggests *Kluyvera* may have gained the *bla*$_{KLU}$ gene after species divergence and suggests most isolates should carry this gene. Expression in the ancestral host is known to be weak and requires a strong promoter. The IS*Ecp1* and IS*CR*1 provide this strong promoter in *Enterobacteriaceae* but have not been found in any environmental isolates of *Kluyvera* (Canton, 2012). β-lactamases have few essential positions; 136 polymorphic positions have been identified with some increasing the spectrum of activity and some restoring activity of previous mutations. The instability of these enzymes suggests evolutionary stasis of these enzymes has not yet been reached and consequently could result in broader, more efficient activity (Canton, 2012).

1.2.2 Aminoglycoside antibiotics and resistance mechanisms

The use of aminoglycoside antibiotics is primarily in the treatment of Gram-negative aerobic bacilli, staphylococci and other Gram-positive bacterial infections (Ramirez and Tolmasky, 2010). They function through disruption of the 30S ribosomal subunit causing disincorporation of amino acids in to peptides which can result in misfolded membrane proteins and resultant accumulation of drugs leading to cell death (Kohanski, 2010). Aminoglycoside resistance is widespread. The global dissemination of resistance to these antibiotics has rendered them virtually useless in the treatment of some infections. Aminoglycoside modifying enzymes have different modes of action with some modifying the –OH or NH_2 groups of the 2-deoxystreptamine nucleus or the sugar moieties. There are three different types of aminoglycoside

modifying enzymes: phosphotransferases, nucleotidyltransferases and acetyltransferases (Ramirez and Tolmasky, 2010). Other mechanisms of aminoglycoside resistance exists with mechanisms included the modification of the target though 16S rRNA or ribosomal protein mutation, the methylation of 16S rRNA, reduction in permeability through outer membrane modification or removal of inner membrane transport export via efflux and drug sequestration through binding to an acetyltransferase of low activity (Galimand et al., 2005, O'Connor, 1991, Hancock, 1981, MacLeod, 2000, Aires, 1999, Magnet, 2003).

1.2.3 Quinolone and fluoroquinolone antibiotics and resistance mechanisms

Quinolones were first discovered in 1962 (Lesher, 1962). They are broad-spectrum antibiotics and are mainly used in the treatment of urinary tract infections (UTI) caused by Gram-negative pathogens They are listed in as important antimicrobials in the treatment of human infections (WHO, 2013). Substitution at C-6 and C-8 positions results in more effective drugs and a substitution at the C-6 position with fluorine results in fluoroquinolone production (Mitscher, 2005, Domagala, 1994, Guo, 2011, Aldred, 2014). The highly effective nature of quinolones combined with a low toxicity makes them highly favourable in the clinic (Sharma, 2009). They are also used in veterinary medicine to treat infections in food-producing animals, in aquaculture and companion animals (Liu, 2006). Because of their extensive use a wide range of resistance mechanisms have been identified. The fluoroquinolones, have been used extensively to treat both Gram-positive and Gram-negative infections. There are three mechanisms by which resistance can occur; mutation of target, plasmid mediated involving efflux and a decreased interaction and chromosome-mediated involving a decreased influx of drug in to the cell (Jacoby, 2005). Quinolone targets class II topoisomerase (GyrA and GyrB) and topoisomerase IV (ParC and ParE) converting drug-enzyme-DNA cleavage complexes in to cellular toxins (Kampranis, 1999). Gyrase and topoisomerase IV work together in replication, transcription, recombination and DNA repair. Quinolone resistance prevents binding of gyrase/topoisomerase, through modifications of serine or acidic residue of amino acids in the water-metal ion bridge which decreases quinolone binding affinity. The location of these amino acid substitutions is termed the quinolone-resistance-

determining-region (Cabral, 1997). Efflux mediated resistance in *E. coli* is mediated by the AcrAB-TolC efflux pump with mutations in the *acrR* gene increasing pump activity (Wang 2001). Mutations inactivate *marR* resulting in increased activation of *acrAB* and *tolC* decreasing in turn the translation of the *ompF* gene ultimately reducing the influx and increasing the quinolone efflux (Cohen, 1989, Alekshun, 1997). Efflux pumps conferring resistance to quinolones have been found in many enteric bacteria as well as *Psuedomonas aeuringosa, Stenotrophomonas maltophilia* and *Acinetobacter baumannii* (Cohen, 1993, Alonso, 2001, Magnet, 2003). Plasmids can encode resistance mechanisms with the *qnr* genes coding for proteins which bind both gyrase and topoisomerase IV resulting in resistance against ciprofloxacin and next generation fluoroquinolones (Tran, 2005, Tran, 2002). Plasmids carrying *qnr* genes usually carry additional resistance genes including $bla_{CTX\text{-}M}$ *ampC*, $bla_{FOX,}$ and bla_{SHV} and are therefore often associated with ESBL producing bacteria (Zhong et al., 2015, Ewers et al., 2010, Dhanji, 2011), (Paterson DL, 2000).

1.2.4 Macrolide antibiotics and resistance mechanisms

Macrolide antibiotics are primarily used in the topical treatment of infections, upper respiratory tract infections and soft tissue infections. They are particularly useful in the treatment of patients with allergic reactions to penicillin. The first macrolide antibiotic to be discovered was erythromycin which was first used in the early 1950's (Lewis, 2013) and are listed by the WHO as critically important antimicrobials for human medicine (WHO, 2013)Erythromycin is useful in treating some intracellular pathogens including *Legionella, Mycoplasma* and *Chlamydia.* However, erythromycin can cause gastrointestinal (GI) intolerance and has a short half-life making them unfavourable. The development of advanced macrolides with an extended spectrum of activity and increased half-life has however resulted in more favourable characteristics for use in the clinic (Zuckerman, 2004). The use of clarithromycin and azithromycin has been extensive since their introduction in treatment of respiratory infections, sexually transmitted diseases and *Helicobacter* or *Mycobacterium avium* complex (Zuckerman, 2004). Ketolides are synthesised by substitution of the α-L-cladinose moiety at the 3 position of the 14-memebered erythronolide A ring which in turn provides greater acid stability and prevents

resistance (Douthwaite, 2001, Shain, 2002). Macrolide antibiotics function by reversibly binding to the 50s subunit of the ribosome blocking protein translation (Sturgill, 1992). The ketolides bind the 23S rRNA enabling binding to macrolide resistance bacteria (Zuckerman, 2004).

Macrolide resistance is usually via multiple mechanisms including ribosomal modification through methylation or mutation, efflux and drug inactivation and resistance (Leclercq, 2002). Methylation of the ribosomal target is the most widespread mechanisms of resistance and was the first detected resistance which arose shortly after the introduction erythromycin in to the clinic in 1956 (Leclercq, 2002). The Erm proteins are responsible for this mode of resistance and function by dimethylating a key adenine residue in the 23S ribosomal subunit which consequently prevents binding of macrolide antibiotics. This effective mode of resistance has resulted in approximately 40 different *erm* genes identified (Roberts, 1999b). Target modification resistance is less frequently detected but involves the modification in *rrn* operons encoding the 23S subunit. The mechanism confers resistance to clarithromycin in the majority of *Mycobacterium avium* and *Helicobacteria pylori* (Leclercq, 2002). Efflux of macrolide antibiotics is primarily via the RND pumps in Gram-negative bacteria and by ABC and MFS pumps in Gram-postive (Leclercq, 2002).

1.2.5 Tetracycline antibiotics and resistance mechanisms

Tetracyclines were discovered in the 1940s (Chopra and Roberts, 2001). Action is via inhibition of the binding of aminoacyl-tRNA to mRNA-ribosome complex to inhibit protein synthesis (Chopra and Roberts, 2001). In addition to their antibacterial properties are also effective against non-bacterial infections including protozoan diseases such as giardiasis and viral infections such as West Nile fever (Katiyar, 1991, Dutta, 2011). They also have been shown to have anti-inflammation, anti-apoptotic and neuroprotective properties which cannot be explained by the current mode of action (Debrah et al., 2006, Michaelis et al., 2007, Elewa, 2006). A recent paper suggested that rather than a specific binding site, tetracycline action is related more to double-stranded structures of RNA which in turn may disrupt normal biological

processes through the inhibition of cleavage by RNase II which in turn prevents processing of RNAs which may in part explain why these antimicrobials infer such abroad range of therapeutic activities (Chukwudi and Good, 2016). Regardless of the mode of action, tetracyclines have been widely exploited in both clinical and veterinary medicine due to their broad spectrum of activity and limited side effects (from bacteriostatic concentrations used in the clinic) (Chopra and Roberts, 2001). They are also used as growth promoters in animal feed in the United States despite the emergence of resistance (Chopra and Roberts, 2001). Resistance to tetracyclines is conferred by the *tet* genes which encode efflux mechanisms to remove tetracyclines from the cell and reduce intracellular concentration. The efflux proteins encoded by the *tet* genes are of the major facilitator superfamily (MFS). In addition to *tet* genes, ribosomal protection proteins can also confer resistance to tetracyclines. These are cytoplasmic proteins able to confer a wide range of resistance to tetracycline antibiotics. They are homologous to the elongation factors EF-Tu and EF-G (Sanchez-Pescador, 1988, Taylor, 1996). The TetM, TetO and OtrA proteins all reduce the susceptibility of ribosomes to tetracycline action. In the presence of TetM and TetO tetracycline is released from the ribosomes (Tamayo. M., 1999).

1.2.6 Polymyxins antibiotics and resistance mechanisms

The emergence of the mobilisable *mcr-1* and very recent (June 2016) emergence of *mcr-2* colistin resistance genes has received a lot of attention (Liu, 2015, Xavier et al., 2016). Polymyxins represent last-resort antibiotics and since the discovery of plasmids carrying *mcr-1* and ESBL genes, pan-resistant bacteria are likely to become more frequent in the clinic (McGann et al., 2016). The dissemination of *mcr-1* is likely underestimated but is increasingly being detected with reports from Singapore, UK and United States confirming the presence of *mcr-1* in isolates less than a year after the initial identification (Liu, 2015, Teo et al., 2016, Anjum et al., 2016, McGann et al., 2016).

Polymyxins were first introduced in 1949 (Catry et al., 2015) but were not considered suitable for clinical systemic use due to their nephrotoxicity (Koch-Weser, 1970).

Instead polymyxin antibiotics have been primarily used as topical antibacterial agents in human medicine and in the treatment of GI infections in agriculture and veterinary medicine (Olaitan, 2014). However, due to the global dissemination of multidrug-resistant (MDR) Gram-negative bacteria, the polymyxins have recently received a lot of attention as last resort antibiotics.

There are two forms of colistin that are available, colistin sulphate and the prodrug CMS (syn. colistin methanesulphate, colistin sulphonyl methate, penta- sodium colistimethanesulphate), which is microbiologically inactive but becomes active after inhalation (Li et al., 2006, Falgas, 2005). Polymyxin B differs from colistin by one amino acid (D-leucine in colistin and phenylalanine in polymyxin B at position 6). Like colistin, polymyxin B is systemically toxic and is therefore only used as a last resort antibiotic (Nord, 1964). Most investigations in to the mechanism of polymyxin action has focused on polymyxin B, because colistin is so similar in structure the mechanism is predicted to be identical (Storm, 1977). Polymyxin B acts by binding to the outer membrane of Gram-negative bacteria through electrostatic interaction which competes with divalent cations for membrane lipid phosphate groups (Dixon, 1986). The attachment of polymyxins causes membrane disruption and release of lipopolysaccharide (LPS) (Peterson, 1985). Resistance mechanisms employed by bacteria include modification of the LPS via covalent modification of lipid A moiety by adding phophoethanolamine (PEtN) and 4-amino-4-depxy-L-arabinose (L-Ara4N), deacylation and hydroxylation (Ernst, 2001, Raetz et al., 2007). Efflux pumps are also used to remove polymyxin from the cell and capsule formation is also utilised (Campos, 2004, Padilla, 2010).

PhoP/PhoQ and PmrA/PmrB are environmentally stimulated two-component systems (TCS) which result in the overexpression of LPS modifying genes (Gunn, 1996, Gunn, 2001, Trent, 2001, Abraham, 2009, Miller, 2011)). The stimulation of the PmrA/PmrB TCS upregulates the *pmrCAB* and *arnBCADTEF-pmrE* operons which mediate synthesis and transfer of PEtN and L-Ara4N t to lipid A. PhoP/PhoQ indirectly activates the PmrA/PmrB TCS through PmrD which once activated, the phosphorylated PmrA binds *arnBCADTEF* operon promoter increasing recognition and binding RNA polymerase leading to lipid A modification (Wosten, 1999). Like

these TCS, the most recent polymyxin resistance conferring genes *mcr-1* and *mcr-2* confer resistance through modification of lipid A (Liu, 2015, Xavier et al., 2016). Capsular polysaccharides from *Klebsiella pneumoniae* have been reported to trap polymyxins preventing the drug from reaching the cell however this mechanism of resistance is debated by some (Llobet, 2008, Clements, 2007). Efflux of polymyxin antibiotics out of the cell has also been observed as a mechanism of colistin resistance with mutated AcrAB pumps making *K. pneumoniae* more susceptible to polymyxin B action (Padilla, 2010).

1.2.7 Sulphonamide antibiotics and resistance mechanisms

Sulphonamides were first introduced in the 1930s and have been used in both clinical and veterinary medicine and are active against bacterial and protozoal infections. Sulphonamide action is via alternative substrate binding, acting as a structural analogue of ρ-amino-benzoic acid binding dihyropteroate synthase (DHPS). DHPS is involved in the folic acid biosynthesis pathway to form dihydrofolic acid, binding of DHPS therefore prevents this formation (Skold, 2000). This mechanism of resistance is effective against Gram-negative and Gram-positive bacteria (Brown, 1962).

Resistance to sulphonamides is widespread and as a result they are usually used in combination with diaminopyrimidines or trimethoprim in the treatment of diarrheal disease (Huovinen, 2001, Perreten, 2003). Resistance arises from mutations in the DHPS gene (*folP*) or acquisition of alternative DHPS genes (*sul*) (Swedberg, 1993, Perreten, 2003, Rådstrom, 1988, Sundstrom, 1988). There are three *sul* genes; *sul1, sul2* and *sul3*. The *sul1* gene is characteristically located on the 3' conserved region of the Class 1 integron, the *sul2* gene was identified on RSK10010 from *E. coli* and has been associated with resistance plasmids and the *sul3* gene was first identified from *E. coli* isolates from pigs (Skold, 1976, Rådstrom, 1988, Perreten, 2003).

1.2.8 Multidrug resistance conferring efflux pumps and their role in antimicrobial resistance

MDR efflux pumps allow export of a diverse range of antimicrobial agents. All bacteria carry multiple MDR genes, however, the mobilisation of these pumps on to

plasmids has resulted in a higher rate of efflux of antimicrobials from bacterial cells due to increased expression (Piddock, 2006). Efflux pumps are able to confer resistance through two mechanisms; increased expression or amino acid substitutions improving efflux efficiency. They can have a broad range of activity, able to export a variety of different antimicrobial classes from the cell, or they can be specific (Piddock, 2006). Efflux pumps can be chromosomal or plasmid encoded.

There are five classes of chromosomally encoded efflux pumps: the resistance nodulation division (RND) family, the major facilitator superfamily (MFS), staphylococcal multiresistance (SMR), multidrug and toxic compound extrusion (MATE) families and ATP binding cassette (ABC). RND pumps are tripartite proton exporters, functioning through exchange of hydrogen ions for drug molecules they consist of a transporter, a periplasmic accessory protein and an outer membrane protein channel (Paulsen, 2003, Piddock, 2006). MFS pumps possess 12 transmembrane domains and also function through proton motive force (PMF) (Paulsen, 2003). MATE pumps have two different energy sources, one is the PMF and the other is a sodium ion gradient. They have similar functions to RND pumps but are not tripartite. ABC transporters have not been found to confer clinically related MDR but are present in pathogenic bacterial genomes. A recent study found a novel tripartite resistance nodulation division (RND) on a IncH1 plasmid which was also carrying the New Delhi metallo-β-lactamase 1 (NDM-1) gene conferring resistance to carbapenems (Dolejska et al., 2013). The RND class of multidrug efflux pumps constitute the most clinically relevant pumps and are able to export a variety of different antimicrobials when overexpressed (Piddock, 2006).

1.3 Persister cells and their role in evading antimicrobial treatment

Not only are bacteria antibiotic resistant, persistent infections are becoming increasingly reported as the knowledge around these bacteria improves. Persister bacteria are defined as those cells that are metabolically quiescent, neither growing or dying when exposed to bactericidal antibiotic concentrations (Lewis, 2013). The persistence phenotype is not inherited and induction of persister cell formation is stochastic (Germain, 2015). Persister formation is generally regarded as a 'bet-

hedging' strategy to evade unpredictable stress allowing a small population to survive in extreme conditions (Losick and Desplan, 2008, Veening, 2008). The number of persisters has previously been reported to increase in biofilms suggesting a high number of persister cells may be present in the environment where biofilms are highly abundant (Maisonneuve et al., 2013).

Often toxin-antitoxin (TA) systems are involved in the development of persister cells (Wen et al., 2014, Gelens et al., 2013, Li et al., 2016, Bertram, 2014, Keren et al., 2004). *E. coli* typically encodes 11 type II TA systems which are involved in persistence, the HipA (high persister protein A) kinase of the *hipBA* model, is one of the best characterised proteins involved in persister cell formation and functions through inactivating glutamyl tRNA synthetase by phosphorylation resulting in high levels of $tRNA^{glu}$, which in turn results in the activation of the stringent response by activating RelA and increasing (p)ppGpp levels (Kaspy et al., 2013) . The induction of the stringent response is linked to persistence of bacteria however the precise mechanisms is unknown.

The function of antibiotics requires cells to be actively dividing, the formation of persister cells allows bacteria to evade killing and start dividing when antibiotic concentration decreases. The requirement for antimicrobial drugs that are able to kill dormant bacterial cells is therefore urgent in the fight against infection.

1.4 Current state of antibiotics

Costs associated with antibiotic resistance are predicted to increase to up to 100 trillion US dollars by 2050 (O'Neill, 2016). As well as huge economic costs, the loss of lives as a direct result of antibiotic resistance is currently estimated at 700000 deaths and could overtake the number of deaths from cancer at ten million by 2050 (O'Neill, 2016).

No major antimicrobial discoveries were made between 1980-2015. The discovery of the novel antimicrobial teixobactin made by Lewis and colleagues was the first new discovery since 1997 when the diarylquinolone bedaquiline was discovered, a narrow

spectrum antibiotic used in the treatment of *Mycobacterium tuberculosis* (Ling et al., 2015, Lewis, 2013). Teixobactin represents a novel class of natural product antibiotics and was a major breakthrough for antibiotic discovery being the first study to successfully identify a novel antimicrobial using the iChip allowing the unculturable fraction of microorganisms to be mined for antibacterial activity (Ling et al., 2015). Teixobactin is active against Gram-positive bacteria including *Staphylococcus aureus* (including methicillin resistant *S. aureus* (MRSA)), *M. tuberculosis, Clostridium difficile* and *Bacillus anthracis* and acts through the inhibition of peptidoglycan biosynthesis by binding a highly conserved motif of lipid I and lipid II (precursors for peptidoglycan and teichoic acid respectively). Although the authors suggest resistance to this antimicrobial agent is unlikely due to the mechanism by which it acts, teixobactin is a natural product and therefore natural resistance will occur in *Eleftheria terrae,* the organism from which it was discovered and therefore will disseminate if teixobactin is used in the clinic (Piddock, 2015).

Livestock usage of antimicrobials is estimated between 50-80 % in many developed nations (Cully, 2014). The large demand for meat production has resulted in a vast amount of antimicrobials being used in treatment of active infections and prophylactically to prevent infections but also for growth promotion purposes. Despite research showing high levels of resistance as a direct result of antimicrobial use as growth promoters as early as 1951, the growth promotion exhibited by prophylactic treatment has continued to outweigh the significant human health costs (at least from an agricultural viewpoint). In the United States the use of antibiotics as growth promoters is still widely carried out with more than half of antibiotics used for this sole purpose (Cully, 2014). The specific use of antibiotics as growth promoters is most significant in pig production in China. China is the largest antibiotic producer and consumer and it was estimated that annual production equates to 210 million kg with 46.1 % of this being used in livestock (Hvistendahl, 2012). 30 % of global pork production comes from China which has resulted in significant pressure to maintain such high production levels. Consequently farmers in China are reported to use 4 times more antibiotics than the US in the production of the same amount of meat (Cully, 2014). As a direct result of this irresponsible overuse of antibiotics one study

found that ARG were enriched by 28000-fold in manure and soil from Chinese pig farms and has resulted in mobilisable colistin resistance (Zhu, 2013, Liu, 2015). Although the numbers of antibiotics used as growth promoters is still high in both China and the United States there have been some changes to reduce this. The first country to made amendments to the number of antibiotics as growth promoters was Denmark when the Danish minister for agriculture and fishing banned the use of avopacin in 1995 (Cully, 2014). The European Union (EU) banned the use of clinically important antibiotics for growth promotion in 2000 and in 2006 invoked a complete ban on antibiotics for agricultural growth promotion purposes (Cully, 2014).

1.5 Antimicrobial production prospects

Currently antibiotic discovery is not an attractive prospect to industry due to the likelihood of AMR. Therefore, incentives were set out in the recent government report regarding tackling AMR to make antibiotic production a more attractive prospect (O'Neill, 2016). As well as providing incentives to industries there were 9 other points made to tackle AMR: 1. Raising public awareness; 2. improving hygiene and preventing the spread of infection; 3. reduce unnecessary use of antimicrobials in agriculture to present dissemination in the environment; 4. global surveillance of AMR for both animal and human consumption; 5. promotion of new, rapid diagnostics; 6. promotion of the development and the use of vaccines and alternatives; 7. increase the numbers, pay and recognition of people working in infectious disease; 8. initiate a global innovation fund for the early-stage and non-commercial research; 9. build a global coalition for real action through the G20 and the UN (O'Neill, 2016).

With the treatment of MDR and persistent bacteria there is an urgent need for novel antimicrobial agents. In the 1940’s the method used to investigate antimicrobial compounds involved the soil-derived streptomycetes and screened for antimicrobial activity by investigating zones of inhibition. This method of screening was extremely effective and resulted in many major classes of antibiotics being discovered that are still used today This was known as the ‘golden era’ of antibiotic discovery and spread throughout the 1940’s to 1960’s (Lewis, 2013). However, the extensive resistance

that arose due to the misuse and overuse of antibiotics has resulted in fewer and fewer antibiotics still useful in the clinic (Figure 1.1).

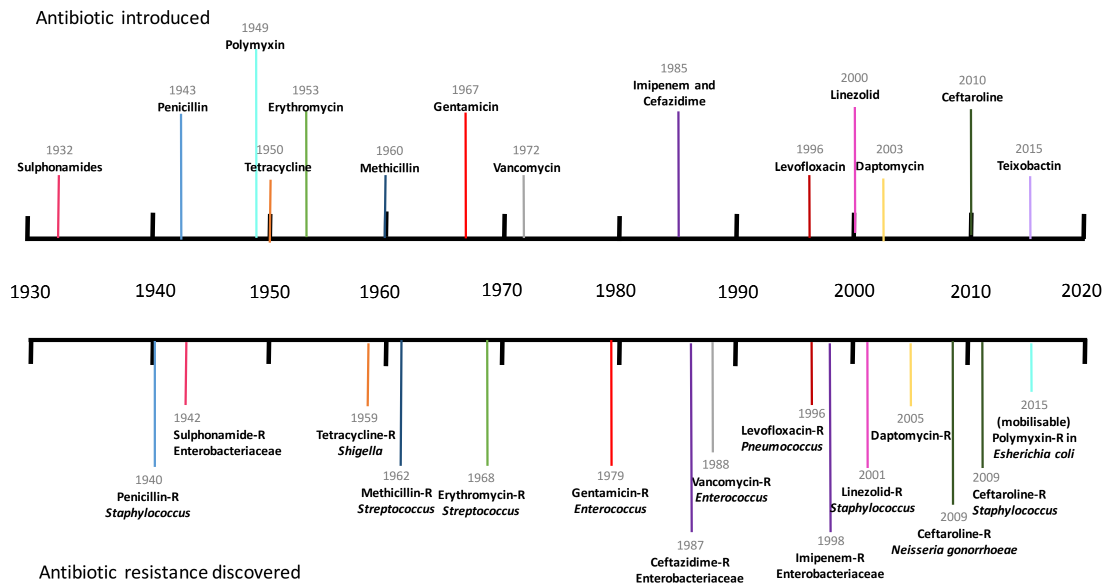


Figure 1.1 Clinical introduction of antibiotics and first reported resistance

Although incentives are to be employed to make antibiotic research an attractive prospect, alternative antimicrobials may be required as the rate of discovery of antibiotics has dramatically decreased since the first discovery of penicillin (O'Neill, 2016). Alternative approaches include phage therapy, predatory bacteria, antimicrobial peptides from plants, animals and fungi, gene-editing enzymes and the use of metals.

1.5.1 Phage therapy

Phage therapy was first introduced in the 1920s but after penicillin was discovered the Western world dismissed research in this field to focus on discovering new and novel antibiotics due to their broad spectrum of activity. Almost eight decades after the discovery of penicillin, research is returning to the idea of using bacteriophage to treat MDR infections. The benefit of using phage is also its major downfall; bacteriophage are highly specific for their target making them useful tools in targeting specific MDR bacteria, however this specificity means unlike antibiotics,

they have an incredibly narrow spectrum of antibacterial activity. The development of phage is potentially less costly than the development of antibiotics which requires rodent studies so may provide an attractive solution to industry. Hamamoto *et al.* suggested silk worm larvae may be suitable hosts for experiments drastically cutting costs of production (Hamamoto et al., 2004). Takemura-Uchiyama *et al.* developed phage to destroy *S. aureus* in successful experiments resulting in survival of the silkworm (Takemura-Uchiyama et al., 2013).

1.5.2 Predatory bacteria

There is interest in the use of predatory bacteria *Bdellovibrio bacteriovorus,* which attacks the bacterial host's inner and outer cell membrane by growing filaments and replicating until the cell bursts (Dwidar et al., 2012). It has even been shown to be effective against *E. coli* O157:H7 (Fratamico and Cooke, 1996). A study has also successfully engineered *E. coli* to kill *Pseudomonas aeruginosa* through production of peptides (Saeidi et al., 2011).

1.5.3 Antimicrobial peptides

Cationic antimicrobial peptides from plants, animals and fungi may provide a novel solution to treating AMR bacterial infections. Reptiles and amphibians are characteristically resistant to some infections therefore investigating peptides with antimicrobial activity from these animals may provide an effective solution to MDR pathogens. Studies investigating animals as a potential reservoir of antimicrobial peptides have already successfully isolated peptides from frogs, alligators and snakes (Flamm et al., 2015, Barksdale et al., 2016, Blower, 2015). A recent study was also able to isolate an antimicrobial peptide from the ant *Tetramonrium bicarinatum* effective against *Staphylococcus* and *Enterobacteriaceae* (Tene et al., 2016). These peptides have the ability to heal infections in mice and some are in clinical trials. They are particularly are useful because their potency can be modified. A recent study carried out by Barksdale *et al.* found antimicrobial peptides active against MDR *S. aureus, E. coli, Pseudomonas aerunginosa* and *Acinetobacter baumannii* (Barksdale et al., 2016). Pexiganan, isolated from a frog, is in phase III clinical trials and could potentially be important in treating diabetic foot ulcers (Flamm et al., 2015).

Antimicrobial peptides, like antibiotics, have the capacity to be broad spectrum and work by damaging the bacterial membrane making resistance difficult (Hancock, 1997). Using antimicrobial peptides therefore is a promising method of combating antibiotic resistance, however, in contrast to phage therapy as an alternative to antibiotics, peptide production is expensive and therefore currently an unattractive prospect to industry.

1.5.4 Gene-editing

Gene-editing using clustered regularly interspaced short palindromic repeats (CRISPR) poses a promising strategy that may help to destroy MDR bacteria. CRISPR is used as a protective system in bacteria against phage, however studies investigating the potential to use CRISPR to destroy bacterial DNA are being conducted. Yosef *et al.* used temperate phage to deliver CRISPR associated (Cas) in to the genome of a AMR *E. coli* to kill only the AMR *E. coli* and not the antibiotic-sensitive strains (Yosef et al., 2015). The Cas9 system has recently been used to treat ESBL-producing *E. coli* by re-sensitizing to antibiotics (Kim et al., 2016). Gene editing using CRISPR presents a promising approach to overcome AMR with the Cas9, perhaps the most promising, a double-stranded DNA nuclease which can be programmed to cleave almost any specific DNA sequence allowing MDR to be re-sensitised to antibiotics (Jinek et al., 2012).

1.5.5 Metals as antimicrobial agents

Metals have also been suggested as potential antimicrobial agents to replace antibiotics. The main concern over using metals to combat AMR pathogens is that metals are often toxic to humans. Therefore, although they are effective against MDR pathogens, they will likely induce significant side-effects. The most promising metal to be used is gallium. Gallium is able to kill the pan-resistant *A. baumannii* due to the inability of proteins to distinguish between Fe^{3+} and Ga^{3+} (Antunes et al., 2012).

1.6 Contributing factors to the global dissemination of ARG

Anthropogenic impacts have no doubt contributed to the widespread dissemination of ARG (Figure 1.2). The primary factor accelerating ARG in the clinic and

environment is currently unknown, but the cumulative effects of poor prescription practice, self-prescription, misuse of antibiotics, veterinary use, agricultural use involving prophylactic use in animals and on crops, manure spreading, industrial waste, and wastewater treatment plant inefficiency all play a role (Wellington et al., 2013).

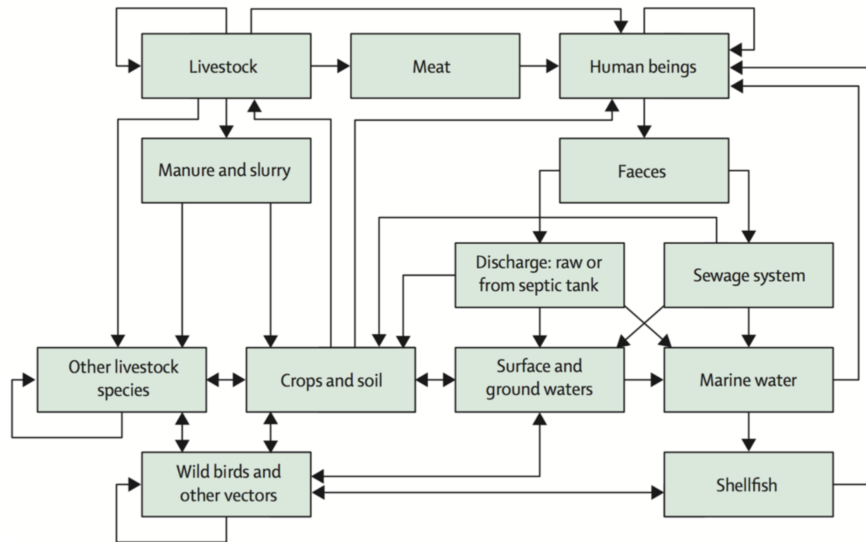


Figure 1.2 The environmental resistome.
Reprinted from the Lancet 13(2):155-165 The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria, Copyright (2016), with permission from Elsevier.

1.6.1 Travel as a factor of ARG dissemination

Over the past twenty years there has been significant increases in the transport of goods between Asia and Europe as well as an increase in travel of the general population (Hawkey, 2015). In a ten year period from 2002 to 2012 the number of flights between the UK and the rest of the world increased from 39.5 to 50 million (Hawkey, 2015). In addition, an increasing number of megacities (defined as cities with populations greater than or equal to 10 million have meant more people are living in close proximity (DESA, 2014). In 2014 there were 28 megacities with a combined population of 453 million people (DESA, 2014). The largest city was Tokyo with 38 million people followed by Delhi which had a population of 25 million. Six of

these megacites are in China. Over 50 % of the global population currently lives in urban areas and it is predicted to increase to 66 % by 2050 (DESA, 2014).

The increase in population and resultant megacities has increased the pressure for effective sewage disposal and safe drinking water (Hawkey, 2015). Consequently, overcrowding has resulted in heavy antimicrobial usage leading to large levels of AMR within these communities. Combined, global travel has become more accessible and has dramatically increased in the past fifteen years meaning that population mixing is happening more frequently and at a higher level compared to twenty years ago. The commensal carriage of resistant bacteria in the intestinal tract consequently has resulted in an increased exposure as a direct result of travel (Hawkey, 2015).

Perhaps the most important ARGs to have disseminated as a direct consequence of increased travel are the $bla_{CTX-M-14}$, $bla_{CTX-M-15}$ and bla_{NDM-1} *genes*. The bla_{CTX-M} genes are believed to have originated from the environmental bacteria *Kluyvera*. Originally genes were chromosomal but the association with *ISEcp1* and *IS903* has mobilised these genes on to plasmids allowing their rapid dissemination, particularly in the *Enterobacteriaceae* (Humeniuk et al., 2002, Hawkey, 2015). The spread of ESBL producing bacteria as a direct result of travel has previously been demonstrated with one study reporting travel outside of Europe resulted in a higher prevalence of ESBL producing bacteria in those travellers compared to in people who had travelled within Europe (Tham, 2010). India had the highest colonisation rate with 79 % of travellers tested reported to carry ESBL producing bacteria. Travellers who had visited India were more likely to carry the $bla_{CTX-M-15}$ gene and travellers who visited China were most likely to carry the $bla_{CTX-M-14}$ gene. Commensal carriage of ESBL producing bacteria has been shown in healthy Chinese persons with studies showing high carriage of *E. coli* with ESBL genes at 50 % and 51 % carriage (Li, 2011, Zhong et al., 2015).

Another study demonstrating the relationship between location and prevalence was a study conducted by Wickramasighe *et al.* who demonstrated that residents in

Birmingham, UK had a higher likelihood of *bla_{CTX-M}* carriage if they came from the Middle East or South Asia (based on the assumption of names indicating origin) than individuals who had names of European origin (and therefore assumed to be of European descent). Those individuals assumed to be of Middle East or South Asia had a 23 % carriage compared to European carriage of 8 % (Wickramasinghe et al., 2012). These results were similar to another study conducted in France which reported people who were born outside of France were more likely to carry *bla_{CTX-M}* genes (Nicolas-Chanoine et al., 2012).

One other noteworthy gene found to have been disseminated as a consequence of travel is the carbapenemase gene *bla_{NDM-1}*. NDM refers to New-Delhi metallo-β-lactamase and was so named due to the location from which it was first detected. The first report of this gene was in 2008 and was identified from a patient of Indian origin. Both *K. pneumoniae and E. coli bla_{NDM-1}* carrying strains were isolated from this one patient (Yong et al., 2009). The spread of this gene has been strongly linked to travel in India, Bangladesh and Pakistan with >50 % of cases from patients who had visited hospitals in these three countries (Yong et al., 2009, Kumarasamy, 2010).

A recent study investigated the microbiomes and resistomes of low income countries. There are currently about 5.8 billion people living in low and middle income countries and 863 million people living in slums (Pehrsson et al., 2016). Human microbiomes and resistome from human faecal samples from two low-income communities were investigated, one being from a rural village and the other a peri-urban shanty-town. Studying these low-income counties is important public health priority due to the large levels of antibiotic usage that occurs in these developing counties (Pehrsson et al., 2016). Functional metagenomics studies revealed 1100 unique AMR genes displaying resistance to all antibiotics tested except meropenem. They found in accordance with previous studies that AMR was correlated with the community composition rather than a random distribution across habitats (Pehrsson et al., 2016).

1.6.2 Agriculture as a factor in the dissemination of AMR

There have been many studies investigating the global importance of the use of antibiotics in agriculture, both in prophylactic use and in the treatment of infection. Studies in China investigating antibiotics in animal manure found incredibly high levels of tetracyclines (hundreds of mg/kg) (Qiao M, 2012, Pan X, 2011). As previously mentioned, the study by Zhu *et al.* highlighted the impact that antibiotic use can have on the selection of ARG with a 28000-fold increase in samples taken from farms using antibiotic supplemented feeds compared to antibiotic-free control samples. In this study 149 unique resistance genes were detected and both antibiotics and heavy metals were found in manure allowing for co-selection. The direct use of antibiotics and metals correlated with an increased ARG level in manure (Zhu, 2013). The majority of veterinary antibiotics are excreted explaining the high levels in manure (Alcock, 1999). Consequently, these antibiotics are then spread on to land when manure is used as fertilizer and resultantly antibiotics and ARG are found in manure-amended soils and surrounding river waters and sediment due to runoff (Ghosh and LaPara, 2007, Pruden et al., 2012). As a direct result of manure application to land, studies have shown an increase in the number of resistant bacteria that can be retrieved from soils amended by manure compared to those not amended with an increased in the number of horizontal gene transfer (HGT) events of *ermF, sul1* and *sul2* and an increase in the prevalence of Class 1 integrons (Byrne-Bailey et al., 2009, Sengelov, 2003, Heuer, 2007, Fahrenfeld et al., 2014, Byrne-Bailey et al., 2011).

Other studies investigating agricultural impacts of antibiotics have shown enrichment of ARG in faeces (Chapman et al., 2006, Skurnik et al., 2006, Kanwar et al., 2014). One metagenomic study investigated 10 different environments investigating the plasmid metagenome. They reported large differences in ARG presence between different sites. In chicken faeces for example there was an enrichment of 12 ARG types highlighting the importance of ARG selection in chicken production (Li et al., 2015a). The ARG profiles of each site were independent of other sites suggesting specific antimicrobial selection pressures at each site.

1.6.3 The impact of wildlife on the dissemination of ARG

The importance of wildlife in the dissemination of ARG has been largely underreported (Huijbers et al., 2015). The presence of ARG within wildlife has been mainly attributed to the overuse of antibiotics in humans and in animals which enter the environment through inefficient wastewater treatment and runoff from land (Arnold et al., 2016). The environmental exposure to both antimicrobials, metals and biocides can co-select for MGE carrying ARG, biocide resistance genes (BRGs) and metal resistance genes (MRG). This results in widespread dissemination of ARG resulting in wildlife that has never been exposed to humans carrying ARB (Fondi et al., 2016). Generally reports have shown animals within close proximity to human populations carry a larger number of ARG than animals from more isolated populations (Arnold et al., 2016). The first MDR bacteria isolated from a wild animal was in 1975 and was from an *E. coli* isolate from a pigeon (Sato, 1978). Other studies have included gorilla populations, elephant seals and iguanas (Rwego et al., 2008) (Stoddard, 2008) (Wheeler, 2012). Birds of prey with clindamycin and erythromycin resistant staphylococci have also been isolated in Portugal and in the UK antibiotic resistant *E. coli* was isolated from magpies and rabbits with resistance to tetracycline, ampicillin, chloramphenicol, kanamycin, sulphonamides (Sousa, 2016 , Livermore, 2001). In contrast to the many reports of AMR bacteria isolated from wild animals one study which took 33 humans and 198 wild animals to investigate *E. coli* prevalence showed no resistance in isolates from wild animals but did find resistance in human isolates suggesting human impact may not be as substantial as other papers have reported (Lescat et al., 2013).

1.6.4 Domestic animals and livestock transmission of ARG

It is easy to assume that wild animals acquire resistance from human impacts in the environment. What is not clear is if animal-human or human-animal transmission is more important in the dissemination of resistance. As discussed previously, the use of antibiotics in livestock production is extensive. The veterinary use of antibiotics for the treatment of companion animals is also extensive and involves the use of broad spectrum antimicrobials including clavulanate-potentiated aminopenicillins, cephalosporins and fluoroquinolones (Lloyd, 2007). Cats and dogs represent the

most likely source of domestic animal transmission of ARG. In EU countries the number of cats and dogs kept as pets is estimated at 70 million and several reports have shown resistant pathogenic species isolated from cats and dogs (Guardabassi et al., 2004, Ewers et al., 2010, Lloyd, 2007). One of the most concerning resistant human pathogens to be found in companion animals is *E. coli* ST131 O25:H4. The study by Ewers *et al.* identified ST131 O25:H4 ESBL producing strains from dogs and horses (Ewers et al., 2010). O25:H4 is a human pandemic strain which is generally characterised by carriage of fluoroquinolone and 3GC resistance (Matsumura et al., 2015, Coque et al., 2008, Zhong et al., 2015, Can et al., 2015, Dhanji, 2011, Rooney et al., 2009). Finding this resistant pathogenic strain in companion animals is therefore concerning and may result in the further dissemination of this pathogen.

Probably one of the most important reports of livestock resistance transmitted to humans is the *mecC* gene. The *mecC* gene was identified in an isolate of what was believed at the time to be *mecA* carrying MRSA from bovine mastitis in 2007. Strains of *S. aureus* carrying the *mecC* gene are characteristically animal adapted lineages suggesting that the transmission of *mecC* likely originated from animals, transferring to humans (García-Álvarez et al., 2011).

1.6.5 The impact of WWTPs on the dissemination of ARG

WWTPs collect 11 billion litres of water from homes, municipal, commercial and industrial premises and rainwater run-off from roads every day in the UK (DEFRA, 2012). There are four stages of wastewater treatment that can be employed in the UK sewage treatment process (Figure 1.3). These are preliminary treatment, primary treatment, secondary treatment and tertiary treatment with tertiary treatment being the most efficient method of wastewater treatment. The level to which wastewater is treated is dependent upon the population equivalent which takes in to account the size of the community and measure the required oxygen level to break down the organic matter in waste water considering if waste is from domestic origin. Other factors taken in to account include the receiving water body and its sensitivity to discharges, considering if discharge is to inland, estuarine or coastal waters

(DEFRA, 2012). In developing countries, the wastewater treatment process is not as extensive and often untreated sewage is released in to water bodies. It has been suggested that the spread of the *bla*$_{CTX\text{-}M\text{-}}$ genes are in part attributed to the poor sewage disposal processes of developing countries (Hawkey, 2015).

Preliminary treatment involves grit removal by flow attenuation and removes large solids from wastewater. Primary wastewater treatment is the minimum treatment. It is only considered for areas less sensitive which include estuarine and coastal waters as the receiving water bodies and is no longer used in the UK. It involves the settlement of suspended solids and is therefore a fairly inefficient removal of waste. Secondary treatment is required for all communities of 15000 or more. The risk associated with major untreated waste arises from the increased oxygen level required for efficient removal of waste (DEFRA, 2012). Secondary treatment involved biological treatment using the activated sludge process involving aeration and agitated bacterial culture liquor. It also uses filter beds which use bacteria-coated aggregate which wastewater is trickled over. The most efficient treatment, tertiary treatment can involve various treatments to increase the quality of effluent water. Methods can be combined methods or individual. Potential methods involved phosphate removal, nitrate removal by chemical process and disinfection either through UV radiation of through filter membranes (DEFRA, 2012).

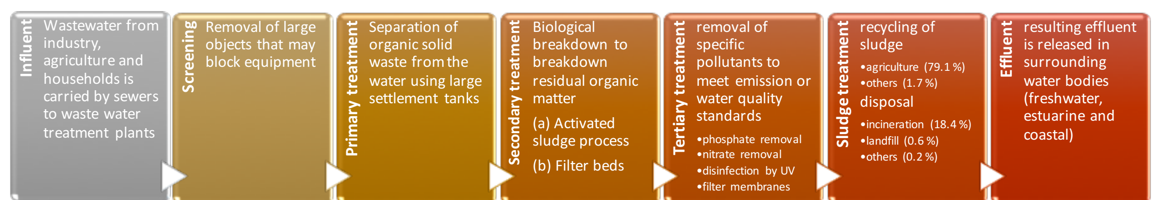


Figure 1.3 The key steps involved in the WWTP process

There has been a lot of work investigating the importance of WWTPs in the dissemination of ARG (Li et al., 2015a, Amos et al., 2014). The general consensus is that they promote HGT as a direct result of increased selection pressures from the mixing of wastes from hospitals, industries, households and agriculture and

resultantly have a high diversity of bacteria present (Berendonk et al., 2015, Berglund et al., 2014, Rizzo et al., 2013). One study reported more than 700 genera and 3000 operational taxonomic units (OTUs) in activated sludge (Zhang et al., 2012b). Rivers impacted by WWTPs represent one of the most extreme examples of human pollution of the environment and consequently has led to river systems contaminated with ARG and ARB. Escalating population numbers have resulted in increased pressure on the WWTP companies to deal with the rising volumes of waste. Recent studies have seen significant levels of coliform bacteria within river systems possessing numerous antibiotic resistances as a consequence (Amos et al., 2014, Auerbach et al., 2007, Berglund et al., 2015, Li et al., 2015a, Wang et al., 2013).

Studies investigating resistance genes before and after treatment through WWTPs have reported a diverse range present before and after but most show a reduction in the abundance of resistance genes in the effluent (Szczepanowski et al., 2009, Li et al., 2015a, Auerbach et al., 2007, Al-Jassim et al., 2015, Yang et al., 2014). The selection pressures exerted in treatment process from the mixing of antimicrobial agents from wastes results in mutations or selective gene transfer events. Much of the antibiotics consumed by animals and humans are excreted with between 30 – 90 % of antibiotics being excreted unchanged (Sarmah et al., 2006). These antibiotics can consequently end up in the WWTPs along with heavy metals and disinfectants (Su et al., 2014b). WWTP do not monitor the levels of biocides and antibiotics in the treatment plants and are not expected to remove these compounds from waste. The only specified metal that must be removed is mercury (DEFRA, 2012). Sublethal concentrations of antibiotics have been shown to increase the rate at which mutations occur with significant genomic and phenotypic changes resulting in increased MDR and in some cases increased fitness (Chow et al., 2015, Morero et al., 2011, Fuzi, 2016, Marcusson et al., 2009). Increased transposon activity has also been observed, with gene rearrangements allowing more efficient expression of the required resistance genes by movement to a position nearer the promoter resulting in an increased level of resistance (Barraud and Ploy, 2015, Hocquet et al., 2012). Recombination has also been shown to increase in frequency with sublethal antibiotic treatment and DNA mobilisation has been reported to increase in the

presence of antibiotics in several studies, with the increase in movement of the IncA/C plasmid and increased HGT events in the presence of sublethal concentrations of tetracycline (Lopez and Blazquez, 2009, Johnson et al., 2015, Jutkina et al., 2016). In the absence of no direct selection induced by the presence of antibiotics in waste, varying abundance of ARG and ARB can be explained by the process of coselection (Randall et al., 2007, Russell, 2003). It has been reported that biocides and metals have the capacity to co-select ARG and with a recent study showing levels of biocide and metals resistance in wastewater at 50-300 times that of ARG there is a huge potential for co-selection (Pal et al., 2015, Baker-Austin et al., 2006, Bengtsson-Palme, 2016). The same study also showed that some metal and biocide resistance genes were enriched in the effluent (Bengtsson-Palme, 2016). One study also showed that over the period of 3 years the exposure to quaternary ammonium compounds (QACs) resulted in an increase in resistance genes in river sediment communities (Oh et al., 2013). Clearly, co-selection is dependent upon the genetic context of the genes, therefore knowing the context of resistance genes and what additional elements are carried by the same integron/transposon/plasmid is important in understanding the extent to which co-selection can occur in WWTPs. A recent study investigated ARG abundance and prevalence during the WWTP taking in to account the concentration of biocides and metals found that although the number of ARG did decrease in the effluent compared to the influent the relative ARG did not decrease to the same extent. It was also found that the WWTP in fact enriched for the carbapenamse gene $bla_{OXA\text{-}48}$ during the treatment process (Bengtsson-Palme, 2016).

Investigating the plasmid metagenome can help elucidate co-carriage of resistance genes. Schluter and colleagues have carried out much of the current work on the plasmid metagenome with the first study in 2008 (Szczepanowski et al., 2008). They published three papers from the same dataset generated from the plasmid genome of cultured bacteria from activated sludge selecting for ARB with 12 antibiotics (Szczepanowski et al., 2009, Schluter et al., 2008, Szczepanowski et al., 2008). The first study found a diverse range of plasmids in these viable resistant bacteria including IncP, IncN and IncT type plasmids with a wide array of resistance genes to all the classes used for isolation (Szczepanowski et al., 2008). The second study

further investigated the plasmid metagenomes and aimed to determine the genetic diversity. They reported 1050 different protein families encoding a range of plasmid-related functions. They concluded that many of plasmids sequenced were mobile from the sequencing signatures and that within plasmids were many transposons encoding highly mobilisable genetic regions (Schluter et al., 2008). The third study focussed on the presence of ARG in the samples taken from activated sludge and final effluent using PCR-based approach to investigate 193 key ARG. They reported a reduction in the prevalence of ARG in the final effluent compared to the sludge with 123 and 140 ARG detected respectively with resistance to all classes of antibiotics and ARG (Szczepanowski et al., 2009). In 2011, a different approach was taken by Zhang *et al.* to isolate plasmids. They employed the transposon aided capture (TRACA) system to isolate plasmids from activated sludge from a WWTP in China (Zhang et al., 2011). This study also reported a large number of ARG and MGEs present in the plasmid metagenome. They identified novel plasmids but fewer ARG than the previous studies, possibly attributable to the enrichment method used by Schluter and colleagues (Zhang et al., 2011). Consistent with Schluter, they identified ARG or a diverse range of antimicrobial classes (Zhang et al., 2011). In 2013, Sentchilo *et al.* reported the first comprehensive analysis of extrachromosomal DNA from WWTPs. They isolated both plasmid and circular phage DNA from activated sludge samples directly reporting a large levels (51-68 %) of uncharacterised coding sequences convincingly showing that plasmids are a clear driving force for innovation of genetic material (Sentchilo et al., 2013). It was also shown that compared with complete metagenomes, plasmid metagenomes carry a significant number of ARG that are detectable in full metagenomes and that plasmids and resistance determinants vary between treatment plants, even those with similar influents and size (Sentchilo et al., 2013). The most recent study of the plasmid metagenome comprises the most extensive study. It was carried out by Li *et al.* and investigated influent, activated sludge, digested sludge from two WWTPs. It identified 323 ARG and 23 MRG with a higher number of resistance genes found in the influent compared to the other samples. Unlike other studies, Li *et al.* compared the plasmid metagenome to corresponding metagenomes of the same samples revealing a higher annotation of plasmid metagenomes compared to the total metagenomes

suggesting plasmids carry genes that may be shared with culturable microorganisms which are better characterised (Li et al., 2015a).

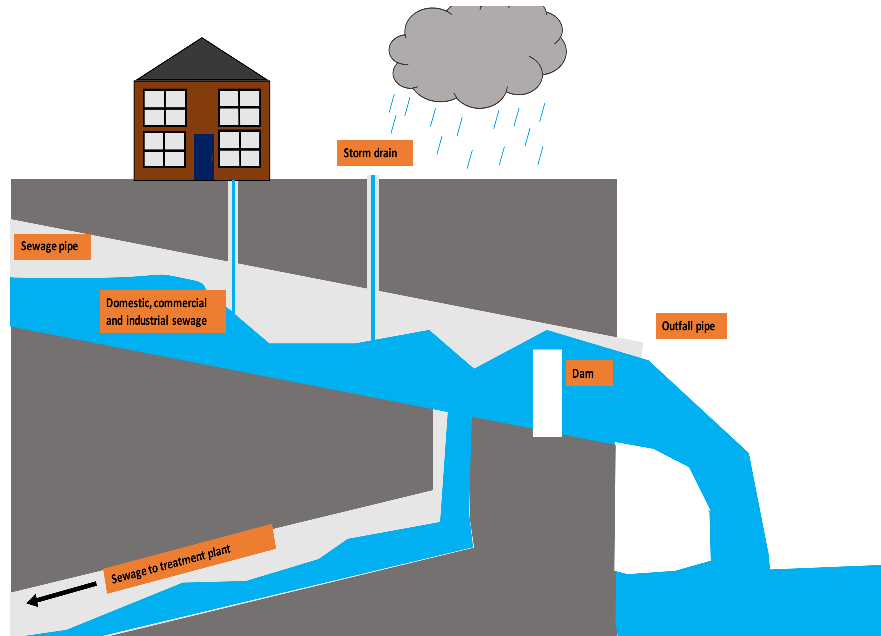
In 2010, 18.4 % of sewage sludge was incinerated, 0.6 % put to landfill and 0.2 % was disposed of through other methods, 1.7 % was redistributed for unspecified uses and the remaining 79.1 % of sewage sludge was re-used for agricultural purposes (DEFRA, 2012). Considering the extensive ARGs and ARBs that have been found in sewage sludge, the substantial amount of sewage that is applied to land is no doubt contributing to the environmental resistome with large numbers of resistance plasmids and ARG found in soils treated with sludge (Martini et al., 2015, Jechalke et al., 2015, Smalla et al., 2000a, Heuer et al., 2012).

In times of heavy rainfall WWTPs are able to release untreated wastewater and sewage in to rivers via combined sewer overflows (CSOs) (Figure 1.4). The number of CSO events that take place is often unmonitored (Robert Huxam, SevernTrent Water, personal communication, 13th July 2016) resulting in an unknown volume of raw sewage entering the UK rivers. Clearly, this will play a role in the ARB and ARG levels at sites impacted by CSO release events. With an unknown number of release events studying CSOs is difficult and consequently there have been few studies that have tried to evaluate the significance of these events on the river environment. Originally, CSOs were introduced in 1855 in an attempt to improve urban life with the removal of cesspool ditches (Tibbets, 2005). CSOs are considered major sources of microbiological and physiochemical pollutants (Madoux-Humery, 2015) yet the extent to which CSOs contribute to environmental pollution has been rarely studied. One study conducted by Madoux-Humery *et al.* attempted to investigate the temporal changes in *E. coli* and micropollutants as a result of CSO release events reporting an increase of three orders of magnitude of *E. coli,* total suspended solids and micropollutants during a release event (Madoux-Humery, 2015). Another study by Jalliffier-Verne *et al.* also investigated the prevalence of *E. coli* in discharges from CSOs during release events by estimating *E. coli* concentrations based on monitoring data and modelling the cumulative effects of CSO releases (Jalliffier-Verne, 2016). CSO release events were surprisingly recorded in periods of dry weather suggesting

an overuse of these emergency outlets. They concluded that the presence of *E. coli* was related to the concentration of discharge rather than the number of overflows and that specific CSO events were more important in determining the presence of *E. coli* in river water (Jalliffier-Verne, 2016). They also reported high *E. coli* abundance in drinking water intakes as a result of the dispersion process resulting from high flow as a direct result of release from the CSO suggesting that high flow and dispersion is more important than dilution factor and consequently is something that should be taken in to consideration by wastewater treatment companies when considering release events (Jalliffier-Verne, 2016).

Release events result in the disruption of river sediment which consequently can also result in increased risk associated with river water at these sites (Jalliffier-Verne, 2016, Madoux-Humery, 2015). There has been little work investigating sediment as a source of antibiotic-resistant pathogens with most studies primarily investigating the water body (Kotlarska et al., 2015, Bonetta et al., 2016, Tang et al., 2016, Amador, 2015, Baquero et al., 2008, Ferreira da Silva et al., 2007, Akter et al., 2012, Hu et al., 2016, MacLellan et al., 2015). A couple of studies have identified *bla*$_{CTX\text{-}M\text{-}}$ genes from sediment however suggesting sediment is an important reservoir of ARG (Amos et al., 2014, Lu et al., 2010). No studies could be found comparing water and sediment ARB to demonstrate the differences in abundance. It is apparent that both carry a significant level of ARG and ARB and therefore must be investigated if correct risk assessments are to be made. Another factor that should also be considered, but is rarely investigated are bioaerosols (Korzeniewska and Harnisz, 2013). Korzeniewska and Harnisz showed that ESBL producing *Enterobacteriaceae* were present in air samples, with higher numbers in samples taken at higher temperatures and wind speeds. Similar results were also reported previously by Gotkowska-Płachta *et al.* and Korzenewska *et al.* who were also able to detect *Enterobacteriaceae* in air samples using culture based methods (Gotkowska-Płachta et al., 2013, Korzeniewska et al., 2009).

(a)



(b)

Figure 1.4 CSO release events (a) Release of untreated water from outlet pipe resulting from high level of wastewater. Dam regulates the flow of water from CSO, in times of heavy rainfall, the wastewater level increases above the threshold for the 'pass forward flow' as defined by the EA and untreated wastewater is released directly in to receiving water bodies. (b) photograph of one of the CSO release sites from the Finham WWTP.

Although most evidence supports the opinion that WWTPs contribute to the surrounding environment, there are a few reports that suggest evidence to the contrary. The recent study by Munck *et al.* investigated the dissemination of WWTP core resistome in the surrounding environment. Seven metagenomes were sequenced over a two-year period and a core resistome was based on the comparison between functional metagenomic results and complete metagenomes. It was suggested that due to the stability within the WWTP communities and core resistomes, HGT is a rare event contrasting with many studies which suggest HGT is a likely event due to the mixing of large quantities of bacteria, biocides and antibiotics which can select directly and indirectly for antibiotic resistance (Munck et al., 2015). Core resistomes were compared with numerous available metagenomes including human gut, cow rumen and permafrost and aquifer finding only 6 of the core genes of the core resistome were present in other, non-WWTP genomes. No river sediment metagenomes were compared so it is unclear from this study if the WWTP metagenome is similar to the river sediment resistome downstream of the WWTP. It also highlighted that only a small fraction of the community exchange genes, however this subpopulation of bacteria that undergo HGT are likely the most important fraction and often, the infectious dose of such microorganisms is low so although they are only present in a small fraction of the total community, their existence is important (Kaper et al., 2004).

1.6.6 Clinical impacts on the dissemination of ARG

In 2014 the World Health Organisation listed *E. coli, K. pneumoniae* and *S. aureus* as concerning pathogens with respect to resistance (WHO, 2014). Specific resistance included 3GC and fluoroquinolone resistance in *E. col,i K. pneumoniae* and MRSA.

MRSA in Europe and the United states decreased between 2007-2015 with reductions of 22 to 18 % and 53 to 44 % respectively of methicillin *S. aureus* isolates. MRSA was first identified in the UK in the 1960s and was generally regarded as a healthcare-associated pathogen (Gelband, 2015). The emergence of community-associated MRSA (CA-MRSA) infections over the last 2 decades however has resulted in infections outside hospitals and acquired AMR in CA-MRSA has resulted in very

similar phenotypes observed between CA-MRSA and healthcare-associated MRSA (HA-MRSA) (Bal et al., 2016, David and Daum, 2010). MRSA differs from methicillin-susceptible *S. aureus* (MSSA) through the acquisition of the staphylococcal cassette chromosome (SCC) element which characteristically carries the *mecA* or *mecC* genes as well as other resistance genes (Bal et al., 2016). The dominant clones of HA-MRSA in the UK are ST22 and ST36 and dominant clones of CA-MRSA in Europe is the ST80 clones. In 2010 the control of MRSA was increased in the UK with the English national policy changed to screen all hospital admissions for MRSA however due to substantial costs associated policy was streamlined to only test patients admitted to high risk units and patients who had previously identified as colonised or infected by MRSA (Robotham et al., 2016, ARHAI, 2014).

The range of antibiotic resistance in *K. pneumoniae* is extensive with isolates carrying carbapenemases (KPC) and oxacilinase 48 (OXA-48) found globally. The carriage of carbapenemase genes often correlated to the carriage of extensive β-lactamase genes and consequently treatment of infections with carbapenem resistant (CR) *K. pneumoniae* is restricted to the last resort antibiotics colistin and polymyxin B (Lee et al., 2016). As a result there is a high mortality rate associated with bloodstream infections with CR *K. pneumoniae* (Munoz-Price et al., 2013). In the UK outbreaks of KPC producing *K. pneumoniae* susceptible to fluoroquinolones began in 2010 with positive isolates carrying CR as a result of an IncFIIK plasmid (Munoz-Price et al., 2013). Isolates of *K. pneumoniae* carrying the metallo-β-lactamse bla_{NDM-1} have more recently been identified (Lascols et al., 2013). The spread of CR within *K. pnuemoniae* is due to the large numbers of plasmids able to transfer resistance. The bla_{KPC} gene has been identified on IncF, IncI2, IncX, IncA/C, IncR and ColE1 plasmids and the bla_{NDM-1} gene has been identified on IncA/C, IncF, IncR, IncH, IncN, IncL/M and IncX plasmid types (Garcia-Fernandez et al., 2012, Lee et al., 2016).

1.7 The role of *E. coli* in human clinical infections

E. coli is typically found as a commensal of the GI tract of humans. It colonises infants only a few hours after birth and usually the relationship is mutually beneficial (Kaper et al., 2004). It is usually found to be most abundant in the colon however its ability

to survive and outcompete other bacterial species in the colon is poorly understood. One proposed mechanism of survival is that it is able to exploit gluconate more efficiently than other species (Sweeny, 1996). Although *E. coli* is generally considered to be a commensal bacterial species, there are some pathotypes that have acquired virulence factors making them opportunistic pathogens and in some cases true pathogenic species. This acquisition of virulence is usually from MGEs which either continue to exist as MGE or could be inserted in to the chromosome (Kaper et al., 2004). These pathotypes can cause diarrhoeal disease and urinary tract infections (UTIs). There are 6 types of *E. coli* that are able to cause intestinal disease, collectively all extraintestinal pathogens are termed ExPEC. The intestinal pathogens include the enteropathogenic *E. coli* (EPEC) enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative (EAEC), enteroinvasive *E. coli* (EIEC) and the diffusely adherent *E. coli* (DAEC).

The most important clinically related pathogenic *E. coli* is the uropathogenic *E. coli* (UPEC) which causes UTIs but increasingly common are extraintestinal infections resulting from meningitis-associated *E. coli* (MNEC). Pathogenesis of *E. coli* is a multi-step process involving the colonisation of a mucosal site, evasion of defence, multiplication and finally host damage which ultimately involves many factors including adhesins, invasins, toxins, autotransporters, siderophores as well as many other virulence determinants (Table 1.1).

EPEC were the first type of pathogenic *E. coli* to be identified in the UK in 1945 as a result of a large outbreak in children. Such outbreaks are no longer common in industrialised countries but still occur in developing countries (Nataro, 1998). ETEC infections cause watery diarrhoea which can range from mild, self-limiting disease to sever purging disease. Like EPEC infections they are responsible for many childhood diarrhoeal disease in the developing world (Nataro, 1998). ETEC strains are also responsible for causing animal disease. These strains express fimbrial intestinal colonisation factors (K88 and K99) which are not present in human infecting strains (Kaper et al., 2004). EHEC strains were first identified in 1982 and cause bloody diarrhoea, non-bloody diarrhoea and haemolytic uremic syndrome. Infections are

associated with the bovine intestinal tract and often outbreaks are associated with undercooked hamburgers (Kaper et al., 2004). The low infectious dose (estimated below 100 cells) has led to many outbreaks of EHEC associated disease. The Shiga toxin producing O157:H7 serotype is the most important EHEC pathogen in the UK, but there are other important serogroups responsible for disease (O26 and O111). EAEC cause persistent diarrhoea in both developed and developing countries. They are currently characterised by adhering to HEp-2 cells and the fact they do not secrete heat-labile or heat-stable enterotoxins (Nataro, 1998) but the characterisation of all EAEC as human pathogens is debated and it is likely non-pathogenic clones are in this group of *E.coli.* EIEC are very closely related to *Shigella* species. They are responsible for causing watery diarrhoea and may play a role in invasive inflammatory colitis (Nataro, 1998). Pathogenesis is related to plasmid-borne type III secretion system which secrete IpaA, IpaB, IpaC and IpaD which are involved in epithelial signalling, cytoskeletal rearrangements, cellular uptake, lysis of endocytic vacuole as well as other actions (Sansonetti, 2000, Tran Van Nhieu, 2000). DAEC are defined as a subgroup based on the diffuse pattern of adherence to Hep-2 cell monolayers. They are able to cause diarrhoeal disease particularly in children over 12 months of age (Nataro, 1998, Bilge, 1989). The majority can be characterised by the production of F1845 fimbrial adhesion. UPEC strains cause urinary tract infections and are the most common bacterial infections which is primarily caused by *E. coli.* There are six O groups associated with the majority of UTI infections (Kaper et al., 2004). These UPEC strains have no distinct phenotypic profile but the Pap adhesion and FIC fimbriae are often associated and are important factors in the colonising of the urinary tract (Johnson, 1991, Nowicki, 1989). UPEC strains typically possess toxin encoding genes including haemolysin, cytotoxic necrotizing factor and autotransporter protease (Sat) and also carry pathogenicity islands (Kaper et al., 2004). MNEC are responsible for causing neonatal meningitis and are the most common cause of this potentially fatal disease with 15-40 % of cases resulting in death and of the survivors it is common that they will suffer from severe neurological defects (Unhanand, 1993, Dawson, 1999). 81 % of such cases are caused by strains with the K1 capsule type. They are spread haematogenously and a higher level in the blood correlates with a higher probability of contracting meningitis. MNEC are able

to translocate from the blood to the central nervous system with no damage to the blood-brain barrier. The infection of the central nervous system does not confer an obvious advantage with respect to selection and transmission as like the other pathotypes they are readily transmitted in urine and faeces (Kaper et al., 2004). The pathotypes causing disease in animals and humans often carry many of same virulence factors making the source of pathogenic *E. coli* often hard to determine. The avian pathogenic *E.coli* (APEC) pathotype however is primarily associated with respiratory infections, pericarditis and septicaemia of poultry (Kaper et al., 2004).

In combination with the extensive virulence factors, *E. coli* typically carry wide range of resistance genes. The emerging human pandemic O25:H4 ST131 clone which is responsible for causing UTI infections in both humans and animals (Can et al., 2015, Hertz et al., 2016, Ewers et al., 2010). Fluoroquinolones are often the first line response to the treatment of this strain of *E. coli,* however there are several reports of strains carrying an IncF-type plasmid with both 3GC and fluoroquinolone resistance genes (Matsumura et al., 2013, Dhanji, 2011, Phan et al., 2015, Rooney et al., 2009). IncF plasmids carrying the $bla_{CTX\text{-}M\text{-}15}$ gene are not exclusive to the ST131 clone but are in fact carried by a range of clones including ST405, ST354, ST28 and ST695 and often carrying additional resistance genes including bla_{OXA} and *aac(6')-IB-cr* (Carattoli, 2009).

Table 1.1 Virulence factors in *E. coli*

Type	Gene	Description	Associated
Adhesin	*paa*	Porcine A/E-associated gene	EPEC
	ompA	Outer membrane protein, heat-modifiable OMP, structural and ion-	MNEC, EHEC, UPEC
	fimH	Type 1 fimbriae, D-mannose-specific adhensin	ExPEC
	csgG	Facilitator of fibronectin-binding curli assembly	Various
	sfmA	Fimbrial-like adhesion protein	Various
	aidA	Adhesion involed in diffuse adherence consisting	DAEC
	fanAB	Fimbrial adhesin	ETEC
	papA-G	P fimbriae, adhesion, includes cytokine expression	UPEC
	F41	adhesin	ETEC
	focA	Formate channel	UPEC
	focG	Pilus tip molecule, F1C fimbriae (sialic acid specific)	ExPEC
	iha	Novel nonhemagglutin adhesion	EHEC
	nfaE	Non fimbrial adhesion I assembly and transport	ExPEC
	sfa2 operon	S fimbriae	ExPEC
	gafD	Chaperone protein	Various
	hra	Heat-resistant agglutinin	UPEC
	tsh	Temperature-sensitive hemagglutinin	APEC
	mat operon	Meningitis-associated and temperature-regulated fimbrail operon	MNEC
	afa	Described as an Afimbrial adhesion but in fact has fine fibrillary structure	ExPEC, many UPEC
	tia	Outer-membrane proteins mediating adhesion to colonic epithelial cells	ETEC
Toxins	*hylA*	α-hemolysin	ExPEC
	sheA	Silent hemolysin gene	ExPEC
	cnf1	Cytotoxic necrotizing factor 1	ExPEC, NTEC
	stx	Shiga toxin, acts through cleavage of ribosomal RNA disrupting protein	EHEC (STEC)
	cif	Cycle inhibiting factor, blocks cell division possibly by inhibition of Cdk1	ExPEC
	map	Two activities: stimulates Cdc42-dependant filopodia formation, targets	EPEC, EHE
	cdt	Cytolethal distending toxin, DNaseI activity blocking cell divison in G2/M	ExPEC
Autotransporter	*agn43*	Biofilm formation, autoaggregation and attachment to cells	EAEC
Siderophores	*fyuA*	Yersina siderophore receptor (ferric yesiniabactin uptake	ExPEC, ETEC
	ireA	Iron acquisition, siderphore receptor	ExPEC, UPEC
	iroN	Iron acquisition, siderophore receptor	UPEC
	iutA	Ferric aerobactin receptor	ExPEC, APEC
	Irp2	Iron repressible protein	APEC
	iucD	Within Aerobactin operon	APEC
	sitD	Iron transport	APEC
	eitA	Iron transport	APEC
Invasins	*ibeA*	Invasion of brain endothelium	ExPEC, MNEC
	ipaH	Invasion plasmid antigen	EIEC
	gimB	Genetic island associated with human newborn meningitis	NMEC, UPEC APEC
Other	*traT*	Serum-resistance associated outer membrane protein	ExPEC
	ompT	Outer membrane protein A and T (protease	ExPEC
	malX	Phosphotransferase system enzyme II that recognises maltose and	ExPEC
	fliC	Flagellin structural protein, H antigen determinant (H19 allele)	ExPEC
	copA	Cu(I)-translocating efflux pump	Various
	cueO	Copper oxidase	Various
	etsA	Putative ABC transport system, strongly associated with NMEC	NMEC
	cvaC	Plasmid borne colicin V production	ExPEC
	pic	Secreted mucinase. Antisense strand encodes Shigella enterotoxin	EAEC and UPEC
	Iss	Increased serum survival	ExPEC
	neuC	Sialic acid synthesis	Various

1.8 Mobile genetic elements

1.8.1 Plasmids

Plasmids and integrons arguably represent the most significant routes of bacterial transmission of ARG and are consequently the most studied method of ARG dissemination (Norman et al., 2009). They have evolved mechanisms that allow the efficient capture, expression and transfer of genes within and between bacterial species allowing the fast proliferation of ARG within the environment and clinic.

The term plasmid was first coined in 1952 by Joshua Leaderberg to describe any "extrachromosomal hereditary determinant" (Lederberg, 1998). Since then, there have been more than a thousand plasmids identified from all three domains of life and from almost every environmental niche (Norman et al., 2009). Approximately 96 % of the plasmids sequenced so far have been bacteria, 2.9 % from archaea and 1 % from eukaryote (Shintani et al., 2015).

Success of many plasmids is due to their ability to replicate independently of host chromosomal DNA and the ability to self-transfer through the active process of conjugation (Carattoli, 2013). Ultimately plasmids act to accumulate and transfer non-essential genes that confer benefit under selective pressures (Norman et al., 2009). The mobilisation of ARGs has given rise to "superbugs" which have evolved extensive methods of resistance to nearly all antibiotics (Partridge et al., 2009, Gaze et al., 2013, Wellington et al., 2013, Marti et al., 2013b). It has been reported that ~14 % of plasmids sequences so far are capable of conjugation (Smillie et al., 2010). Plasmids able to undergo conjugation in Gram-negative bacteria typically carry origin of transfer (*oriT*), relaxase, type IV coupling protein (T4CP) and type IV secretion system (T4SS), which allow double stranded plasmid DNA to undergo cleavage at the *orit* site by the relaxase protein which then covalently binds *oriT* DNA which is transported to the recipient cell via the T4SS and single-stranded DNA is transferred by the T4CP (Shintani et al., 2015). In Gram-positive bacteria plasmid transfer is less well understood but is known to occur via two mechanisms, the first involves the single stranded plasmid DNA transported by a T4SS and second through conjugative

systems that are similar to segregation of chromosomal DNA in cell division mediated by a FtsK-like protein (Shintani et al., 2015).

The first record of plasmid-mediated ARG transfer was in 1959 by Ochiai *et al.* who transferred resistance from *Shigella* to *Escherichia coli* (Ochiai, 1959). The process of conjugation is generally regarded to be genetic exchange between bacteria, but it has been documented on a few occasions that transkingdom transfer can occur. Plasmid transfer from *E. coli* to *Saccharomyces cerevisiae* was first demonstrated by Heinemann and Sprague back in 1989 (Bates et al., 1998, Hayman and Bolen, 1993). This route of transmission is not well studied in the environment but may provide another viable route of plasmid dissemination.

A study investigating conjugative transfer from strains collected in the 'pre-antibiotic era' and strains collected 25 years later found that the same plasmid incompatibility (Inc) types were detected in both collections suggesting ARG mobilise to already favourable plasmids (rather than selection of rare plasmids) (Datta and Hughes, 1983, Hughes and Datta, 1983). This is evident when examining the dissemination of the IncF plasmids within *Enterobacteriaceae.* The IncF-type plasmids have been commonly detected in both clinical and environmental isolates of *Enterobacteriaceae* and pose one the most significant threats to transmission of ESBL genes. These plasmids are highly present in *E. coli* isolates and are often found to carry $bla_{CTX\text{-}M\text{-}15}$. They often possess virulence determinants making them favourable in the environment possibly contributing to increase fitness (Carattoli, 2009).

One of the first records of plasmid detection outside of the clinic was in the 1970's with plasmid-possessing strains of *Enterobacteriaceae* being isolated from eggs by Lakhotia *et al.* in 1973 (Lakhotia and Stephens, 1973). From the 1970's the environment was highlighted as a recognisable source of ARG and investigations in to the routes of ARG dissemination initiated. At this point, transmission routes were still widely unknown and most plasmid-focussed work was based in the laboratory (Cullum et al., 1978). Since then plasmids have been detected frequently in the environment through exogenous capture, highlighting the importance of IncP

plasmids, carriage of plasmids in environmental isolates and more recently plasmidome studies have investigated the WWTP environment to determine the fraction of the metagenome that is mobilisable (Fox et al., 2008, Schluter et al., 2007, Jechalke et al., 2015, Amos et al., 2014, Li et al., 2015a).

One of the first experiments investigating the environmental reservoir of ARG was in 1976 which investigated plasmid transfer associated with human waste. The study investigated plasmid possession and transfer of ARG from hospital and domestic waste isolates (Fontaine and Hoadley, 1976). Later, experiments pinpointing wastewater treatment plants as a potential source of ARG and multidrug-resistance gene-containing plasmids began in the early 1980's. Two separate studies, both conducted in 1982 were perhaps the first studies to link plasmid-mediated ARG transmission to WWTP in what is now considered a known "hotspot". Altherr and Kasweck investigated the effects of WWTPs on the effects of conjugation of a 60-megadalton plasmid possessing antibiotic resistance genes (Altherr and Kasweck, 1982). They showed that transfer occurred only in raw sewage and not in the effluent-receiving waters. This was one of the first studies demonstrating that ARG could transfer in situ. At the same time Mach and Grimes at the University of Wisconsin-La Crosse were also working on plasmid transfer in wastewater treatment plants (Mach and Grimes, 1982). They transferred multidrug resistance-gene containing plasmids from *Salmonella enteritidis, Proteus mirabilis* and *E. coli* possessing resistance to ampicillin, chloramphenicol, streptomycin, sulfadiazine and tetracycline to susceptible *E. coli* and *Shigella sonnei.* They demonstrated the plasmid-mediated transfer of resistance in the absence of antibiotics (Mach and Grimes, 1982).

Plasmid persistence varies significantly between environment and host. There are four basic principles that underlie persistence: efficient plasmid segregation during cell division, addiction systems including plasmid-encoded toxin-antitoxin systems, conjugative transfer of plasmids to infect new cells, and a low plasmid cost to prevent host rejection of plasmid and reduce fitness costs (Ebersbach and Gerdes, 2005, Hayes, 2003, Stewart and Levin, 1977, Ponciano et al., 2007). Plasmid persistence can

be gained through acquisition of TA systems which concurrently can provide a larger host range and thus are favourable in environmental conditions where plasmids may be transferring to a diverse range of hosts (Loftie-Eaton et al., 2015, Hayes, 2003, Goeders and Van Melderen, 2014). TA systems in plasmid maintenance is via post-segregational killing which relies on the different stability of the toxin and antitoxin where the toxin is more stable than the antitoxin. Daughter cells that do not acquire a plasmid will have toxin in their cytoplasm but no mechanism to produce the antitoxin resulting in cell death (Goeders and Van Melderen, 2014). Therefore, plasmid carriage is advantageous to daughter cells and will disseminate in the bacterial population (Goeders and Van Melderen, 2014).

The mechanisms underlying plasmid possession in non-selective conditions is of particular interest with respect to plasmid persistence within the environment. In laboratory conditions it is generally observed that plasmids from the environment are highly stable and not readily lost. However, it is unlikely that plasmids are as stable in the natural environment with the expectation that if it were so, with time all bacterial cells would possess one plasmid from each plasmid incompatibility group (Sorensen et al., 2005). Studies investigating plasmid stability in the environment are however limited due to the laboratory methods required to determine plasmid loss which involve replica plating, selective markers and PCR detection (Smalla et al., 2000b).

Investigating the rate at which plasmids disseminate consequently determines how successful plasmids are. It is of particular interest and concern with respect to the future prevention of environmental spread of ARG. Understanding the rate of transfer may provide better models for transmission rate. The majority of experiments aiming to determine the rate of transfer rely on estimating the rate of transfer in model organisms with well-known genetics in highly controlled environments (Aminov, 2011). They rely upon simple cultivation needs, tolerance to high nutrient levels and ease of genetic manipulation, which makes them more amenable to plasmid transfer. Although useful as a reference these experiments are unlikely to be a true interpretation of environmental rates of transfer. Studies with

these microorganisms can only partly predict the level of transfer in the environment. All these experiments rely on the culturable fraction, which is known to be less than 1% of all bacteria (Amann et al., 1995, Torsvik et al., 1990). Investigations using "wild" environmental strains are more likely to give more realistic transfer rates occurring in the environment. However, replicating mating conditions in the laboratory is difficult due to several factors which all vary the transfer rate, for example, whether mating is liquid or surface based, the incubation period and temperature, oxygen availability, chemotaxis and pH (Smalla et al., 2015). Any factor affecting the metabolic activity of the plasmid donor will affect the transfer rate. The incompatibility group will also affect the transfer rate in broth and surface matings. This was demonstrated by Bradley *et al*. who investigated the transfer frequencies of different incompatibility groups in *E. coli* K-12 on surface and broth showing that some plasmids are more likely to transfer on solid or liquid surfaces (Bradley et al., 1980). The rate of transfer also depends on the environment, and thus microcosm experiments can only accurately estimate the rate of transfer in a that particular environment. It has been predicted that the rates of transfer detected in the laboratory are significantly lower than in the environment (Sentchilo et al., 2013, Sorensen et al., 2005).

Plasmid host range is also important when considering rate of transfer with IncP and IncA/C typically classified as broad-host range plasmids and IncF, IncX and IncH are narrow spectrum (Carattoli, 2009). The GC content is also important for determining host range with GC contents similar to host likely involved in compatibility (Rocha, 2002).

Incompatibility (Inc) has defined plasmid groups since the 1970's (Shintani et al., 2015). It is based on the observation that plasmids of the same ancestry are not compatible due to the same requirements for replication and partition (Novick, 1987). There are 3 groups of Inc types at present, 27 within *Enterobacteriaceae*, 14 in *Pseudomonas* and 18 in *Staphylococcus* (Shintani et al., 2015, Carattoli, 2009, Carattoli, 2013). Incompatibility is determined by the amino acid sequences of the replication initiation (Rep) protein which is often confirmed by demonstrating *in vitro*

the incompatibility in the same group. Generally, this method of grouping is successful however there are drawbacks which include the fact that plasmids often carry multiple replicons making classification in to a defined Inc group difficult and because the information currently available on Inc groups and Rep types is limited, especially in *Enterobacteriaceae*, (Carattoli et al., 2005, Shintani et al., 2015). To overcome the problems of typing according to replicon type, grouping according to mobilisation has been suggested with 6 groups currently defined according to mobility (MOB) type (MOB_C, $MOB_{F,}$ $MOB_{H,}$ MOB_P, MOB_Q and MOB_V) (Smillie et al., 2010). Grouping based on MOB type may improve typing because it can define whole microbial plasmids and is based on the relaxase gene which is rarely carried in multiple copies on plasmids, however, as the name would suggest, it is only able to type transmissible plasmids (Garcillan-Barcia et al., 2011, Garcillán-Barcia et al., 2009).

Investigating *in vivo* transfer rate and host range is incredibly difficult. Reporter genes can be inserted in to plasmids allowing detection but this method cannot give a true interpretation of transfer rates of the native plasmid. It does provide a useful tool to investigate transmissibility under lab conditions. Klumper *et al.* investigated plasmid transfer in complex communities from three donor species (Klumper et al., 2014). They used Nycodenz™ to extract the cell community from soil and carried out plate mating experiments to investigate the transfer of these GFP-tagged plasmids. They discovered plasmids could transfer to 11 different bacterial phyla, both Gram-negative and Gram-positive. Prior to this experiment, most investigations in to plasmid host range were conducted with pure strains, which cannot exist in the environment, meaning that the question of if plasmids can transfer to another species is not entirely relevant, instead if plasmids will transfer is of greater significance, but ultimately cannot be determined without introduction of genetically modified organisms in to the environment.

It was first documented in 1970's that *Enterobacteriaceae* are highly persistent in the aquatic environments since then there has been a lot of work focussed on prevalence with respect to ARG carriage on mobilisable elements within this family particularly those isolated from the environment (Godwin and Slater, 1979). One recent paper

identified for the first time the prevalence of mobilisable *bla*$_{CTX-M-15}$ in *E. coli* within river systems in UK and highlighted the impact of WWTP in the presence of these genes (Amos et al., 2014). Clinical significance of drug-resistant Enteric bacteria has increased dramatically. In fact the most recent, and most concerning discovery of mobilisable colistin resistance was found in *E. coli* isolates from pigs in China in 2015 (Liu, 2015). This polymyxin resistance mechanism was shown to be mobilised to *E. coli* with a frequency of 10^{-1} to 10^{-3} and was shown to be stable in a range of hosts including *K. pneumoniae* and *Pseudomonas aeruginosa*, suggesting a potentially threatening environmentally stable resistance to last-resort antibiotics.

Gram positive bacteria do not readily take up genetic information, however in response to stress, competence is increased, suggesting information may be more readily received through transformation in the environment (Claverys et al., 2006). The stress response in both Gram negative and Gram positive is induced by solar UV radiation, partly explaining why aquatic environments are hotspots for genetic exchange, and particularly near to wastewater treatment plants which may have selective agents entering the water systems including chlorinated compounds which may be involved (Hader, 2000, Sentchilo et al., 2003). In 1998 microcosm experiments conducted by Ravatn *et al.* concluded that specific substrates may be required for genetic transfer to occur (Ravatn et al., 1998). Stress response may be triggered by many different factors, some of which may not directly affect DNA. This was one of the first papers to talk about the idea that there may be other selective agents for plasmid transfer.

Marcusson *et al*. discovered that induction of certain resistance mutations provided a statistically significant selective fitness advantage over strains with fewer resistance gene mutations. Some combinations of mutations conferring fluoroquinolone resistance resulted in reduced-drug susceptibility as well as increased fitness, for example, mutations in the *parC* gene compensated for fitness costs induced by mutations in *gyrA* genes (Marcusson et al., 2009). This accumulation of particular fluoroquinolone resistance genes suggests plasmid possession may not

always incur a fitness cost but instead the opposite and may provide fitness advantages under non-selective conditions (Marcusson et al., 2009). Hence plasmids may in fact enhance fitness within the environment through carriage of certain resistance genes making these plasmids particularly favourable because they both reduce susceptibility to antimicrobials and induce a selective fitness advantage.

The IncK plasmid (pCT) encoding *bla*$_{CTX\text{-}M\text{-}14}$ incurs no fitness cost when introduced in to new strains of *E. coli* (Cottell et al., 2012). The main associated plasmid of the *bla*$_{CTX\text{-}M\text{-}14}$ gene is via the IncK plasmids within *E. coli* (Valverde et al., 2009). The spread of CTX-M genes (conferring resistance to extended spectrum β-lactams) was first noted in the 1980's. The first discovery of *bla*$_{CTX\text{-}M\text{-}14}$ in *E. coli* in Korea in 1996 by Pai *et al.* (Pai et al., 2001). A mobilisable *bla*$_{CTX\text{-}M\text{-}14}$ gene originated from the *Kluyvera* chromosome with 8 separate mobilising events to plasmids from *Kluyvera* since they diverged from their most recent common ancestor (Barlow et al., 2008). The widespread dissemination of *bla*$_{CTX\text{-}M}$ genes can be attributed in part to the common association of these genes with insertion sequences (IS), ISE*cp1* and IS*CR1,* which have promoted the dissemination of the two most prevalent types of CTX-M gene; *bla*$_{CTX\text{-}M\text{-}14}$ and *bla*$_{CTX\text{-}M\text{-}15}$. The *bla*$_{CTX\text{-}M\text{-}15}$ gene is most commonly associated with FII type plasmids (Coque et al., 2008), other CTX-M enzymes have been associated with IncN, IncI and IncL/M which often carry other resistance genes including *qnr* and *aac(6')-Ib-cr* genes conferring resistance to fluoroquinolones and aminoglycosides respectively (Bado et al., 2010, Canton, 2012), hence the movement of one plasmid may infer multiple resistances.

In addition to enhanced promiscuity induced by IS elements, often these transposable genetic elements will enable continuous expression of resistance genes within cell due to location near a promoter. Typically, these elements are between 0.8 and 2.5 kb and will only encode the genes required for transposition and are able to insert in to plasmids and chromosomal DNA (Depardieu et al., 2007). They are usually bounded by inverted repeat regions of ~40 bp which are specific for each IS. They may in addition carry partial or complete promoters which are normally in an outward orientation and therefore capable of activating the expression of

neighbouring genes (Mahillon, 1998). It has been suggested that they play a role in the expression of genes conferring resistance to β-lactams, aminoglycosides, quinolones, glycopeptide, imidazoles and tetracyclines and as a result have been observed to increase resistance levels (Depardieu et al., 2007). This IS mediated expression of ARG may result from the insertion in to regions carrying weak/incomplete/no promoter resulting in the generation of a complete IS-borne promoter with the -35 and -10 regions which will form the consensus sequences TTGACA and TATAAT with 17bp spacing region (optimal promoter activity for *E. coli* (Lisser, 1993).

1.8.2 Integrons

Plasmids are able to transfer multiple resistance gene cassettes due to efficient capture by integrons. Integrons have evolved to capture and express gene cassettes. They were first discovered in clinical isolates but have since been found in a variety of environments including wastewater treatment plants, fish farms, on-farm biopurification systems, soil, manured soil, pig slurry, lakes poultry litters, estuaries and reed beds (Marathe et al., 2013, Agerso and Petersen, 2007, Dealtry et al., 2014, Ma et al., 2013, Tennstedt et al., 2005, Ghosh et al., 2009, Holmes et al., 2003, Byrne-Bailey et al., 2009, Tennstedt et al., 2003, Zhang et al., 2011, Du et al., 2014, Flach et al., 2015, Byrne-Bailey et al., 2011, Agerso and Sandvang, 2005, Heuer et al., 2012, Gaze et al., 2005, Nandi, 2004, Lu et al., 2015, L'Abee-Lund and Sorum, 2001, Ferreira da Silva et al., 2007). Integron structure allows for efficient insertion and expression of a gene cassette in to a genome without disruption of the genome making them highly favourable both in clinical and environmental conditions.

Integrons were first identified by Stokes and Hall in 1989 over 30 years after the first Japanese studies investigating plasmid-mediated transferable antibiotic resistance in 1950's (Stokes and Hall, 1989, Ochiai, 1959). They consist of three key components: the integrase gene (*intI1*), a recombination site (*attI*) and a promoter (Pc) (Figure 1.5). All integrons will possess these three elements to enable the capture and expression of gene cassettes with minimal disruption to the genome (Labbate et al., 2009). They are able to capture genes from diverse backgrounds and hence act as genomic

diversity “hotspots” (Boucher et al., 2007, Hall and Collis, 1995). Over 130 different antibiotic resistance gene cassettes have been found on integrons providing resistance to most antibiotics used in the treatment of Gram-negative bacteria (Partridge et al., 2009, Centron and Roy, 2002, Falbo et al., 1999, Koeleman et al., 2001, L'Abee-Lund and Sorum, 2001, Maguire et al., 2001, Nordmann and Poirel, 2002, van Belkum et al., 2001). There are five known classes of integrons, class 1 through to 5 (Cambray et al., 2010). Classes 1, 2 and 3 are the most readily detected integrons with classes 4 and 5 having only been detected once (Hochhut et al., 2001). They are classified based on sequence homology of the integrase protein with 40-58% identity (Mazel, 2006).

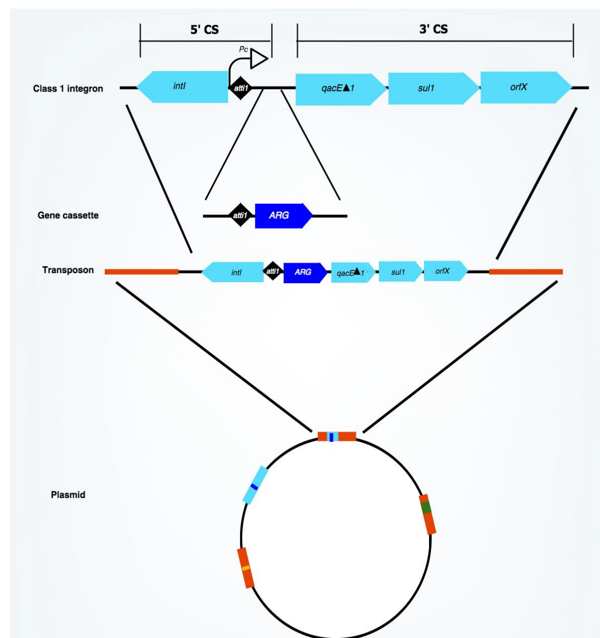


Figure 1.5 Structure of Class 1 integrons, capture by transposons and plasmid insertion.

The most commonly detected integron in both the clinic and environment is the class 1 integron (Deng et al., 2015). The suggestion that class 1 integrons may act as a marker for antibiotic resistance has been considered for over a decade, with the first suggestions that antibiotic resistance in *Enterobacteriaceae* is strongly related to integrons in 2003 (Leverstein-van Hall et al., 2003). The study conducted by Leverstein-van Hall *et al*. found the multidrug-resistance phenotype was attributed

to the presence of integrons within strains and that 100 % of strains possessing integrons expressed resistance to at least 1 antibiotic tested.

Recent work into class 1 integrons as a proxy for ARG has been carried out to investigate how efficient a marker it is. Amos *et al.* proposed a model to predict 3GC resistance prevalence based upon class 1 integron prevalence combined with environmental metadata (Amos et al., 2015). Furthermore, Gillings *et al*. suggested the class 1 integron-integrase gene may act as a marker for anthropogenic pollution based on the observation of high abundance of this gene in polluted environments in addition to many pathogenic and commensal bacteria in the humans and animals (Gillings et al., 2015, Goldstein et al., 2001, Stokes and Gillings, 2011, Amos et al., 2015, Berglund et al., 2015, Marathe et al., 2013, Gaze et al., 2011).
They also represent potential monitoring mechanisms due to their assembly, which has been influenced by human activities resulting in the accumulation of BRG and ARG on the same genetic element (Gillings et al., 2008). If the integrase gene is to be used as a marker for antibiotic resistance, care must be taken to ensure all integrons are detected. A study conducted by Dawes *et al*. found that of 79 isolates known to possess class 1 integrons, only 31 of them could be detected using standard PCR primers which typically amplify the 3' region (Dawes et al., 2010).

Class 1 integrons are commonly associated with Gram negatives, but they have also been detected in Gram-positive bacteria. In 1998 a class 1 integron was found in *Corynebacterium glutamicum.* Interestingly this integron showed higher expression in this host than in *E. coli* suggesting an Gram-positives may be important reservoirs of ARG-carrying integrons (Xu et al., 2011). Other Gram-positive bacteria, in which class 1 integrons have been found include *Staphylococcus, Corynebacterium*, and *Aerococcus* (Nandi, 2004, Nesvera et al., 1998, Xu et al., 2007, Xu et al., 2008b, Xu et al., 2008a).

Class 1 integrons have been associated with many bacterial families in both commensal and pathogenic bacteria and have been found in a variety of environments possibly as a result of location on *Betaproteobacteria*, which has

allowed efficient transfer from the environment in to the human food chain. Consequently this has led to a sub class of class 1 integrons that are found in human-dominated ecosystems (Gillings et al., 2015). This clinically related class 1 integron is characterised by a 3' conserved region, a truncated *qacEΔ1* and a *sul1* gene. These components are believed to have arisen through capture of an environmental betaproteobacterium integron containing *qacE* biocide resistance gene and subsequent *sul1* sulphonamide resistance gene which led to a truncated *qacE* which was captured by a Tn402 transposon which targets the *res* region of plasmids and hence allows the class 1 integron-transposon hybrid to transpose in to a wide range of plasmid and hence become high mobile (Minakhina et al., 1999).

1.9 Hypotheses

The majority of work investigating WWTPs as a reservoir of ARG and ARB has focussed on the water column but recent studies have suggested that river sediment may also contribute a significant reservoir of resistance. In the current study it was hypothesised that the sediment would in fact carry a greater abundance of ARB and ARG than the river water and that due to seasonal release from CSOs, the wetter winter months would carry a greater level of ARB compared to the drier summer months.

Combined, it was hypothesized that the sediment provides an unknown reservoir of pathogenic bacteria and aimed to investigate the pathogenic and resistance potential of *E. coli* isolates.

1.10 Aims

1. Investigate the prevalence and abundance of ARG within planktonic and sediment river samples.
2. Determine if sediment or planktonic samples carry more Gram-negative AMR bacteria.
3. Determine if climatic features affect the number of viable antibiotic resistant Gram-negative bacteria
4. Evaluate diversity within AMR *E. coli* and consider the genetic potential for pathogenicity.

Chapter 2
Materials and Methods

2.1 Site selection for Sowe sampling

For results chapters 5 and 6 sampling was carried out at the River Sowe. River sediment samples were collected from two sites near to the Finham WWTP (see figure 2.1): one site was CSO impacted and the other was CSO and WWTP impacted.

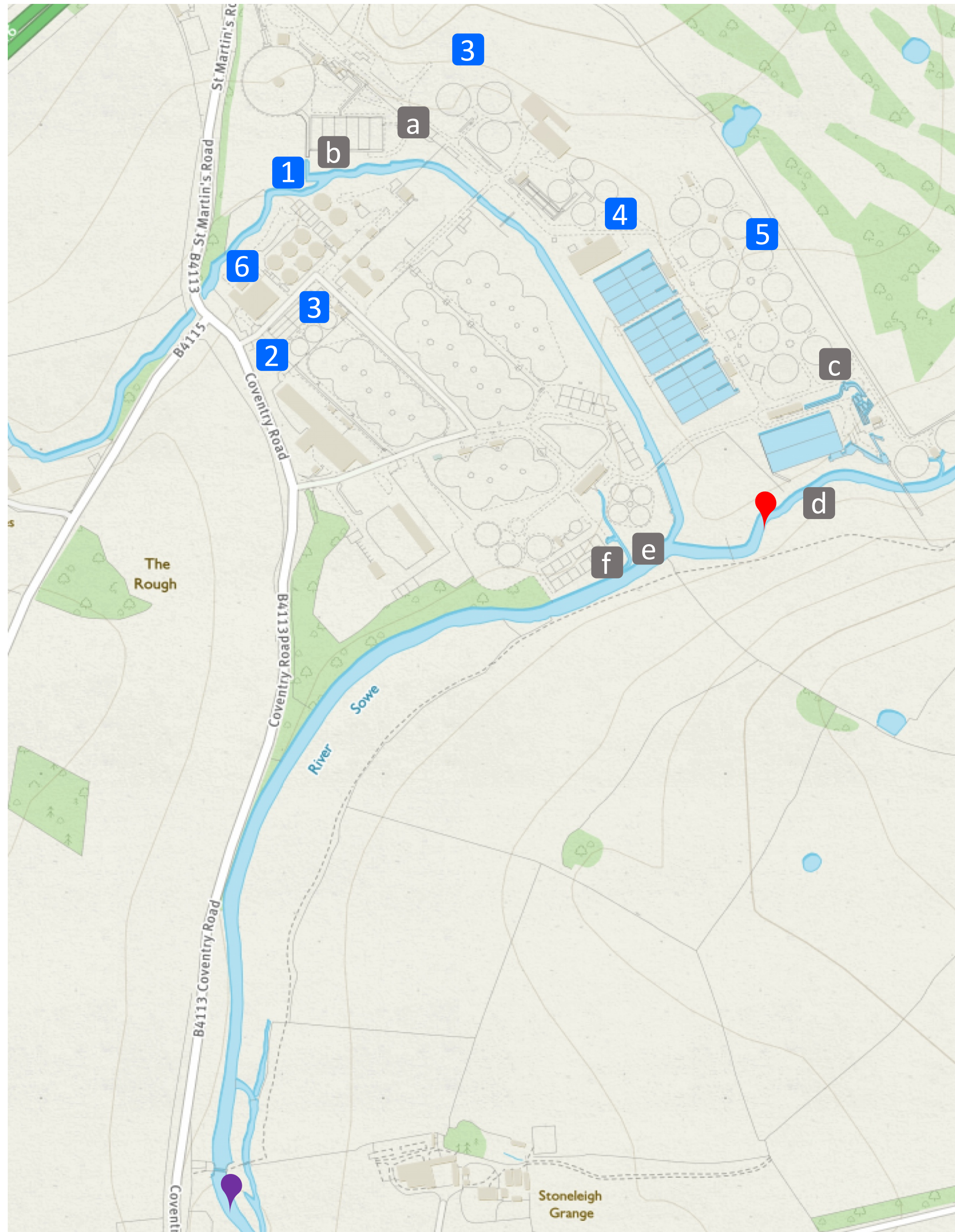


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Figure 2.1 Sampling sites near the Finham WWTP and key components of the treatment plant. CSO impacted site (red point) and combined CSO and WWTP impacted site (purple point) from the Finham WWTP on the River Sowe. Numbers in blue squares represent the components involved WWTP process: (1) Digesters, (2) centrifuge, (3) Primary settlement tanks, (4) Activated sludge plants, (5) Final settlement tanks, (6) Combined heat and power plant. Grey squares represent inlets and outlets from the treatment plant: (a) Sherbourne inlet sample point, (b) Sherbourne settled storm sewage sample point (to Fihnam Brook)/ Sherbourne settle storm sewage outlet 2 (to Finham Brook), (c) Sowe inlet sample pount, (d) Sowe settled storm sewage sample point (to river Sowe)/ Sowe settle storm sewage outlet 3 (to river Sowe), (e) Sample point/final effluent sample point/flow monitoring point, (f) Final effluent outlet 1.

2.2 Sowe sampling regime

Approximately 20 g of river sediment was collected in 50 ml falcon tubes at four seasonal time points between February 2015 and February 2016. Samples were taken in biological triplicate and were processed on day of collection.

Weather metadata including daily temperature, average rainfall over the month and average sunshine hours were recorded (data taken from MetOffice) between February 2015 and February 2016.

Weather summaries were taken from the Met Office website and reports regarding temperature and rainfall were recorded. Weather data was scored on a scale of very high, middle, low and very low based on manual relative measure. Metadata was added to mapping file for 16S Miseq analysis.

Table 2.1 UK Weather summaries. Taken from Met Office, http://www.metoffice.gov.uk/climate/uk/summaries/2015

Month/Year	Average month temperature (^{o}C)	Average rainfall (mm)	Hours of sunshine
Winter 2015	6.5	72.9	77.5
Spring 2015	7.9	46.3	212
Summer 2015	18.5	109.5	164.8
Autumn 2015	7.9	230	29.8
Winter 2016	7	114.2	85.3

2.3 Site selection for Thames sampling

For results chapters 3 and 4, the river Thame and river Kennet were chosen as sampling sites. The sample sites within the Thames catchment were chosen based upon agricultural and anthropogenic impacts (see table 2.2). The river Kennet was chosen primarily for agricultural impacts and the Thames for anthropogenic activity with a greater number of farms and communities respectively in each River catchment. The sites can be found in Figure 2.2.

All sites were downstream of at least one WWTP, however sites were labelled upstream and downstream based on nearest WWTP location. Small WWTP were

considered as plants serving a population less than 10000 and any plant serving over 10000 was considered large. WWTP impact was determined based on size of the nearest WWTP and distance from the plant. Impact was determined based on distance away from the WWTP.

Table 2.2 Summary table of sampling sites in Thames catchment

River	Site no.	Coordinates	Nearest WWTP (upstream/ downstream)	Population served by nearest upstream WWTP	Agricultural impact
Thame	3	51.81478, -0.88781	Downstream	104000	Cattle,
Thame	5	51.75693, -0.98053	Downstream	11000	Cattle
Thame	7	51.67514. -1.1322	Upstream	>10000	Cattle
Thame	8	51.67512, -1.13157	Downstream	30000	Cattle
Kennet	2	51.4144, -1.78448	Downstream	<10000	Sheep
Kennet	7	51.42445, -1.69324	Downstream	<10000	Sheep, fish farm
Kennet	8	51.4301, -1.53687	Downstream	<10000	Pig and fish farm
Kennet	11	51.41197, -1.49562	Upstream	<10000	Cattle and fish

2.4 Thames sampling regime

Samples were collected in September 2015. Sampling was carried out using specially made sampling poles to allow access across the river bed. Sediment samples were collected in 50 ml falcon tubes and stored on ice till time of plating. Samples were collected at each site in triplicate. Water samples were collected in 500 ml screw cap bottles. 3L was collected from each site replicate (9L in total for 3 replicates).

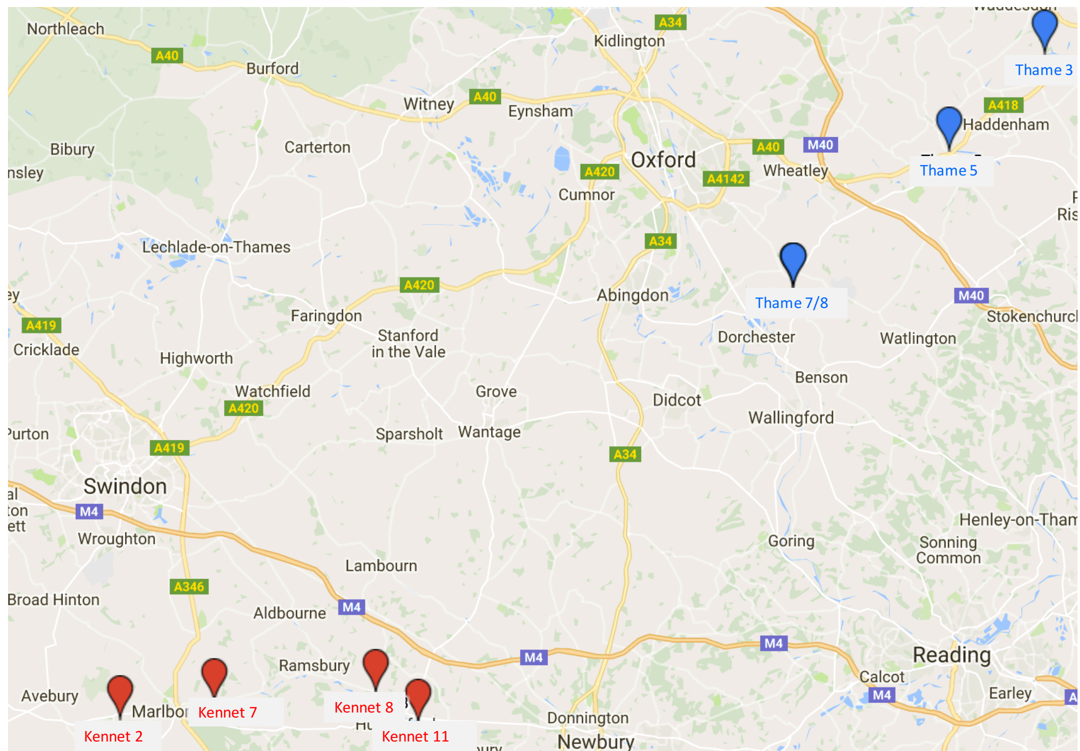


Figure 2.2 Thames sampling sites. Blue markers represent Thame sampling sites and red markers indicate Kennet sampling sites

2.5 Media preparation

HiCrome^{TM} coliform agar (HiCA) plates were made as specified by manufacturers protocol with the exception of CTAB containing plates which was added prior to autoclaving Antibiotic containing plates were made by autoclaving HiCA prior to addition of antibiotic. Plates without any antibiotic or biocide were also made. A summary of the antibiotics used and concentrations are listed in Table 2.3.

HiCA was chosen because it allows the simultaneous detection of coliforms and specific identification of *E. coli* through metabolic profiling using Salmon-GAL and X-glucoronidase to identify coliforms and *E. coli* respectively. Coliforms produce β-D galactosidase which cleaves Salmon-GAL to produce salmon-red colonies. *E. coli* also produced this enzyme as well as β-Dglucuronidase which cleaves X-glucoronidase. The combination of breakdown of Salmon-GAL and X-glucoronidase results in dark blue-violet coloured colonies allowing quick and easy identification.

Table 2.3 Concentration of antimicrobials used in HiCA plates. *used in the Thames study only, ** used in Sowe monitoring study only. EUCAST clinical breakpoint data taken from: http://www.eucast.org/clinical_breakpoints/

Antibiotic/Biocide	EUCAST clinical breakpoint (µg/ml)	Concentration in HiCA plates (µg/ml)
Cefotaxime	2	2
Ciprofloxacin	1	1
Meropenem**	4	4
CTAB	N/A	128
Erythromycin*	N/A	8
Tetracyline *	N/A	10

2.6 Sediment sample processing

Sample processing was carried out on the same day of sampling (within 12 hours of collection). Plating was done in technical duplicate for each biological replicate. Sediment samples were diluted in PBS from 10^{-1} to 10^{-5} with 1 g sediment (wet weight) diluted in 9 ml PBS and spread plated (100 µl) in triplicate on to HiCA agar with and without antimicrobials. Plates were incubated for 48hrs at 30°C.

2.7 Water sample processing

3L of water was passed through a 0.45 µm filter. The filter was cut in half, half was stored at 4 °C for later processing (see section 2.11) and DNA extraction and the other half was placed on antibiotic/biocide HiCA plates and incubated 30°C for 48 hours.

2.8 Plate counts

Blue and pink colonies (representing *E. coli* and presumptive coliforms excluding *E. coli* (PCE) respectively) were recorded for each condition tested. At least three plates for each condition were recorded and counts averaged.

2.9 *E. coli* isolation for whole genome sequencing

Dark-blue/violet colonies were picked from plates and streak plated to purification. One colony was taken from each plate dilution and replicate where possible. Purification involved re-streaking isolates on to respective antibiotic containing HiCA

agar plates at least 4 times until only dark-blue/violet colonies appeared on the plates after overnight incubation.

2.10 Sediment and water sample collection of biomass for DNA extraction

After 48 hours colonies were scraped using plastic loops in to 1.5 ml microcentrifuge tubes. 250µl PBS was used for scrapings. Cell suspensions were frozen at -20 ^{o}C prior to DNA extraction. Technical duplicates were scraped together for each antibiotic and biocide condition.

2.11 DNA extraction from sediment and cultured biomass

DNA extraction was carried out using the FastDNA™ spin kit for soil following manufacturers protocol. For sediment samples 0.5 g of sediment (wet weight) was used, for cultured DNA extractions (from both water and sediment samples, where specified, 0.5g soil was replaced with 250 µl of suspended colonies. 2 extractions were done for each sample to make a total of 500 µl colonies were extracted from. DNA was pooled after extraction in 75µl to give total DNA in final volume of 150µl

2.12 DNA extraction from water

DNA was extracted from filtered water samples (as specified in 2.7) the PowerWater® kit from MO BIO was used as stated by manufactures protocol.

2.13 qPCR conditions, primers, probes and oligos

qPCR reactions were set up using 12.5 µl environmental Taqman (Fischer Scientific), 1 µl BSA (10 mg/ml), 1 µl primers (100 µM), 0.75 µl nM probe (100 µM) 1 µl DNA template made up to 25 µl with DNA free water. A list of primers and probes used can be found in Table 2.4. For sediment DNA extractions, DNA was diluted 1:10 prior to qPCR.

Conditions for each reaction were the same, the cycle was as follows: 95°C for 10mins, [95°C for 15 seconds, 60°C for 30 seconds, 72°C for 40 seconds] (40 cycles), 95°C for 60 seconds.

Table 2.4 Primers and probes used for qPCR

Target	Primers	Probe	Product size	Reference
$bla_{CTX-M-1}$ (Group 1	ACCAATGATATTGCGGTGATCTGCGTTCTGTTGCGGCT	6FAM-TCGTGCGCCGCTG-MGBNFQ	85	Pitkanen *et al* (Colomer-Lluch et al., 2011)
qnrS	CGACGTGCTAACTTGCGTGAGGCATTGTTGGAAACTTGCA	6FAM-AGTTCATTGAACAGGGTGA-MGBNFQ	118	Colomer-lluch *et al* (Colomer-Lluch et al., 2014)
tetM	GGTTTCTCTTGGATACTTAAATCAATCRCCAACCATAYAATCCTTGTTCRC	6FAM-ATGCAGTTATGGARGGGATACGCTATGGY-TAMRA	93	Peak *et al* (Peak et al., 2007)
ermF	TCTGGGAGGTTCCATTGTCCTACTTTCAGGACCTACCTCATAGA	6FAM-TCGAGGAAGCTTTACAATCCATATACCGT-TAMRA	106	This study, collaborators at University of Exeter
intI1	GCCTTGATGTTACCCGAGAGGATCGGTCGAATGCGTGT	6FAM-ATTCCTGGCCGTGGTTCTGGGTTTT-TAMRA	193	Baraud *et al (Barraud et al., 2010)*
qacE	CGCATTTTATTTTCTTTCTCTGGTTCCCGACCAGACTGCATAAGC	FAM-TGAAATCCATCCCTGTCGGTGT-TAMRA	70	Jechalke *et al* (Jechalke et al., 2013)
E. coli 23S	GGTAGAGCACTGTTTtGGCATGTCTCCCGTGATAACtTTCTC	6FAM-TCATCCCGACTTACCAACCCG-TAMRA	88	Pitkanen *et al (Pitkanen et al., 2013)*
16S	CGGTGAATACGTTCYCGGGGWTACCTTGTTACGACT	6FAM-CTTGTACACACCGCCCGTC-TAMRA	124	Csekalski *et al.* (Czekalski et al., 2014)

For qPCR standards, oligos was ordered were from TIB chemicals. Table. 2.5 lists oligos used in this study. For *E. coli* 23S, 16S, and $bla_{CTX-M-1}$ standards were made from strains and PCR products.

Table 2.5 Oligos used as standards

Gene	Oligo
qnrS	CGACGTGCTAACTTGCGTGATACGACATTCGTCAACTGCAAGTTCATTGAACA GGGTGATATCGAAGGCTGCCACTTTGATGTCGCAGATCTTCGTGATGCAAGTT TCCAACAATGCC
ermF	TCTGGGAGGTTCCATTGTCCTTCAATTAGAACCTACACAAAAGTTATTTTCGA GGAAGCTTTACAATCCATATACCGTTTTCTATCATACTTTTTTTGATTTGAAA CTTGTCTATGAGGTAGGTCCTGAAAGTA
tetM	GGTTTCTCTTGGATACTTAAATCAATCGTTTCAAAATGCAGTTATGGAAGGGA TACGATATGGCTGTGAACAAGGATTGTATGGTTGG
qacE	CGCATTTTATTTTCTTTCTCTGGTTCTGAAATCCATCCCTGTCGGTGTTGCTT ATGCAGTCTGGTCGGG
inti1	GATCGGTCGAATGCGTGTGCTGCGCAAAAACCCAGAACCACGGCCAGGAATG CCCGGCGCGCGGATACTTCCGCTCAAGGGCGTCGGGAAGCGCAACGCCGCTG CGGCCCTCGGCCTGGTCCTTCAGCCACCATGCCCGTGCACGCGACAGCTGCT CGCGCAGGCTGGGTGCCAAGCTCTCGGGTAACATCAAGGC

2.14 Normalisation of qPCR results

16S standards were from *E. coli*. *E. coli* has a median 16S copy number of 7 (data taken from the ribosomal RNA operon copy number database (rrnDB) (Stoddard et al., 2015). For 16S results were multiplied by 7 and divided by 2.5 (the average number of 16S copies).

Cultured DNA extraction 16S were also multiplied by 7 because the average 16S copies in *Enterobacteriaceae* is 7. Values were multiplied by 150 as DNA was diluted in 150 ul water to give copies per total scraped plate content.

Results were normalised then averaged between the three biological replicates and standard error calculated. For sediment samples, results were calculated per gram, for cultured results, results were made relative to 16S.

2.15 qPCR statistical analysis

Boxplots generated in R studio to determine outliers. Outliers identified in excel via sorting and determined if true outliers by firstly finding the 1st and 3rd quartile and then finding the statistical 50% (interquartile range) of the data by subtracting the 3rd quartile from the 1st quartile. Outliers were determined by multiplying the IQR by

1.5, if results were higher than this value, individual results were checked and excluded based on manual curation.

Statistical tests were performed in RStudio (version 0.99.893). Pearsons correlation analysis was used to determine whether populations were independent. Correlograms were generated using the R library corrgram (Wright, 2015) to present correlations between ARG and explanatory variables. Heatmaps were generated using the R package gplots (Warnes, 2016), to show relative abundance at each site/season/condition.

Data collected for all ARG targets was log transformed to overcome the assumption of analysis of variance (ANOVA) that the dataset will show homogeneity of variance. Raw qPCR data was $\log_{10}$ transformed and a constant of 0.0000001 was added to overcome the zeros in the dataset. This number was chosen because it was smaller than any of the recorded values. Two-way ANOVAs were performed to evaluate differences in ARG prevalence across different sample types, rivers and sites within rivers.

2.16 Comparison of antimicrobial treatment of cultured DNA extractions

Data was separated out in to different antibiotic treatments to compare different treatments with qPCR ARG targets. Non-parametric tests were conducted for each qPCR target to compare whether changes occurred in target prevalence with different conditions. Non-parametric tests were performed because data was not normally distributed.

2.17 16S Amplicon targeted metagenomics

16S amplicon targeted metagenomics was carried out on all river samples: total DNA extractions from sediment and water as well as DNA extractions from cultured fraction.

16S targeted metagenomics was carried out as specified in Illumina Miseq guide for 16S metagenomics library preparation (Illumina). Briefly, 16S V3 and V4 region was

amplified using the forward primer TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and reverse primer GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. PCR clean up using the Ampure XP beads was then carried out and 2nd stage PCR to add index primers. Second stage PCR clean-up was then performed and samples qubited and some were run on the bioanalyser to check product size was correct.

Libraries were then pooled to 4nM checking with Qubit®. Once libraries were at 4nM, library denaturation and MiSeq sample loading was performed.

2.18 Analysis of 16S amplicon targeted metagenomics

Analysis of 16S sequencing included demuliplexing of samples through merging paired samples and quality filtering. Quality filtering parameters were set to truncate reads with quality scores less than 7 (p error= 0.19953) and to allow only 5 mismatches in the overlap region of the paired reads. Reads with expected errors of more than 0.5 were discarded and reads with less than 300 bp of sequence were discarded. Sequences were then dereplicated and size sorted (minimum size was set to 2). OTU clustering was carried out at 97 % similarity and chimeras filtered out using UCHIME (Edgar et al., 2011, Edgar, 2010). OTUs were assigned using RDP classifier 2.2 (McDonald et al., 2012, Werner et al., 2012, Wang et al., 2007) and reads mapped back to OTUs to create OTU tables.

QIIME (Caporaso et al., 2010b) was then used to further analyse sequences. Briefly, sequenced were aligned (Caporaso et al., 2010a) and a phylogenetic tree made using the filtered FASTA files (Price, 2010). Taxonomy was added to the biom table and taxa summarized. Samples were rarefied and core diversity analyses was performed separating by metadata categories. Simpsons inverse test was used to analyse OTU diversity for rarefied and unrarefied communities.

Beta diversity was analysed using the rarefied OTU tables and 2D PCoA plots were constructed with weighted and unweighted UniFrac distance matrices (Lozupone

and Knight, 2005) to determine whether sample communities differed based on relative abundance of taxa or true presence/absence of taxa (Lozupone et al., 2007). PCoA plots were visualised in EMPeror (Vazquez-Baeza, 2013). Community structural similarity was determined using analysis of similarity (ANOSIM) analysis run for 999 permutations. The core microbiome was determined for samples separately to determine differences in OTUs among categories.

2.19 Antimicrobial phenotypic screening

Disc diffusion assays were used to determine phenotypic resistance for all 77 *E. coli* isolates. Oxoid^{TM} antibiotic containing discs were used to determine phenotypic resistance profiles. Antibiotic discs used can be found listed in Table 2.6 Oxoid Iso-sensitest^{TM} Agar CM0471 was used to determine phenotypic resistance. Agar was made as per manufactures instructions.

Table 2.6 Antibiotics used in phenotypic assays

Antibiotic	Antibiotic concentration (μg/ml)	Antibiotic class
Ampicillin	25	β-lactam
Cefotaxime	5	Extended-spectrum β-lactam, third generation
Imipenem	10	β-lactam, carbapenem
Tetracycline	10	Tetracycline
Erythromycin E	10	Macrolide
Chloramphenicol	30	Binds 50S subunit of ribosome
Sulphafurazole	300	Sulfonamide
Nalidixic acid	30	Quinolone

2.20 DNA extraction of pure cultures

Once colonies were purified, a single colony was picked and inoculated in to 10 ml LB (with appropriate antibiotics where necessary) and incubated overnight at 37 ^{o}C with shaking at 150 rpm. Cultures were then centrifuged for 10 minutes at 1500 rpm and supernatant discarded. Pellets were resuspended in 500 μl PBS. The MPBio FastDNA^{TM} spin kit for soil was used to extract DNA replaced 0.5 g soil with 500 μl

resuspended cells. DNA extractions were carried out as specified by the protocol set out by MPBio.

2.21 DNA quantification

DNA was quantified using the Qubit® using the ThermoFisher Scientific dsDNA BR Assay Kit following protocol. DNA samples that could not be determined using the dsDNA BR Assay Kit were discarded. Remaining samples were aliqouted to 40 μl and dried down using the speed vacuum till completely dry (~45 minutes).

2.22 Whole genome sequencing

Dried down DNA samples were sent to University of Technology, Sydney for sample preparation and whole genome sequencing using the standard Illumina protocol for Miseq which was performed by Dr Michael Liu. Briefly DNA was suspended in nuclease free water. DNA was then fragmented to produce 5' and 3' recessed, overhang and blunt ends and tagmented using the Illumina Nextera kit which combines fragmenting DNA with tagmentation of adaptors in one step: 8 μl tagmentation buffer, 5 μl gDNA and ddH_2O, 4 μl Nextera tagmentation mix. The reaction was stopped using 4μl NT buffer after holding at 55 ^oC for 5 minutes and room temperature for a further 10 minutes. Samples were incubated with NT buffer for 5 minutes at room temperature to ensure the tagementation step has stopped. The library was enriched through PCR amplification using 20 μl of the tagmented library, 22μl KAPA PCR master mix, 1μl index 1 (i7) primers, 1 μl index 2 (i5) primers and then vortexing to mix. The cycle for tagemented library amplification is as follows: 72 ^oC for 3 minutes, 95 ^oC for 30 seconds, 12 cycles of 95 ^oC for 10 seconds, 55 ^oC for 30 seconds and 72 ^oC for 30 seconds, then 72 ^oC for 5 minutes and infinite hold at 10 ^oC. Tagmented and amplified DNA samples were run on the BioAnalyzer to check sizing and quantification of libraries.

After confirmation of DNA libraries solid phase reversible immobilisation (SPRI) bead clean-up was performed on pooled samples to select for right and left side. Samples were once again run on the bioannalyser to check cleaned sample. DNA library was

then denatured and prepared according to Illumina MiSeq guidelines using 20 pM of each the sample and PhiX control. MiSeq reagent kit 2 x 150 bp v2 was used for sequencing of *E. coli* isolates to generate 250 nucleotides long paired end reads with approximate total output of 4.5 Gb. With *E. coli* sized genomes, the average coverage if one bacterial genome was run on one cell would be 978 times (4500000000/4600000). Therefore, to obtain the minimum level of coverage of 30 time, (978/30), 32 samples can be run on with one v2 kit.

77 isolates were obtained from the Severn Trent catchment (16 CSO impacted site, 61 combined CSO and WWTP effluent impacted site). An initial 30 DNA samples were run on the Miseq at University of Technology, Sydney. The remaining 47 samples were run on the Hiseq at the Garvan institute of medical research.

2.22.1 Generating rough assemblies

Raw reads were assembled using the revised A5-miseq *de novo* assembly pipeline (Coil, 2015) which consists of five steps: 1) read cleaning, 2) contig assembly, 3) crude scaffolding, 4) misassembly correction, 5) final scaffolding. Assembly summary statistics and base-call estimates are produced at stage 5. Rough assemblies were carried out at UTS by Professor Aaron Darling.

Preliminary annotations were generated using the automated annotation software RAST (Overbeek et al., 2014, Aziz et al., 2012). Annotation of ARG was performed using the Resistance Gene Identifier (RGI) Version 2 on the Comprehensive Antibiotic Resistance Database (CARD) website (McArthur et al., 2013) Contigs were also run through CARD database to look for any resistance genes missed by the antibiotic resistance database.

2.22.2 Phylosift analysis

Phylogenetic analysis of genomes was performed using PhyloSift (Darling et al., 2014) which is based on phylogenetically correlating based on 37 “elite” marker gene families that have largely congruent phylogenetic histories. These genes represent ~1% of an average bacterial genome. Phylosift results were to generate phylogenetic trees using FastTree and were analysed using FigTree (found at

http://tree.bio.ed.ac.uk/people/) which is a graphical viewer for phylogenetic trees. Trees with and without references strains were performed (Table 2.7).

Table 2.7 Reference strains. Taken from the GenBank database used for comparative genomics

Description	Accession number
E. coli 042 (EHEC)	FN554766.1
E. coli 536 (UPEC)	CP000247
E. coli O26:H11 11368 (EHEC)	AP010955
E. coli O55 H7 CB9615	CP00184
CD306 ST131	CP013831.1
CFT073	AE014075.1
E2348/69	NC_011601.1
E24277A	CP000800.1
EC958 ST131	NZ_HG941718.1
EDL933 O157:H7 (EHEC)	NZ_CP008957.1
JJ1886 ST131	CP006784.1
JJ1897 ST131	CP013837.1
K12 MG1655	U00096.3
LF82 (AIEC)	CU651637.1
NA114 ST131	NC_017644.1
O26:H11	AP010953.1
O103:H2 (EHEC)	AP010958.1
O111:H- (EHEC)	AP010960.1
O157:H7 Sakai	BA000007.2
E. coli O103:H2 str. 12009 plasmid pO103	AP010959.1
RM12579 O55:H7 (EPEC)	CP003109.1
S. boydii ATCC_9210	CP011511.1
S. flexineri 2a str. 301	AE005674.2
S. flexneri 2a str. 2357T	NC_004741.1
S. sonnei FORC_011	NZ_CP010829.1
UMNK88 (ETEC)	CP002733.1
UTI89 (UPEC)	NC_007946/1
W3110 K12	AP009048.1
ZH063 ST131	NZ_CP014522.1

2.22.3 Comparative genomic and MLST analysis

The move contigs function in MAUVE (Darling et al., 2011) was used for whole genome alignment to reference genomes (Table 2.7). It identifies conserved segments which appear free from genome rearrangements. These regions are referred to as Locally Collinear Blocks (LCBs), the fewer the LCBs, the better the alignment. The best alignments were chosen based on the highest weight score which is a measure of how likely the predicted rearrangement is and whether it in fact exists. Scaffolds unable to align with the reference sequence are referred to as the "accessory genome". Scaffolds identified in MAUVE as regions of interest were further investigated using BLASTn® searches were carried out against databases made at University of Technology, Sydney for plasmid possession, virulence genes, antibiotic resistance genes, phylogrouping, O- and H-antigen typing to characterise strains. Progressive MAUVE alignments were performed between strains of the same sequence time using the arranged contig FASTS files generated using the move contigs function. PubMLST (http://pubmlst.org/) was used to identify sequence types of *Escherichia* isolates using the Achtman *E. coli* MLST scheme (Achtman et al., 2012) (http://mlst.warwick. ac.uk/mlst/). BRIG (Alikhan, 2011) was used for visual comparisons of resistance and virulence genes between key strains.

2.22.4 Investigating virulence, antimicrobial resistance and plasmid incompatibility type

BLAST searches against a databases of virulence genes, AMR genes and plasmid incompatibility groups were carried out using BLAST databases provided by UTS.

2.22.5 Toxin-antitoxin searching

TA finder was used to search the TADB (Shao et al., 2011) was used to determine whether strains possessed toxin-antitoxin systems to help elucidate whether persistence formation was involved in the mechanisms behind significant *E. coli* isolates being found in the environment.

Chapter 3

Community analysis of sites selected in the Thames catchment from the River Thame and River Kennet

3.1 Introduction

Thames Water serves approximately 14 million people over the Thames catchment, which includes a range of diverse environments including highly urbanized areas and large agricultural regions. It comprises approximately 66500 miles of sewer, 350 WWTPs (including the UKs largest plant at Beckon, East London) and treats 4.2 billions of sewage every day (ThamesWater, 2001-2011, Baggs, 2015). In London alone 39 million cubic square meters of raw sewage is released in the Thames river from CSOs each year with release events occurring more than once a week (ThamesWater, 2015b). This has prompted improvements across the Thames catchment. The Thames tideway tunnel is the most important change to be made and will be the largest wastewater project in the UK since Sir Joseph Bazalgette built London's combined sewage and drainage system in the 1850s (Baggs, 2015). Extensive work will improve waste release from CSOs through a new 25 km tunnel to

store and take sewage for treatment to be completed by 2023 (BazalgetteTunnelLimited, 2016). In 2014 a £675 million project was completed in London to improve treatment plants and £20 million is to be invested in the next few years to improve drainage across the catchment to reduce the release from CSOs (ThamesWater, 2015a). Thames water manages 1 million tons of sludge each year. Currently Thames water recycles part of this to provide 12 % of power needs for the treatment plants which is planned to increase to 33 % by 2015-2020. In total ~60 % of sludge is recycled to farms with ~32000 hectares of farmland used for recycling sludge every year and the remaining 40% is incinerated and used as energy (Baggs, 2015).

Numerous studies have been conducted determining anthropogenic impact on this river catchment including studies on ARG levels, chemical pollution, vertebrate and invertebrate studies making it a suitable location for further studies to elucidate full human impact (Whitehead, 2016, Singer et al., 2014, Dhanji, 2011, Amos et al., 2015). Previous experiments have shown human contamination of the environment contributes to the dissemination of resistance through agriculture via run-off from land and wastewater treatment (Chow et al., 2015, Korzeniewska and Harnisz, 2013, Tang et al., 2016, Amos et al., 2014). Previous work in our lab initiated investigations exploring the impact from WWTPs discovering effluent contributes to the dissemination of the ESBL gene $bla_{CTX\text{-}M\text{-}15}$ in sediment (Amos et al., 2014).

The 2016 review on AMR, identified the environment as an important reservoir in the dissemination of ARB (O'Neill, 2016). Future predictions regarding global warming, land-use and population expansion are resulting in increased pressures on river systems to prevent the spread of water-borne disease (Whitehead, 2016, Alcamo et al., 2007). The growth, survival and transport of enteric bacteria has been predicted to increase therefore, it is important now to understand the factors involved in maintaining the resistant bacterial community within both river water and sediment to reduce the threat of such water-borne diseases becoming untreatable (Liu et al., 2013).

This study investigated areas with known WWTP impact. River pollution from WWTPs has been previously linked to an increase in resistant-pathogens downstream of effluent release, however the true extent is not fully understood with the size of treatment plant, type of treatment plant and location all having effects on river pollution (Kotlarska et al., 2015, Garcia-Armisen et al., 2014, Amos et al., 2014). The WWTP process removes the majority of bacteria from wastewater, however some bacteria will survive the process, some of which may be pathogenic and carry ARG. As a result waters receiving effluent from WWTPs are likely to carrying elevated risks compared to rivers without impact (Marti et al., 2013a, Kumaraswamy et al., 2014, Tang et al., 2016). Knowledge about WWTP impact must be expanded to involve other environmental variables which may contribute to environmental pollution. Agricultural pollution contributes to environmental pollution through manure spreading but animal impact on river system ARB levels has not yet been evaluated in combination with WWTP effects (Wichmann et al., 2014, Rogers et al., 2011, Musovic et al., 2014, Agerso and Sandvang, 2005).

In this study the aim was to investigate the importance of WWTPs in the dissemination of ARB between two river systems to take in to account the size of the treatment plant and any potential agricultural impacts in the surrounding area. Previous studies investigating bacterial community composition at sites impacted by WWTPs are largely underrepresented in the literature with few investigating the culturable fraction and none investigating changes in the resistant culturable fraction with changes in environment (Garcia-Armisen et al., 2014, Servais et al., 2007). This study represents one of the first studies to compare factors between the resistant-culturable fraction and the total community structure with the aim to elucidate the main environmental factors leading to ARB persistence in the environment.

The bacterial communities across two river systems were determined to elucidate whether population changes were related to site, WWTP impact and/or farming impact. Both river sediment and water samples were collected to resolve whether bacterial communities were similar in different sample types and if water/sediment communities are impacted by environmental factors. The culturable fraction of

sediment and water samples was also collected and subject to 16S amplicon sequencing to determine how environmental factors affect ARB and whether factors affecting the total fraction correlate with changes in the culturable fraction. The aim of the study was to understand the key factors involved in determining the ARB population and if they also determined the total community structure. Two rivers were chosen for this study from the Thames catchment; the River Kennet and the River Thame. The River Kennet is a chalk stream which flows through the Marlborough downs and is home to wild brown trout. Predominantly, is a rural area and mainly impacted by agriculture rather than urban waste (but the lower regions pass through some urban areas). The River Thame is also predominately rural but has a larger urban influence with elevated phosphate levels in some regions due to WWTP impact (Ascott, 2016). Comparisons between the Thame and Kennet were used to determine whether differences between catchments with similar profiles differed according to increased anthropogenic impact in the River Thame or whether increased agricultural impact at the Kennet sites.

3.2 Aims

The overarching aim was to look at community structure in different sample types in an intensively sampled area representing a whole catchment. The nature of the cultured population on selective plates was investigated to consider the AMR *Enterobacteriaceae* and to investigate the impact of antibiotics on the population to determine which component of this subpopulation was most impacted in the selective medium. The aims can be summarised as follows:

1. Explore the impact of antibiotics on faecal coliforms and other Gram-negatives.
2. Investigate if cultured population differs with site/river.
3. Determine community structure of planktonic and sediment samples.

3.3 Results

Chromogenic media was used to isolate the Gram-negative culturable fraction (CGNF). Identification of *E. coli* and PCEs and other Gram-negative bacteria is based on metabolic capacity resulting in blue/purple colonies, pink colonies and colourless colonies respectively. Media contains X-glucuronide and Salmon-GAL and relies on the ability of *E. coli* to produce β-D-glucuronidase to break down both X-glucuronide and Salmon-GAL resulting in blue/purple colonies, PCEs to break down only Salmon-GAL and other Gram-negatives to break down neither.

3.3.1 Sample sequencing

Illumina MiSeq 16S targeted amplicon metagenomic sequencing data from sediment and water samples from the Thames catchment was collected. DNA from total sediment, total water, cultured fraction from sediment and cultured fraction from water were run. Cultured fractions were obtained as specified in Section 2.4. Each sample was run in biological triplicate: In total there were 48 DNA from total extractions and 288 DNA samples from cultured fraction of samples making up the resistant quotient of river sediment bacterial communities with respect to cefotaxime, ciprofloxacin, erythromycin, tetracycline and CTAB. Analyses were carried out separately for the total DNA and the cultured DNA to compare community differences.

3.3.1.1 Sequencing depth of total DNA extractions

933742 sequences were obtained from the total water and sediment DNA samples with a median sequence number of 17165 sequences per sample. Rarefaction analysis curves determined cut off level (Figure 3.1).

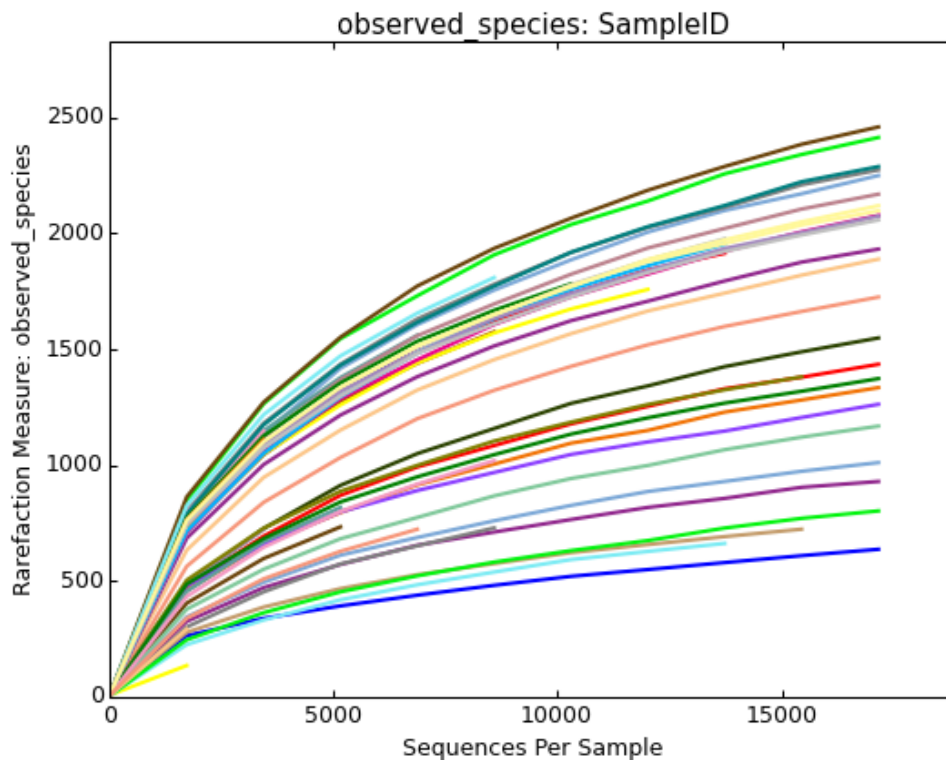


Figure 3.1 Number of sequences and observed species per sample for total DNA extractions.

One sample had only 19 sequences and was excluded from further analysis. 22 samples had fewer sequences than the median. The second lowest count was 2092 and was chosen as the level for rarefaction. Simpsons inverse measure ranged between 5.83-500.66 for total non-rarefied communities, rarefied communities ranged from 5.65-440.64 confirming no substantial loss of diversity when rarefying at 2092.

3.3.1.2 Alpha diversity of total DNA extractions from water and sediment samples

Simpsons inverse measure ranged from 17.19 - 500.66 in non-rarefied sediment communities and 5.83 – 395.08 in water communities and 22.76 to 440.64 in rarefied sediment communities and 5.65 to 336.08 in water communities. Significant differences were found in alpha diversity in sediment and water samples ($p < 0.05$) in both rarefied and unrarefied communities suggesting differing species richness.

3.3.1.3 Composition of water and sediment samples

4243 OTUS were found in both sediment and water samples. Substantial variation in the phyla and order presence in water and sediment samples was observed (Figure 3.2). The *Proteobacteria* were the most dominant phyla in both samples, accounting for 40.13 % in sediment and 60.70 % in water. *Bacteroidetes* (accounting for 11.62 % in sediment and 13.35 % in water), *Cyanobacteria* (accounting for 10.56 % in sediment and 5.81 % in water) and *Actinobacteria* (accounting for 11 % in sediment and 9.84 % in water) were among the most abundant phyla.

Chloroflexi accounted for 6.53 % in sediment and 1.36 % in water showing some variation. Abundance of *Pseudomonales* accounted for only 0.4 % in sediment and 20.3 % in water samples. *Burkholderiales* also varied with sediment samples possessing 3.9 % and water samples 8.7 %. The *Enterobacteriaceae* fraction was not detected in sediment samples but was in water samples accounted for 0.7 % of total abundance.

Group significance tests were conducted to determine which OTU group abundances varied between water and sediment samples. There were 65 OTUs that had significantly different abundances between water and sediment, the most significant being members of the *Betaproteobacteria* and *Gammaproteobacteria*. Chao1 species richness test and observed species were significantly different between water and sediment samples (Kruskall-Wallis $p < 0.05$)

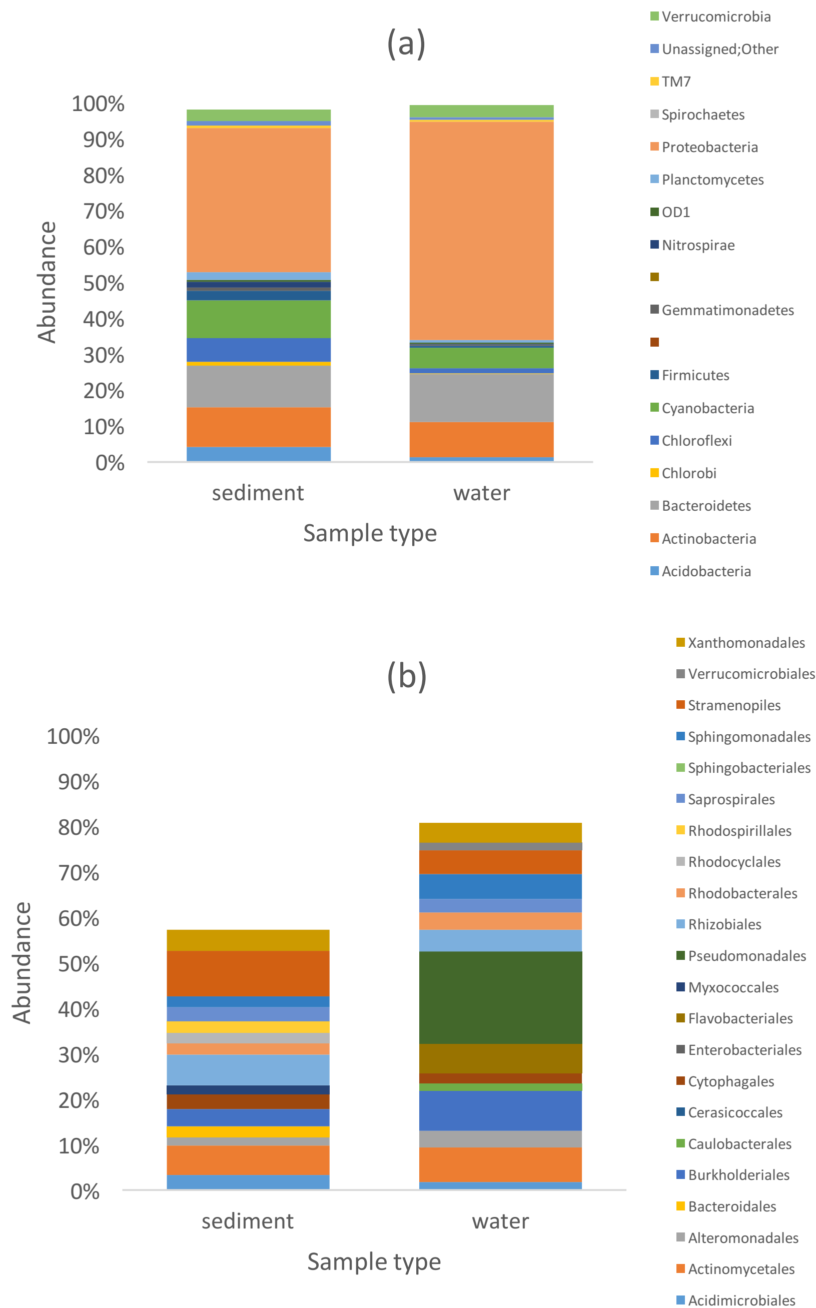


Figure 3.2 The relative abundance of the top 15 OTUs of water and sediment samples. (a) phyla, (b) orders

3.3.1.4 PCoA plots for sample type

Rarefied water and sediment samples were analysed using PCoA plots (Figure 3.3) to determine if samples of the same origin cluster together when weighted and unweighted. Weighted samples explain a greater proportion with greater separation suggesting difference in taxa abundance is more important than changes in taxa presence/absence between sediment and water. Unweighted samples also show

large separation suggesting, although not as important, changes in taxa presence/absence partly explain differences between water and sediment bacterial communities.

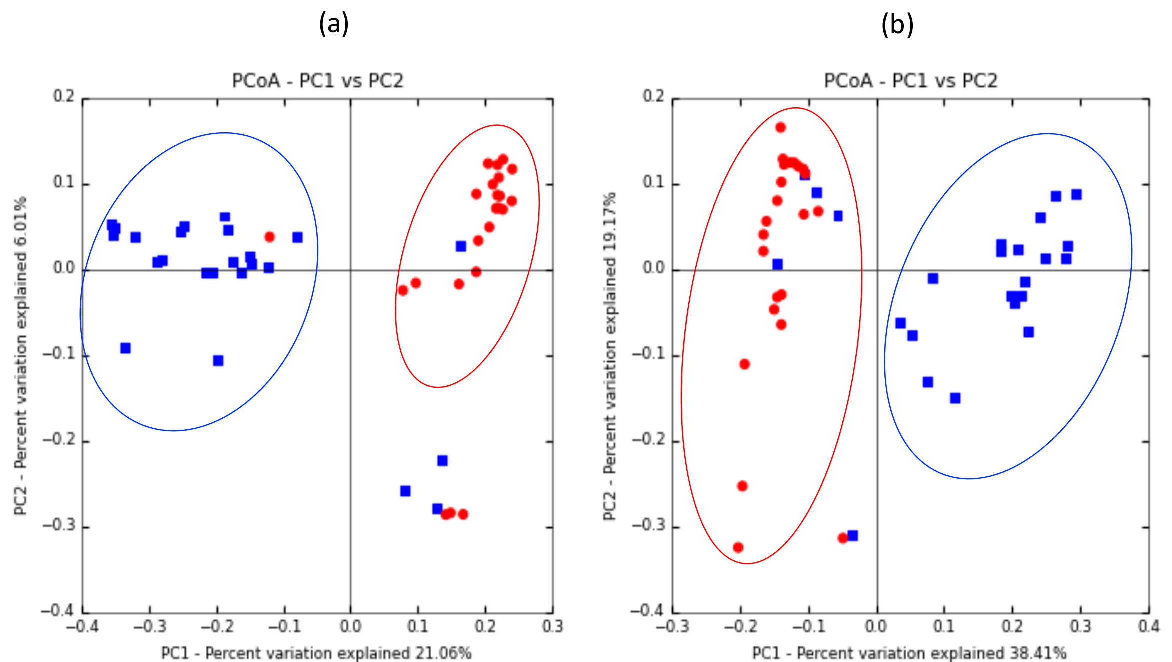


Figure 3.3 PCoA plots for sediment and water samples. (a) unweighted and (b) weighted red = sediment, blue = water

The presence/absence of OTUs and abundance of OTUs varied significantly between water and sediment samples (R = 0.5922, p = 0.001) and taxa abundance (ANOSIM R =0.5201, p = 0.001).

3.3.1.5 Variation in OTUs with farming influence

Total DNA samples were separated according to farming influence to determine if OTU distribution and abundance varied with different types of farming. Simpsons inverse measure revealed changes in community diversity between sites affected with different farming practices. Diversity measure for sites impacted predominantly by cattle ranged between 22.76 to 440.62, for fish farms diversity ranged from 9.28 to 280.94, pig impacted sites ranged from 5.65 to 212.95 and sites predominantly impacted by sheep values ranged between 8.01 to 27.06. Diversity measures may

underestimate diversity of sheep impacted sites due to only 3 samples analysed in the current study.

The predominant phyla in all sites were *Proteobacteria* (accounting for 48.03 %, 54.66 %, 62.32 % and 64.97 % for cow, fish, pig and sheep impacted sites respectively), *Cyanobacteria* (accounting for 9.2 %, 4.3 %, 7.68 % and 3.44 % for cow, fish, pig and sheep impacted sites respectively) and *Bacteroidetes* (accounting for 13.3 %, 11.8 %, 9.98 % and 12.47 % for cow, fish, pig and sheep impacted sites respectively).

Orders of bacteria with obvious differences in abundance included *Alteromanadles* (accounting for 3.96 % in cow separated samples, 1.1 % in fish separated samples, 1.12 % in pig separated samples and 0.5 % in sheep separated samples) and *Rhizobiales* (6.79 % in cow separated samples, 4.18 % in fish separated samples, 2.57 % in pig separated samples and 1.1 % in sheep separated samples).

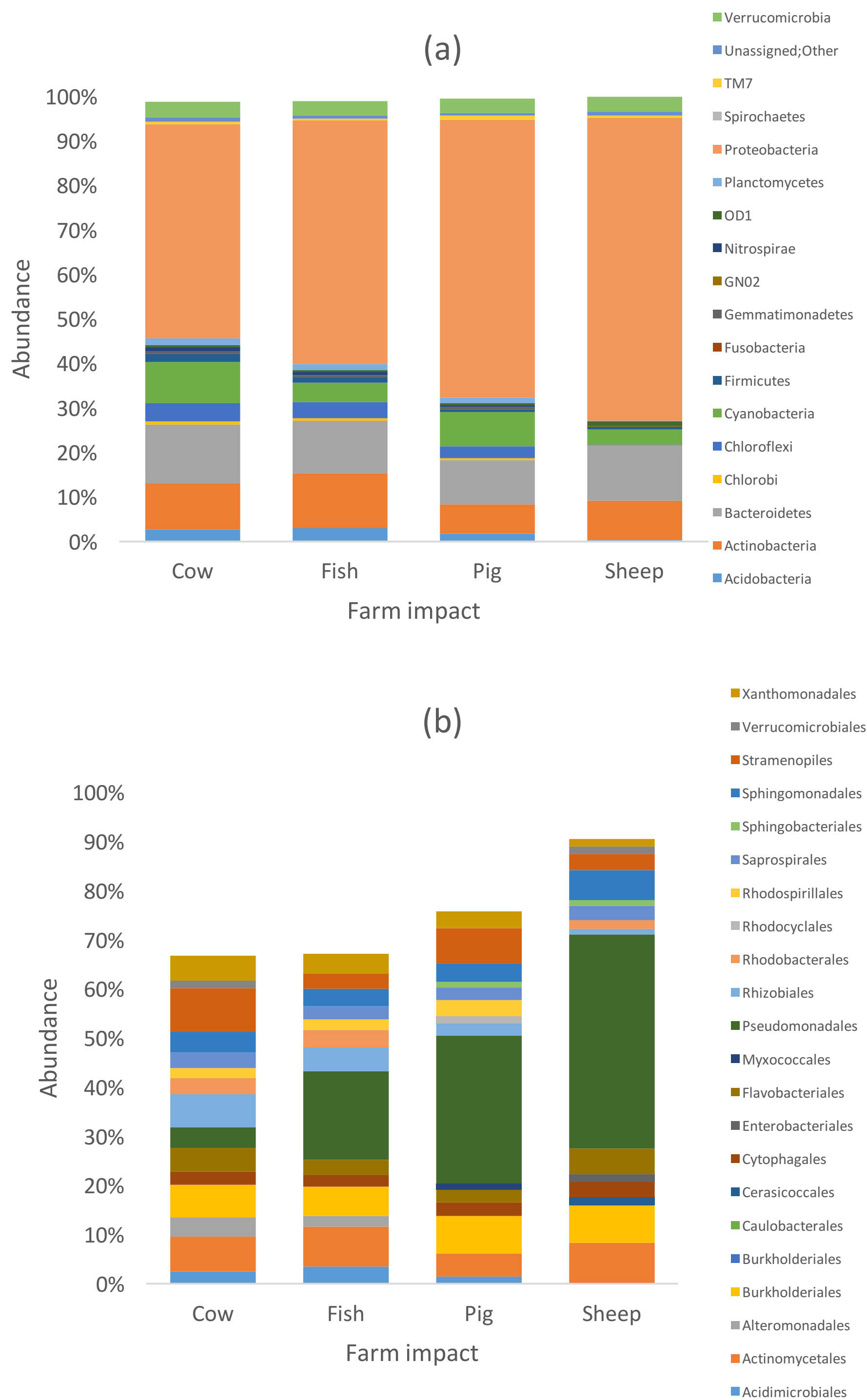


Figure 3.4 Relative abundance of the top 15 OTUs in samples separated by farm influence. Bar charts are shown for (a) phyla and (b) order.

3.3.1.6 Beta diversity of samples separated according to farming influence

Rarefied communities were used to generate weighted and unweighted PCoA plots to determine if different farming practices contribute to clustering of samples for total DNA extractions from both water and sediment samples (Figure 3.5). Clustering

did occur for both weighted and unweighted samples with significant differences between presence/absence of taxa (ANOSIM R = 0.221, p = 0.004) in unweighted communities and weighted communities (R = 0.1430 p = 0.025). The R value for weighted communities is smaller than for unweighted suggesting presence/absence of taxa is more important in clustering of samples according to farming impact than abundance, however there was not a large amount of separation suggesting that there is a lot of overlap between samples with respect to taxa presence and abundance.

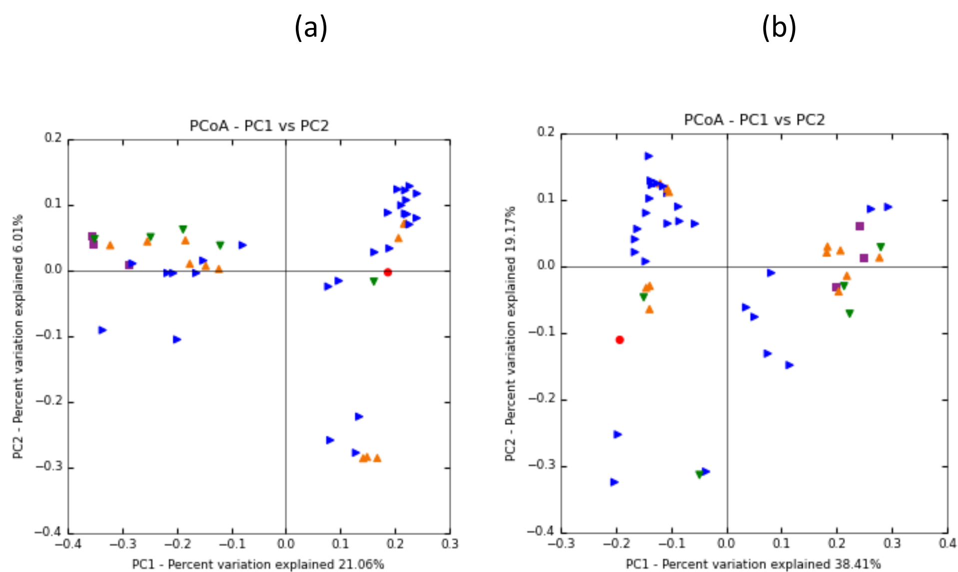


Figure 3.5 PCoA plots of samples separated according to farming influence. (a) unweighted and (b) weighted clustering of samples separated by farming influence. Blue = cow, Orange = fish, Green = pig and Red = sheep impacted samples

3.3.1.7 WWTP impact of alpha diversity within total DNA extraction of samples

The predominant phyla when samples were split by WWTP were the same as specified for samples according by farming impact. There was little variation between samples grouped by WWTP with similar bars presented for most phyla. Species diversity did not change with respect to WWTP impact with diversity measure ranging from 9.28 to 440.64 for samples without WWTP impact and from 5.65 to 405.38 for samples with WWTP impact.

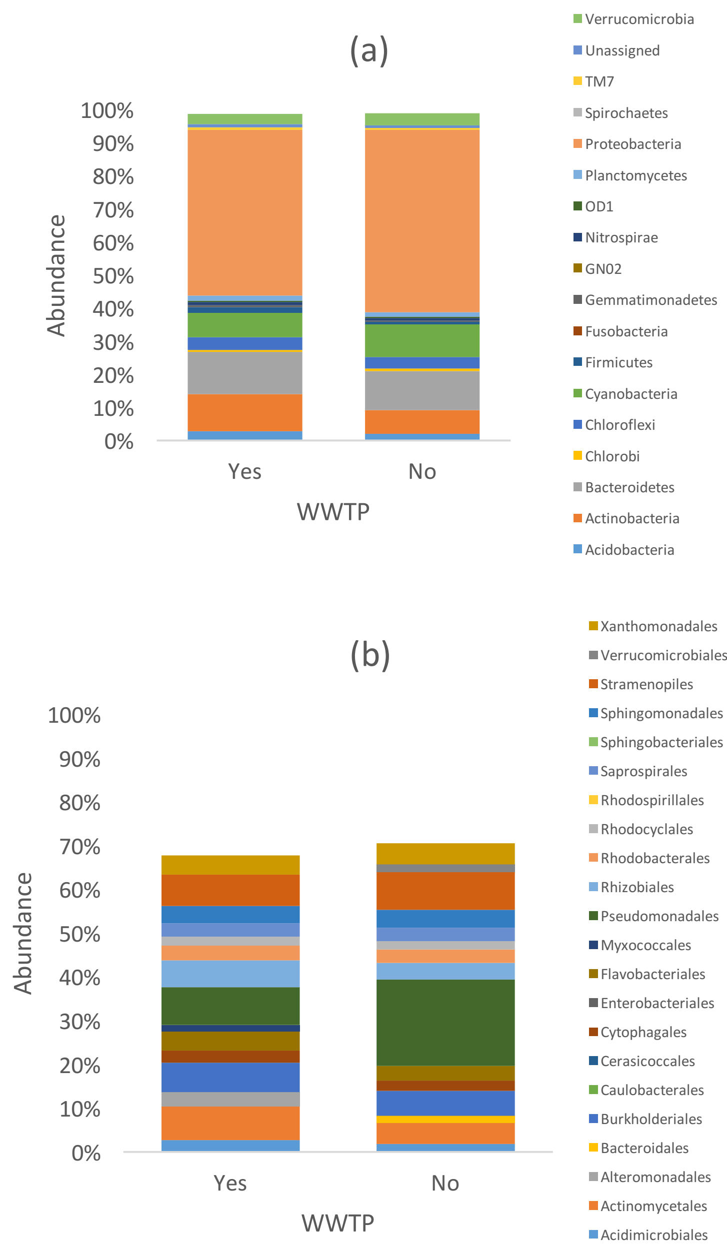


Figure 3.6 Relative abundance of the top 15 OTUs in rarefied communities separated according to WWTP impact. Bar charts are shown for (a) phyla and (b) order

3.3.1.8 Effect of WWTP on beta diversity

There was no sample clustering according WWTP impact (Figure 3.7) for unweighted samples (ANOSIM R = -0.0756, p = 0.889) and weighted samples (R = -0.0988, p = 0.935) suggesting neither taxa presence/absence nor taxa abundance is impacted by the presence or absence of WWTP effluent release

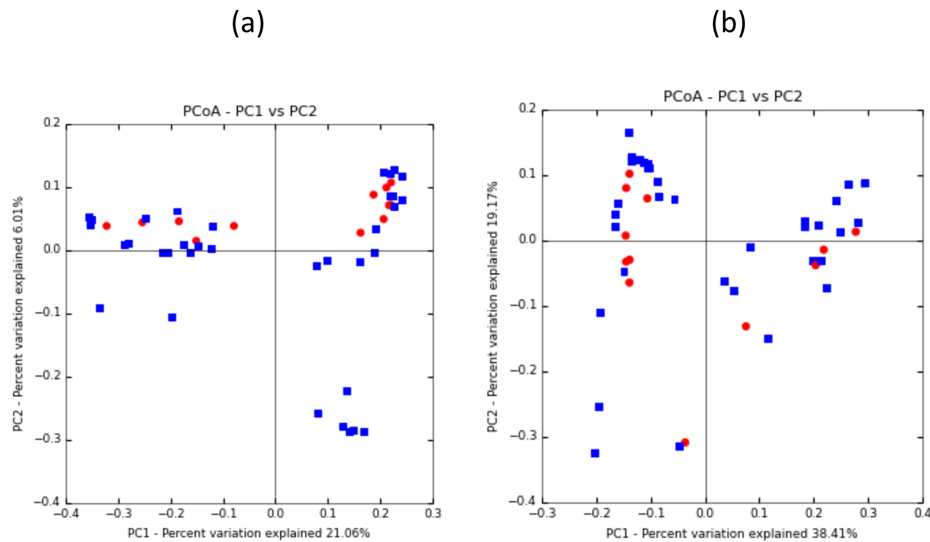


Figure 3.7 PCoA plots for samples separated by WWTP impact (a) unweighted and (b) weighted total DNA samples coloured according to WWTP presence (Blue) and absence (Red)

3.3.1.9 Alpha diversity of samples separated by site

Alpha diversity at the phyla level between sites varied most between the abundance of *Cyanobacteria*: the highest abundance accounted for 22.5 % of diversity (Kennet 2) and the lowest accounted for only 0.6 % (Kennet 7). The most abundant genus was *Perlucidibaca* which accounted for 11.72 % at Kennet 2, 7.59 % at Kennet 7, 29.55 % at Kennet 8, 24.51 % at Kennet 11, 2.34 % at Thame 3, 0.93 % at Thame 5, 9.5 % at Thame 7 and 3.1 5 at Thame 8 (Figure 3.8).

Diversity measure was calculated using Simpsons inverse; values ranged from 7.81 – 117.96 for Kennet 2, 13.97 – 341.06 at Kennet 7, 5.83 – 236.38 at Kennet 8 and 9.28 – 151.20 at Kennet 11. At the Thame river sites, Simpsons inverse measure ranged from 33.20 – 305.25 at Thame 3, 204.28 – 395.08 at Thame 5, 32.27 – 475.16 at Thame 7 and 51.06 – 500.66 at Thame 8.

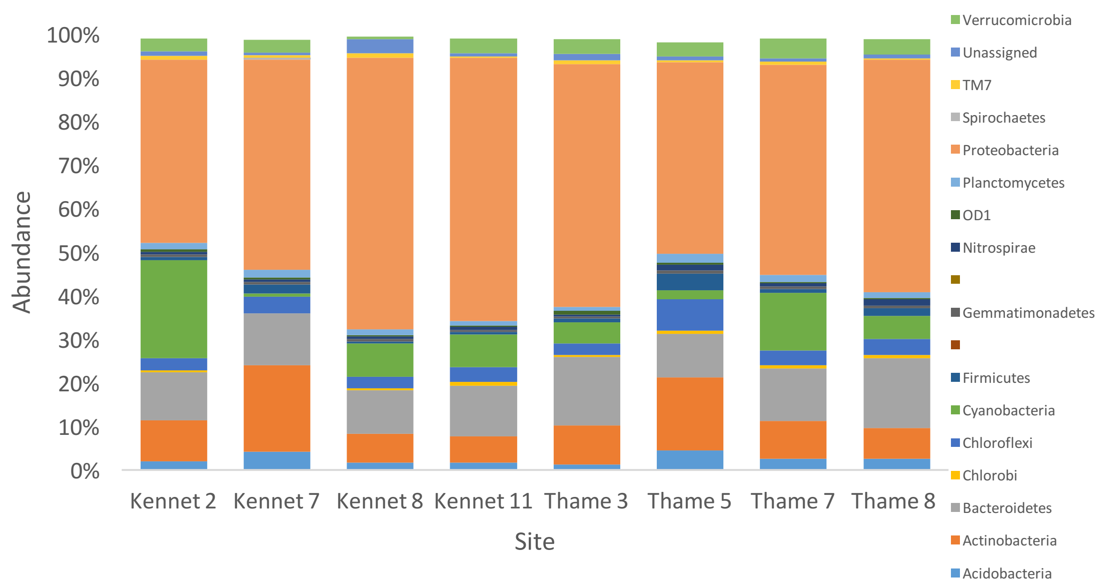


Figure 3.8 The relative abundance of the top 15 genera at each site

3.3.1.10 Beta diversity of samples separated by site

No obvious clustering of samples was observed for unweighted or weighted samples suggesting no clear separation of samples according to the differential presence/absence of taxa or abundance of OTUs between samples. Samples cluster together with a small level of separation between site (ANOSIM R = 0.1369, p = 0.008 for unweighted and R = 0.1499, p = 0.005 for weighted) suggesting some differences in presence/absence and abundance of OTUs exist but there is also a lot of overlap between samples.

3.3.2 Analysis of total sediment and water samples separately

Water and sediment communities differ significantly. To determine if water and sediment communities are affected by WWTP, farming and site, samples were split according to sample type and reanalysed.

3.3.2.1 Alpha diversity of sediment and water DNA samples split according to WWTP impact, farming and site

Water with no obvious WWTP impact had Simpsons inverse diversity measures ranging between 9.28 – 235.27 and 5.83 – 395.08 in samples affected by WWTP. Sediment diversity ranged from 130.45 - 475.16 for sediment samples with no WWTP

impact and 30.57 – 500.66 from sediment samples with WWTP impact. When samples were split according to WWTP size diversity was highest at sites affected by large WWTPs for both water and sediment samples. Samples that had no impact from a WWTP or impact from a small WWTP had similar levels of diversity ranging from 5.83 – 27.39 in water and 25.49 - 475.16 in sediment. Sites impacted by large WWTPs highest diversity measure was 396.08 in water and 500.66 in sediment.

Samples split according to farming type revealed highest diversity in samples taken from sites impacted by cow and lowest diversity for sheep impacted sites in both sediment and water samples suggesting farming influence affects sediment and water communities in the same way and does not substantially affect one community more than the other.

3.3.2.2 Beta diversity of sediment and water samples split according to WWTP presence, WWTP size, site and farming

There were no obvious differences for both sediment and water samples in changes in abundance between sites impacted by WWTP and sites not affected by WWTPs, however when samples were split according to size it was apparent that WWTPs do impact sediment and water bacterial communities, but only when the treatment plant serves more than 10000 people, with small WWTP and sites with no WWTP clustering together both in weighted and unweighted communities for sediment and water samples.

Table 3.1 ANOSIM analysis conducted on distance matrixes to investigate factors important in community structure and abundance for planktonic and sediment communities

	Sediment	Water
WWTP		
Weighted	R = -0.151, p = 0.940	R = -0.0340, p = 0.573
Unweighted	R = -0.621, p=0.962	R = -0.0784, p = 0.695
Farm		
Weighted	R = 0.2464, p = 0.043*	R = 0.1738, p = 0.068
Unweighted	R = 0.3734, p = 0.006*	R = 0.2488, p = 0.017*
Site		
Weighted	R = 0.7159, p = 0.001*	R = 0.4974, p -= 0.001*
Unweighted	R = 0.7013, p = 0.001*	R =0.5906, p = 0 .001*
WWTP size		
Weighted	R = 0.3093, p = .001*	R = 0.2736, p = 0.008*
Unweighted	R = 0.3990, p = 0.001*	R = 0.2341, p = 0.014*

3.3.3 DNA extractions from viable culturable Gram-negative fraction

DNA extractions were carried out for plate scrapings for 6 conditions including one biocide (CTAB) condition and unamended HiCrome™. 4 different classes of antibiotics were used for isolation of the viable resistant culturable fraction based on the key resistances in *Enterobacteriaceae* as specified by CDC, WHO and Berendonk *et al (Berendonk et al., 2015)* that have specified urgent need for monitoring of ARG conferring resistance to these key antibiotics. The antibiotics used in this study were from the classes: tetracycline, macrolide (erythromycin) and fluoroquinolones (ciprofloxacin) and 3GCs (cefotaxime).

3.3.3.1 Sequencing depth of DNA samples from cultured extractions

A total of 15718295 sequences were obtained from sequencing of the cultured fraction with a median number of 42775 sequences per sample. The highest number of sequences obtained from one sample was 541585 and the minimum was 40. 40 is too low to obtain significant insight in to community structures so was excluded from

the analysis. Rarefaction was chosen at 2402 because it allowed the most samples to be kept in the analysis and allowed for significant species diversity (Figure 3.9) as calculated by the Simpsons inverse calculation for species diversity. Cultured non-rarefied community diversity ranged from 1.31-22.10, rarefied communities to 2402 ranged from 1.31 -22.42. From Figure 3.7 it can be seen that by approximately 2402 the number of new observed species is starting to level off. As a result of rarefying to 2402 there were 7 samples excluded from the overall analysis.

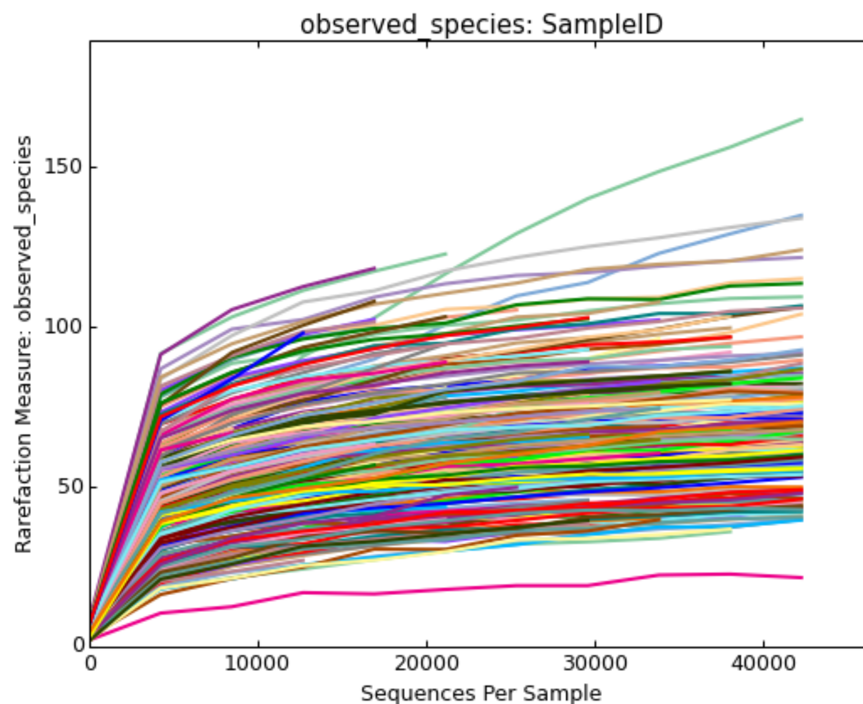


Figure 3.9 Number of sequences obtained per sample of the cultured DNA extractions against observed species.

3.3.3.2 Alpha diversity of cultured DNA samples split according to water and sediment

Samples were split according to sample type to determine if resistant culturable fraction were significantly different between water and sediment. The dominant phyla were *Proteobacteria* for both sediment and water samples accounting for 99.81 % of reads in samples. The predominant genera were *Pseudomonas* which accounted for 61.11 % and 32.87 % of genera identified in sediment and water culturable fraction respectively. The other dominant family were the

Enterobacteriaceae which accounted for 14.1 % and 41.39 % of variability in genera in sediment and water culturable samples respectively. Other dominant families/genera included *Aeromonadeae* which composed 5.82 % of diversity in sediment samples and 3.01 % in water, *Achromobacter* (2.88 % in sediment and 6.4 % in water samples)

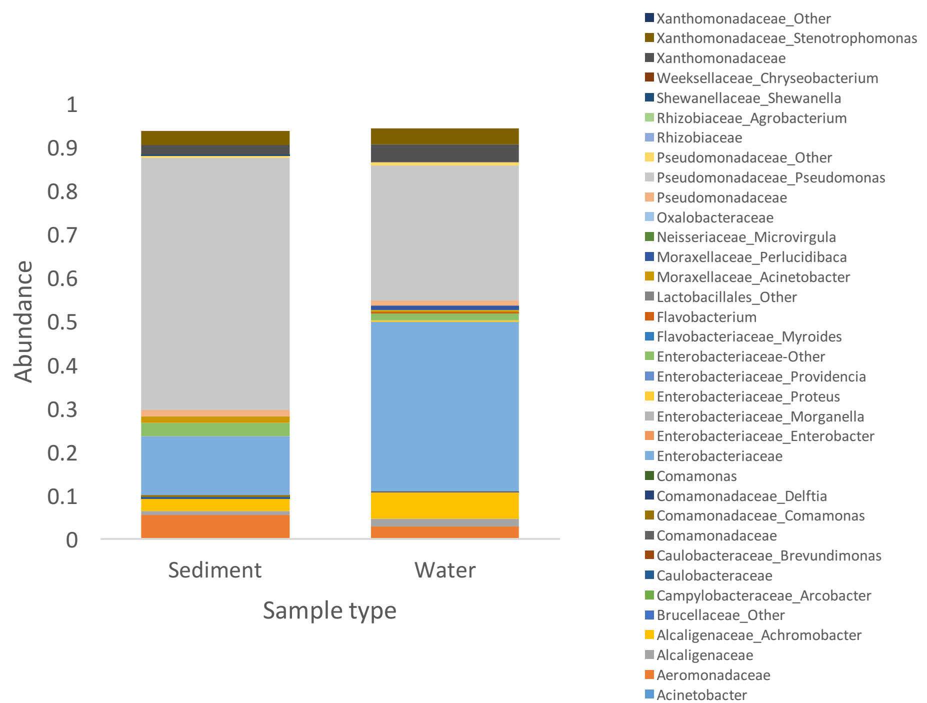


Figure 3.10 Relative abundance of the top 15 OTUs from the CGNF in planktonic and sediment communities. Top 15 order/genus are shown for each sample type with lowest possible taxonomic rank shown. OTUs listed in the same order in the key as shown in the chart.

Samples from sediment culturable fraction had a Simpsons inverse value ranging from 1.66 to 22.41. Samples from water culturable fraction has Simpsons inverse values ranging from 1.31 to 15.11 suggesting a slightly higher level of diversity in sediment samples than in water.

3.3.3.3 Investigating beta diversity of cultured samples split according to sample type

Beta diversity of samples split according to sample type was carried out (Figure 3.11). Unweighted and weighted samples did not cluster separately (ANOSIM R = 0.043, p

= 0.001 for unweighted, R = 0.0864, p = 0.001 for weighted) suggesting neither OTU presence/absence or species abundance was significantly different between water and sediment samples.

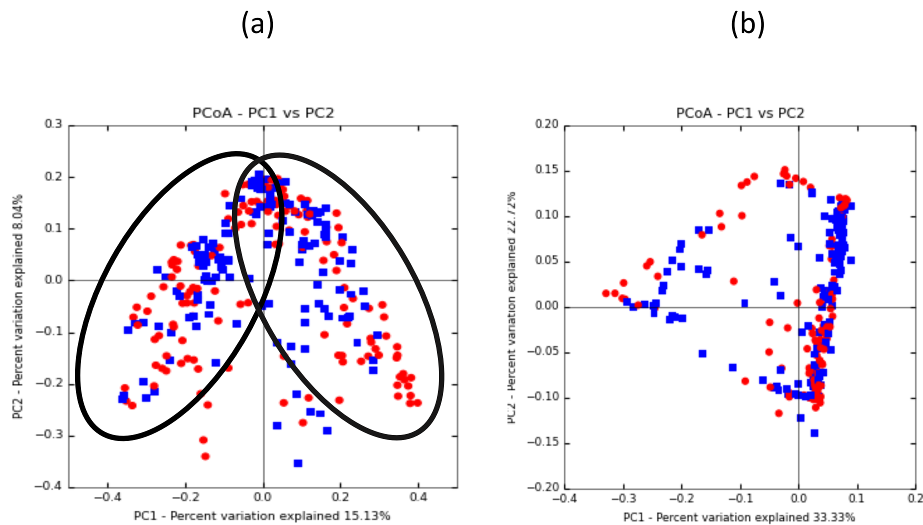


Figure 3.11 PCoA plots of planktonic and sediment CGNF communities: Red= sediment, Blue = water. (a) unweighted samples, (b) weighted samples

Although no significant clustering was observed between samples split by type (Figure 3.11), the unweighted samples show a cone-shaped pattern (as indicated by black ovals) suggesting samples do cluster in to two groups based on OTU abundance, however this is not explained by the sample type. Another variable is therefore likely involved in sample clustering.

3.3.3.4 Alpha diversity of samples split according to farm influence

The most abundant family and genera were *Pseudomonas* which accounted for 42.92 %, 38.21 %, 39.81 % and 60.8 % in samples split according to cow, fish, pig and sheep respectively and *Enterobacteriaceae* which accounted for 28.73 %, 33.20 %, 30.12 %, 11.69 % in samples split according to cow, fish, pig and sheep respectively.

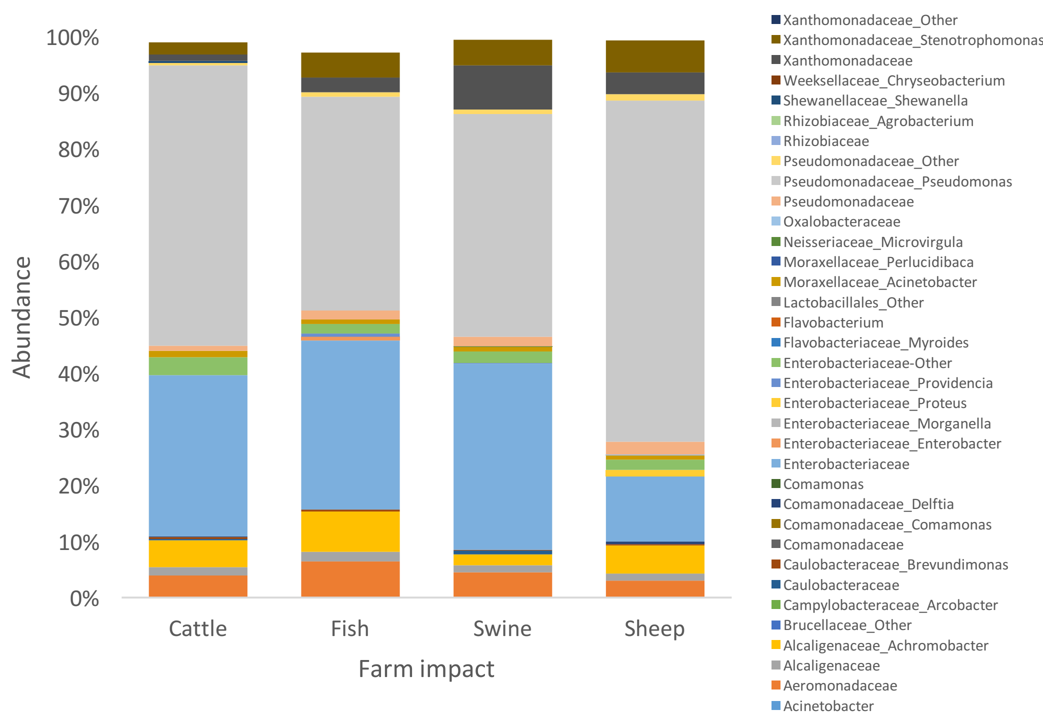


Figure 3.12 Lowest taxonomic rank of the top 15 order/genus from CGNF communities separated according to farming impact.

Simpsons inverse measure ranged between 1.66 – 15.84 for cow impacted samples, 1.54 – 22.42 for fish impacted samples, 1.56 – 14.76 for pig impacted samples and 1.31 – 16.71 for sheep impacted samples. Results suggest sites impacted by swine may be most affected and sites impacted by fish are least affected when taking in to account OTU diversity.

3.3.3.5 Beta diversity of samples split according to farm influence

No distinct clustering of samples occurred (Figure 3.13). Both unweighted and weighted ANOSIM analysis revealed that samples did not cluster (R = 0.0584, p = 0.019, for unweighted samples and R = 0.0274, p = 0.179 for weighted samples), according to farming influence suggesting high level of similarity between viable culturable fraction with little impact from farming influence.

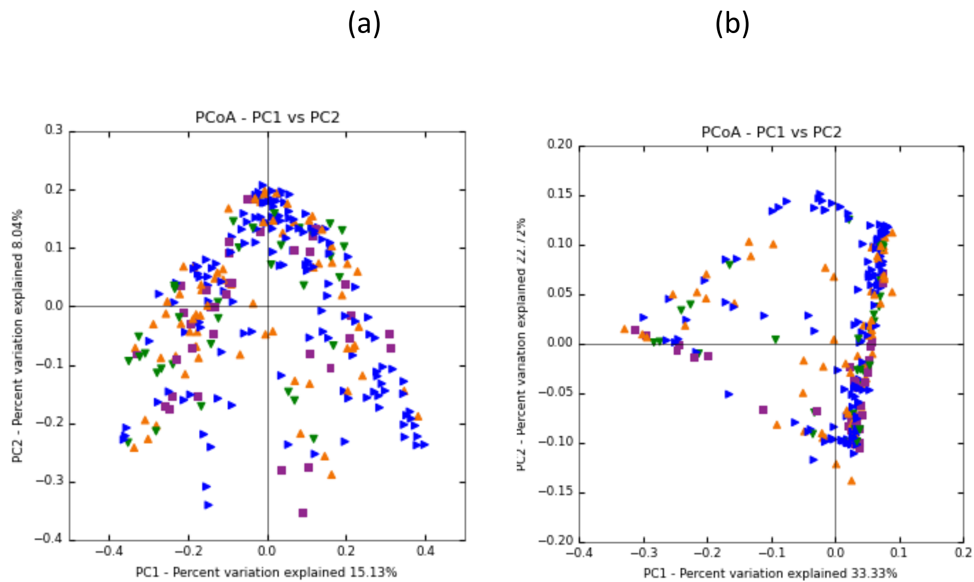


Figure 3.13 PCoA plots of CGNF communities separated according to farming influences. (a) Unweighted and (b) weighted samples separated according to farming influence. Blue = cow impacted Orange = fish impacted Green = pig impacted and Purple = sheep impacted

3.3.3.6 WWTP impact on alpha diversity of viable CGNF

No obvious WWTP impact was found between samples separated according to the presence/absence of a WWTP near to the sampling site (Figure 3.14). Dominant families and genera were *Pseudomonas* (34.75 % in sites with no WWTP impact and 49.23 % in sites impacted by WWTP), *Enterobacteriaceae* (32.41 % in samples not impacted and 26.97 % for sites impacted). Less dominant families and genera included *Achromobacter* (8.64 % in samples with no impact from WWTP and 3.85 % in sites impacted) and *Aeromonadacae* (8.10 % in sites not impacted and 3.70 % in impacted sites).

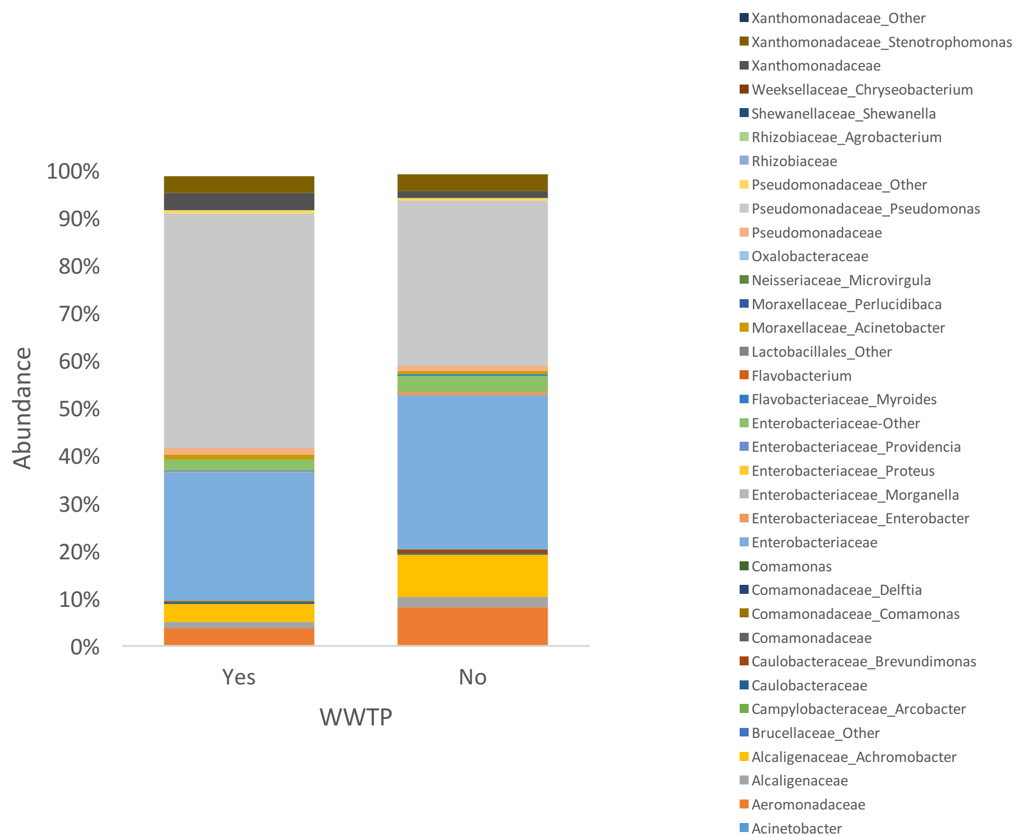


Figure 3.14 Lowest taxonomic rank of the top 15 order/genus from CGNF communities separated according to WWTP influence.

Diversity measures ranged from 1.66 to 14.49 in sites with no WWTP impact and 1.31 to 22.42 in sites impacted by WWTP suggesting a slight increase in the diversity of bacterial species present with WWTP impact.

3.3.3.7 Beta diversity of samples separated according to WWTP impact on the CGNF

No significant clustering was observed in samples split according to WWTP impact in unweighted samples (ANOSIM R = -0.0619, p = 0.969) or for weighted samples (R = -0.0007, p = 0.465) (Figure 3.15) suggesting that the presence of a WWTP alone does not significantly affect the community structure of the ARB subpopulation.

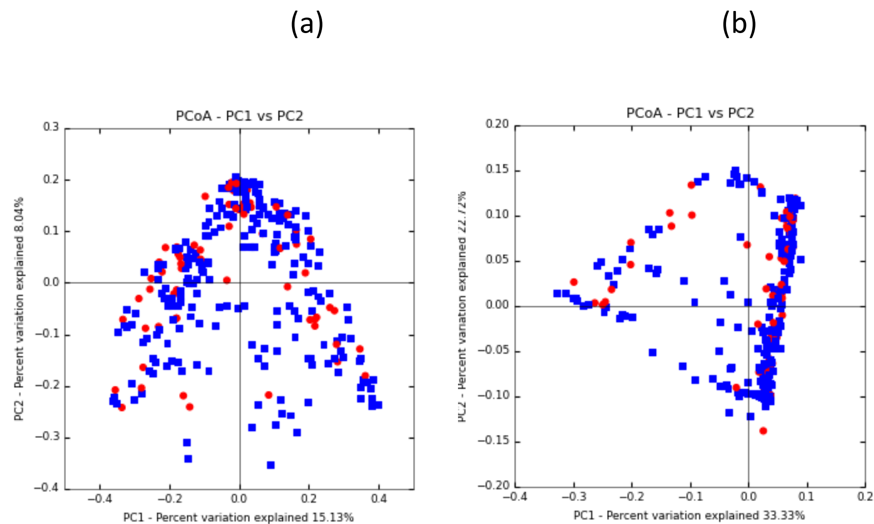


Figure 3.15 PCoA plots of CGNF communities separated according to WWTP impact. (a) Unweighted (a) and (b) weighted samples. Blue = WWTP impacted, Red = no WWTP impact

3.3.3.8 Antimicrobial treatment impact on viable cultured samples

The ciprofloxacin resistant community appears to have the most diversity in genera abundance (Figure 3.16). The most abundant taxa in ciprofloxacin communities were *Achromobacter* (23.3 %), *Stenotrophomonas* (18.09 %), *Enterobacteriaceae* (17.74 %) and *Xanthomonasdaceae* (15.15 %). The most abundant taxa within communities' resistant to cefotaxime, CTAB, erythromycin, tetracycline and none were dominated by *Pseudomonas* (65.39 %, 60.52 %, 58.91 %, 49.53 % and 33.2 % respectively) (in ciprofloxacin isolated samples *Pseudomonas* accounted for only 2.8 %), and *Enterobacteriaceae* (18.6 %, 31.2 %, 35.5 %, 34.2 % and 48.2 % respectively). *Achromobacter* accounted for 4.2 %, 23.3 %, 0.4 %, 0.5 %, 0.1 % and 1.5 % abundance in samples separated according to cefotaxime, ciprofloxacin, CTAB, erythromycin, none and tetracycline respectively.

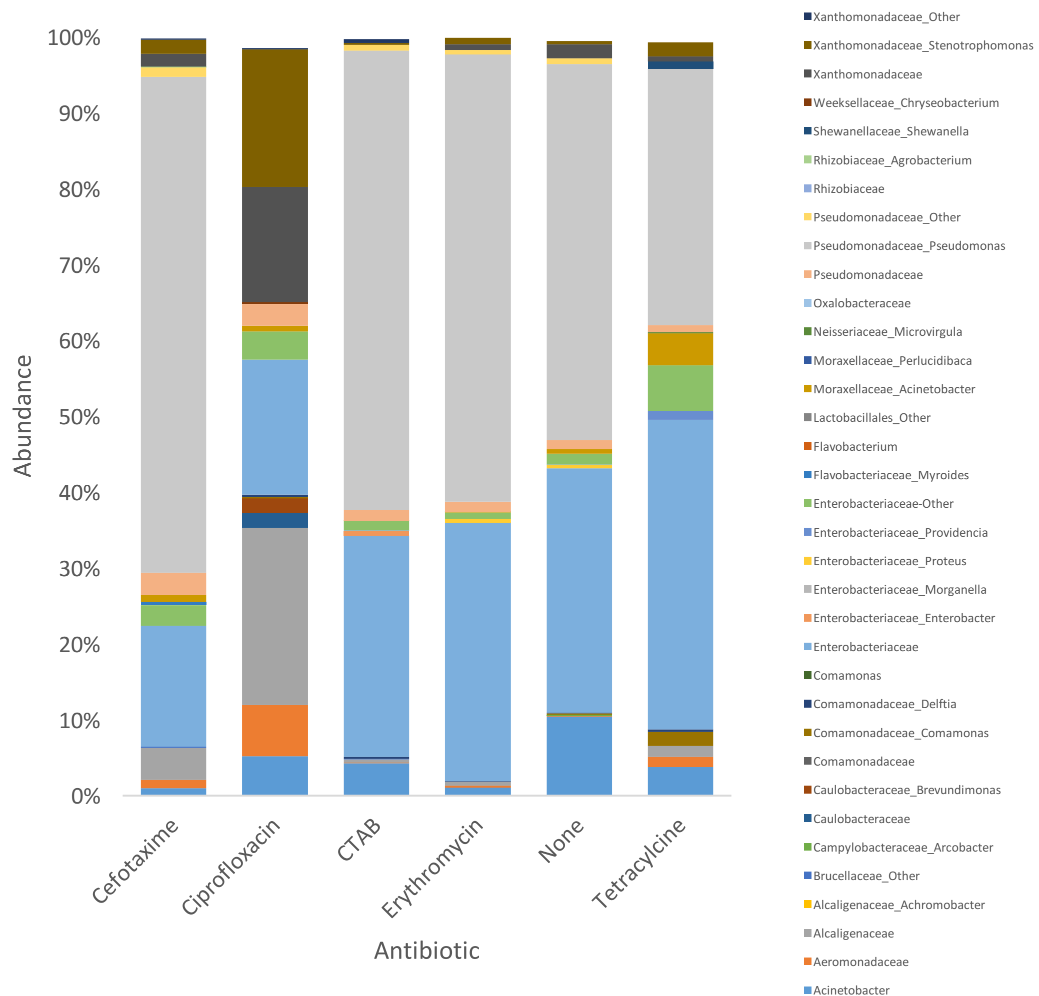


Figure 3.16 Lowest taxonomic rank of the top 15 order/genus from samples separated according phenotypic resistance of the CGNF

Diversity measures did not vary greatly between antimicrobial treatment with cefotaxime-resistant culturable bacteria community diversity ranged from 1.66 - 15.38, for erythromycin-resistant communities it ranged from 1.54 – 14.62, for ciprofloxacin-resistant communities it ranged from 1.51 – 11.59, for CTAB values ranged from 1.99 – 17.92, for no antimicrobial it ranged from 2.02 – 22.47 and for tetracycline values ranged from 1.32 – 13.61.

3.3.3.9 Beta diversity of samples split by phenotypic AMR profile

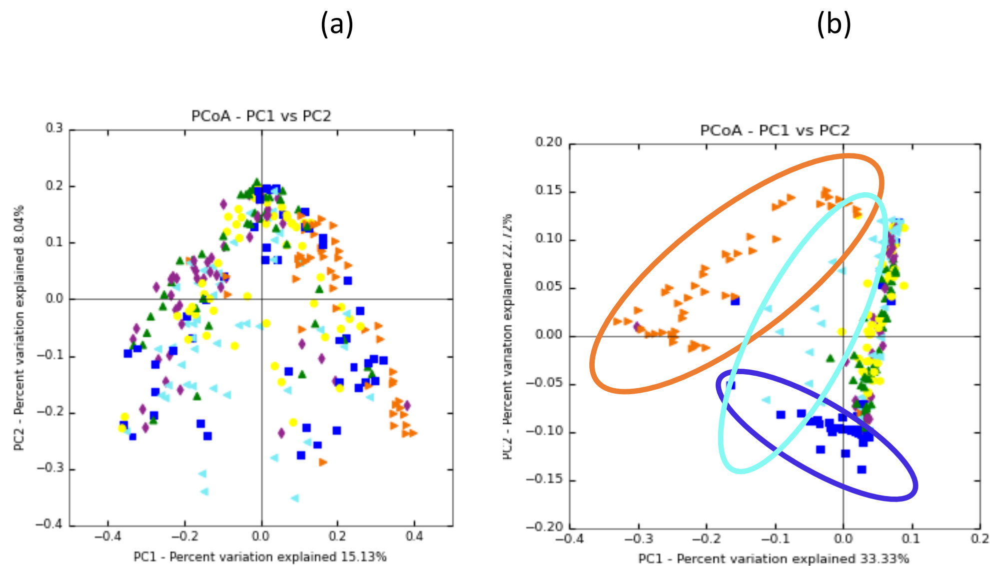


Figure 3.17 PCoA plots of CGNF separated according to phenotypic resistance. (a)Unweighted and (b) weighted. Turquoise = tetracycline, Orange = ciprofloxacin, Purple = erythromycin, Blue = cefotaxime, Green = CTAB and Yellow = none

Communities isolated by their antimicrobial profile do not cluster separately in unweighted communities (ANOSIM, R = 0.0584, p = 0.021). Weighted communities present some clustering, however when comparing all samples against each other the clustering is not significant (R = 0.274, p = 0.162) suggesting communities do not cluster according to species presence, but that the abundance of different taxa may play a role in the clustering according to different antibiotics (Figure 3.16). Ciprofloxacin selected CGNF clearly clusters away from all other antimicrobial-selected communities. Although this group isn’t enough to demonstrate that antimicrobials select for different community compositions, it does demonstrate differences between treatment with weighted communities clearly presenting different abundances of certain OTUs compared to other treatments. There may also be differences in the abundance of OTUs in the cefotaxime and tetracycline communities which show some clustering from other samples but clearly not as much as ciprofloxacin resistant communities.

3.3.3.10 Alpha diversity of samples from different sites

Predominant genera between sites from the cultured samples was *Pseudomonas,* accounting for 60.83 % at Kennet 2. 43.53 % at Kennet 7, 39.80 % at Kennet 8, 32.43 % at Kennet 11, 32.54 % at Thame 3, 49.06 % at Thame 5, 36.5 % at Thame 7 and 71.5 % at Thame 8. The family *Enterobacteriaceae* accounted for 14.7 % at Kennet 2, 31.6 % at Kennet 35.4 % at Kennet 7, 35.4 % at Kennet 8, 35.7 % at Kennet 11, 49.9 % at Thame 3, 36.2 % at Thame 5, 37.3 % at Thame 7, 11.7 % at Thame 8, with the majority at each site from unknown genera, but ones identified include *Enterobacter, Proteus* and *Providencia.*

Simpsons inverse diversity measure ranged from 1.32 – 16.71 at Kennet 2, 1.56 – 22.10 at Kennet 7, 1.54 – 14.43 at Kennet 8 and 2.43 – 13.65 at Kennet 11. The river Thame sites, Simpsons inverse ranged from 1.98 – 9.55 at Thame 3, 1.74 – 15.83 at Thame 5, 1.70 – 13.76 at Thame 7 and 1.96 – 15.09 at Thame 8.

3.3.3.11 Beta diversity of samples from different sites

No significant separation was observed for both weighted and unweighted communities separated by site (unweighted $R = 0.0331$, $p = 0.001$ and weighted $R = 0.357$, $p = 0.001$) suggesting that the AMR culturable fraction of bacterial communities within river water and sediment is not dependent on site.

3.4 Discussion

The work presented here provides the groundwork for part of a larger project. It was carried out as part of the Thames catchment-scale work to evaluate ARG within river systems. Using metataxonomic approaches, the current study demonstrates a diverse viable antibiotic-resilient community. Communities isolated on media with different antimicrobials were similar with respect to OTU presence suggesting a subpopulation of the community is responsible for the ARG phenotype. The ability to grow on antibiotic-containing plates, at clinically relevant levels, suggests that this subpopulation would likely survive antimicrobials entering the river through WWTP effluents.

Metagenomic studies have enabled sequencing of the unculturable bacteria allowing insight in to the diversity and abundance of ARG and pathogens within the river. A caveat to metagenomic approaches is that it cannot identify which bacterial species are responsible for dissemination ARG and with such a large microbial diversity within sediments (possibly higher than soil) it is impossible to tell with these studies alone where ARG are carried (Gibbons et al., 2014). In the current study the aim was to determine bacterial species that are phenotypically resistant to both clinically and veterinary relevant antimicrobials and to investigate how this subpopulation diversity is impaired on plates with regard to competition.

Previous studies have identified key phyla within river sediment but have not investigated in depth the resistant fraction (Chao et al., 2013, Tang et al., 2016). Investigating the culturable resistant fraction helps to identity viable strains where ARGs are harboured to identify the potentially clinically important AMR bacterial fraction within river sediment to provide insight in to the risk associated with environmental ARG. A key drawback of environmental monitoring studies is that they often overlook the importance of host and therefore cannot suitably assess the risk of ARG presence in the environment. Investigating the CGNF has shown that these ARG reside in potentially pathogenic species and therefore are likely to pose a risk to human health if ingested.

In the current study it was found that bacterial community composition was different between water and sediment, with greater bacterial diversity in sediment than water. This data is consistent with results by Ibekwe *et al.* who reported differences in communities within water and sediment following WWTP effluent impact (Ibekwe et al., 2016). Analysis of differences in bacterial prevalence between samples showed significant clustering of samples suggesting, as expected, that water and sediment communities differ in species prevalence.

No differences were observed in species presence between the CGNF from water or sediment suggesting this subcommunity exists in both sediment and water and is therefore resilient against environmental factors that affect the indigenous population. The WWTP process induces a significant amount of stress on bacteria, therefore the species surviving the process are likely to be adapted to harsh conditions possibly explaining why non-indigenous bacteria are found in river samples (Baquero et al., 2008, Pruden, 2014). Another possible explanation for isolation of this subpopulation of antimicrobial-containing plates is that, rather than a persistent community, accretion from the constant influx of effluent results in the isolation of ARB.

Dominant families in the resistant cultured fraction were *Enterobacteriaceae* and *Pseudomonadaceae*. Consistent with previous studies the resistant viable population constituted only a small proportion of total river communities with 0.8 % of abundance in sediment and 1.9 % in water suggesting water communities carry a higher proportion of resistant bacteria but not necessarily a higher abundance. *Enterobacteriaceae* and *Pseudomonadaceae* consist of many pathogenic species which can cause disease in the human population with both multidrug-resistant *Pseudomonas aeringinosa* and ESBL producing *Enterobacteriaceae* labelled as serious threats (Tang et al., 2016, Amador, 2015, Korzeniewska and Harnisz, 2013, Slekovec et al., 2012, WHO, 2014). Determining the factors involved in abundance and persistence of these subpopulations within river communities is ultimately important to reducing resistance levels in the clinic. The *Enterobacteriaceae* constituted a large proportion of the cefotaxime-resistant community, but the

proportion of *Pseudomonas* dominated. Resistance in *Pseudomonas* has been identified as a problem in hospital-acquired infections due to its characteristic ability to acquire resistance to a variety of antibiotics making treatment difficult. The high levels of phenotypically resistant *Pseudomonas* found here in river samples is concerning, particularly the large proportion of phenotypically cefotaxime-resistant *Pseudomonas* detected. Previous detection of *Pseudomonas* in the environment has not elucidated the point source of resistant strains in the environment (Schwartz et al., 2006).

Consistent with results from previous studies *Proteobacteria* dominated all samples (Tang et al., 2016, Ye and Zhang, 2013, Marti et al., 2013a, Ibekwe et al., 2016, Gibbons et al., 2014). Inconsistent with Chao *et al.* who found predominant phyla were *Alphaproteobacteria*, the current study found the dominant phyla were *Gamaproteobacteria* and *Betaproteobacteria* in samples with WWTP effluent impact (Chao et al., 2013). The sample community distribution was more comparable to the raw water communities recorded possibly indicative of significant WWTP effluent and CSO impact on these sites investigated in this study (Chao et al., 2013).

Previous work has demonstrated that sample site is an important factor in determining bacterial community composition (Gibbons et al., 2014). In this current study site variation was most substantial with respect to cyanobacterial abundance. The highest and lowest recorded relative abundance was from Kennet two and seven. Kennet two is directly downstream (130 m) of Lockeridge WWTP and is therefore likely to have a large human impact as well as significant agricultural impact partially explaining the high abundance. Kennet two was recorded as having high levels iron, and zinc (unpublished data, provided by Centre for Ecology and Hydrology) which could explain in part why there was such a high level of cyanobacteria at this site which require high amounts of both these metals to survive (Jiang et al., 2015, Napolitano et al., 2012). Cyanobacteria are naturally-occurring, however anthropogenic activities increase the abundance within freshwater bodies with previous studies showing that human and animal wastes (including effluent release from WWTPs) contribute to the level of cyanobacteria within the river (Davis

et al., 2009). The abundance of cyanobacteria within river systems is predicted to increased urbanization and more exploitation of surround land in addition to climate change (Paerl and Paul, 2012). The most abundant genera at the majority of sites was *Perlucidbaca* of which there is currently only one species identified *Perlucidbaca piscinae.* Only one paper could be found on *Perlucidibaca* which characterised *Perlucidbaca piscinae*, a member of the *Moraxellaceae* family, which was isolated from a eutrophic pond in 2007 (Song et al., 2008). At Kennet eight and eleven *Perlucidibaca* accounted for approximately 25 % of genera in the river communities suggesting a significant role in river community structure which should therefore be further investigated.

The presence of WWTPs did not impact the total community structure with comparable diversity measures from sites with and without WWTPs. WWTPs did impact the resistant cultured fraction with increased diversity measures at sites affected by WWTP effluent. This suggests, in accordance with numerous published studies (Garcia-Armisen et al., 2014, Servais et al., 2007, Madoux-Humery, 2015, Jalliffier-Verne et al., 2015, Jalliffier-Verne, 2016, Marti et al., 2013a, Kumaraswamy et al., 2014, Tang et al., 2016), that WWTPs are likely to contribute to river levels of ARB but contrasts to studies which found a decrease in diversity at WWTP effluent impacted sites with this study finding an increase in diversity in the resistant-culturable fraction (Atashgahi et al., 2015, Drury et al., 2013, Lu and Lu, 2014). WWTP size is important in determining beta diversity, with sites not impacted by WWTP effluent and small WWTP impact clustering separately from sites impacted with large WWTP impact in both sediment and water total communities. This is consistent with previous studies which have determined that the size of the population served by the treatment plant affects river pollution and suggests further work should investigate the extent to which WWTP size impacts ARB and whether size is proportional to ARB in rivers (Atashgahi et al., 2015)

Farming practices affected diversity between total DNA samples. Sites predominantly impacted by cattle had the highest diversity measure whereas sheep impacted samples showed considerably less diversity. Different communities existed

between samples but sample evenness did not vary. This suggests farming practices affect community composition with some exposures leading to a reduction in the number of OTUs detected. There was a small level variation in diversity observed in cultured samples split according to farm impact. The greatest difference was between cow and fish impacted sites which had the highest and lowest measures of diversity respectively. No clustering of samples was observed in both weighted and unweighted samples suggesting the resistant quotient is not affected by the farming practices and that other factors including industrial effluent impact, WWTP effluent impact, temporal changes and CSO events may be more important. Changes in microbial communities related to agricultural impact have been previously recorded by Van Rossum *et al.* who determined that although changes occurred in community, seasonal variation significantly affected these changes and is more influential in determining community structure (Van Rossum et al., 2015), a point which is further discussed in Chapter 5.

Rarer genera detected within the cultured fraction included *Achromobacter* which are ubiquitous environmental organisms and opportunistic pathogens under some conditions (Swenson and Sadikot, 2015). Global significance of *Achromobacter* remains unclear but there has been an increase in reports over the past ~15 years of MDR strains causing infection, many with phenotypic resistance to ciprofloxacin and β-lactams (Adam et al., 2014, Chandrasekar, 1986, Asano et al., 2005, Derber, 2011, Gomez-Cerezo et al., 2003, Swenson and Sadikot, 2015, Amoureux et al., 2013, Doi et al., 2008). Characteristically they have intrinsic resistance to arsenic and other toxic metals and can degrade aromatic compounds and plastics allowing them to thrive in otherwise inhospitable environments (Swenson and Sadikot, 2015, Jin et al., 2015). They have been isolated from oil contaminated environments displaying biosurfactant producing abilities, in Antarctic soils and have been isolated from a copper mine in Poland (Tambekar, 2012, Cowan and Tow, 2004, Dziewit et al., 2015). Primarily studies have isolated *Achromobacter xyloxidans* (both in the clinic and environment) suggesting this is the dominant and therefore the most successful species within this genus able to survive extreme environments and cause human infection (Gomez-Cerezo et al., 2003, Doi et al., 2008, Chandrasekar, 1986,

Tambekar, 2012). It has previously been found that *Achromobacter* may interact with *E. coli* in UTI infections providing an increase in fitness and resilience against antibiotic agents (Azevedo et al., 2014) (Azevedo et al., 2016). It has been suggested that due to their ability to degrade components of plastics they may sustain *E. coli* in catheters leading to nosocomial UTI infections (Jin et al., 2015, Azevedo et al., 2014). Azevedo *et al.* demonstrated the ability of *Achromobacter* to increase fitness of *E. coli* in dual-species biofilms in 96-well tissue culture plates and pre-colonisation of surfaces with *Achromobacter* appeared to promote *E. coli* adhesion and that these dual-species biofilms required a higher concentration of antibiotics to eradicate *E. coli* from the biofilm (Azevedo et al., 2014, Azevedo et al., 2016). These studies also demonstrated that although alone *Achromobacter* was more resistant to the antibiotics used to eradicate biofilms, when dual-species biofilms were treated, it was *E. coli* that survived at a higher rate (Azevedo et al., 2016). This protective role may be explained by 3 hypotheses; 1. *Achromobacter* are able to transfer ARG on MGE to *E coli;* 2. *E. coli* may be induced into a different physiological state whereby antibiotic uptake is reduced; 3. *Achromobacter* provide a protective role through degrading antibiotics in the biofilms (Azevedo et al., 2016). This enhanced survival of *E. coli* in the presence of *Achromobacter* in these *in vitro* experiments may explain why this genus was detected on HiCA (which should be specific for coliforms) and may play a role in *E. coli* persistence in river water and sediment. Evaluating the contribution of *Achromobacter* in the environmental resistome by elucidating whether the resistance they carry is intrinsic or on MGE should be investigated in future studies to determine if the transfer from these predominantly environmental bacteria to pathogenic species is likely to occur.

Investigating 16S diversity of total communities did not capture all diversity with no detectable level of *Enterobacteriaceae* present within the river sediment samples which from the culture DNA samples cannot be accurate. The CGNF contained a significant presence of *Enterobacteriaceae* which were completely absent from the diversity analysis of sediment clearly showing the value of selective enrichment of rare populations which are likely to contain pathogenic species (Munck et al., 2015). Using metataxonomic approaches for total community analysis misses rare families

and genera in environmental samples and therefore does not capture true diversity (Vos et al., 2012). The less prevalent groups may represent the most interesting populations within the communities and underestimating the prevalence of these groups, due to sequencing, is something that must be considered in future sampling regimes. In this study a focus of the component of the resistome proved that in the *Enterobacteriaceae* and Gram-negative bacteria were viable and therefore may be able to disseminate the resistome further. A problem of culture dependent studies is that, although they are more sensitive than culture independent methods at determining rarer populations, they are considerably more biased, deselecting difficult to culture groups.

The bacterial community did not vary significantly with WWTP impact but ciprofloxacin clearly had an impact on the cultured component, particularly on the abundance of *Pseudomonas.* Ciprofloxacin is still used to treat clinical infections of *Pseudomonas* with relatively low levels of clinical resistance, at approximately 30 %, in this genus which may explain why few *Pseudomonas* were isolated on ciprofloxacin containing plates and why relatively larger levels of *Enterobacteriaceae* were isolated on these plates in place of this genus suggesting a wider environmental dissemination of clinically important *Enterobacteriaceae* rather than clinically important *Pseudomonas* species (Su et al., 2010). The relative increase is most likely due to the combined results of higher carriage of resistance to ciprofloxacin and a decrease in competition on plates (Frank, 2011, Livermore, 2002). The selective effects of other antimicrobial components produced similar impact in species diversity suggesting this community of ARB is a stable component of the total microbial community.

This study found water and sediment populations vary significantly for total communities but the CGNF which remains constant with respect to same OTUs. It also concluded that alone WWTP effluent release is not significant in determining the community structure but that intuitively the size of the population served by the WWTP is important with a larger population contributing more substantially to ARB environmental pollution than WWTPs serving smaller communities. The work

presented here clearly indicates that a dual approach combining culture independent and culture dependent methods is required to fully elucidate where ARG persist and how they are disseminated.

Chapter 4

Analysis of ARG in water and sediment communities in the Thames catchment

4.1 Introduction

To assess the potential risks of environmental contamination by ARB it is critical to determine both abundance (defined as the absolute count) and prevalence (defined as the count relative to 16S copies) in microbial communities of river systems. Continuing the work on the Thames catchment the next stage was to determine the prevalence of selected ARG to evaluate levels of dissemination and determine if ARG levels correlated to various environmental factors including both agriculture and waste processing.

AMR has previously been studied in the Thames catchment reporting an average prevalence of the class 1 integron integrase gene at ~4 % in river sediment. The association of this MGE with ARG (specifically ESBL genes) has been implied and this integrase gene has previously been recommended as a proxy for environmental ARG presence (Amos et al., 2015, Dhanji, 2011, Gillings et al., 2015). Few studies have attempted to correlate the prevalence of the class 1 integron with ARG, however the relationship remains tenuous.

To date no direct analysis has been done on ARG using culture independent methods in the Thames catchment. In the current study the aim was to further investigate AMR with respect to ARG in both culture independent and culture dependant enrichments of the antibiotic resistant CGNF to determine which factors impact the prevalence of ARG in rivers. The effects of WWTP effluent and agriculture were investigated to determine which play a dominant role in shaping ARB communities.

Berendonk *et al.* reviewed a wide range of studies on ARG in the environment and recommended a list of indicator targets and bacterial groups to be monitored to achieve comparability to monitor global prevalence of a standardised core set of clinically relevant genes (Berendonk et al., 2015). Targets included *E. coli*, *intI1, bla$_{CTX-M}$, qnrS, ermF* and *tetM*. This study used these recommendations and additionally *qacE,* to further study ARG in the Thames catchment at the eight sites previously discussed in Chapter 3. The chosen genes confer resistance to a range of antimicrobials including antibiotics (*bla$_{CTX-M}$, qnrS ermF* and *tetM*) and biocides (*qacE*) and the suggested anthropogenic pollution marker; the integrase gene from class 1 integrons was also investigated (Amos et al., 2015, Gillings et al., 2015). Emphasis was placed the integrase gene because of the suggestion it may act as a proxy for AMR by acting as a genetic platform for gene capture and is commonly characterised to carry the *qacEΔ1* gene (which is detected by the *qacE* primers). It has been found to be prevalent in clinical environments where biocides are used very frequently resultantly co-selecting ARG on the same mobile genetic element.

WWTPs have previously been linked to the dissemination of ARG in the environment and present one of the most important routes of anthropogenic pollution (Jalliffier-Verne, 2016). Although there have been contrasting studies about the overall contribution WWTPs play, most evidence suggests that they do play a significant role contributing to higher levels of ARB in river water and sediment downstream compared with upstream sites (Munck et al., 2015, Amos et al., 2014, Berglund et al., 2015, Li et al., 2015a, Szczepanowski et al., 2009). Both water and sediment samples were collected from sites downstream of WWTP effluent release at varying distances from the effluent release site. Samples were taken from regions heavily impacted by

agriculture and areas with significant urbanisation. The same samples were used for both community analysis (Chapter 3) and evaluating the extent of ARG contamination in this chapter.

Agriculture can have a significant impact on the prevalence of ARG in specific environments (O'Neill, 2016, Van Boeckel et al., 2014). A number of studies indicate a strong correlation between AMR prevalence and antibiotic usage in farm livestock where waste, such as manure, is commonly used as fertilizer. Many reports have demonstrated the link between ARG and manure spreading showing significant increases in ARG as a direct consequence (Sengelov, 2003, Agerso and Sandvang, 2005, Fahrenfeld et al., 2014). A key problem in the dissemination of ARG relates to the fact that >50 % of antibiotics (in most countries) have a dual use in veterinary and human medicine (Boucher et al., 2009). Within the UK, the use of antibiotics for growth promotion was banned by the European Union in 2006, however reports of antibiotic resistance in livestock are still frequently reported, the most notable is the detection of the mobilisable colistin-conferring resistance gene *mcr-1* from swine (Casewell et al., 2003, Anjum et al., 2016).

The cumulative effects of WWTP effluent and farming is unknown with most studies focussing on only one variable when evaluating environmental ARG (Munck et al., 2015, Smalla et al., 2000a, Byrne-Bailey et al., 2011, Byrne-Bailey et al., 2009, Szczepanowski et al., 2009, Li et al., 2015a). For example, the study by Munck *et al.* investigated the resistome of the WWTP and its dissemination by mapping reads from WWTP core resistome to metagenomes from human gut, cow rumen, permafrost and aquifer. They showed little dissemination of the resistome, with only 8 % of reads from the WWTP core found in non-WWTP metagenome, contrasting to many studies suggesting the opposite, and detected less than 10 % of the core resistance genes found in the treatment plant in the environment (Munck et al., 2015). They considered farms as a producer of waste and the potential consequences of waste disposal to the treatment plant but only investigated the waste to be treated at the plant and did not investigate the effects of surrounding farmland and the impacts they might have on the river resistome. Studies investigating farming

impacts on the environmental resistome consequently find ARG in manure and the surrounding environment. The study by Smalla *et al.* showed that resistance genes in manure were also found in farm soils demonstrating the dissemination of ARG as a direct result of manure spreading (Smalla et al., 2000a). Within a river catchment it is important to look at all anthropogenic impact's including WWTPs, farming and urbanisation. Industrial wastes are more carefully monitored as part of EA activities however no monitoring of disposal of ARG and AMR in rivers is recorded. Only monitoring of biochemical oxygen demand (BOD) for water quality and biological pollution is considered (which is related to organic effluent rather and biological pollution) (DEFRA, 2012).

There are many possible routes by which ARG disseminate in to human populations, these include contamination of the food chain, tourism and travel. ARG contamination within the food chain can occur via several routes; primarily the use of antibiotics in food production can result in active selection of ARG, however intentionally added bacteria (i.e as starter cultures and for bioconserving purposes) can also contribute, as can cross-contamination in food processing (Verraes et al., 2013). One study predicted the likelihood of exposure to one thousand colony forming units of *E. coli* at approximately 1.5 % when consuming chicken (Depoorter et al., 2012). Notable transfer from livestock to humans include the transfer of the *mecC* gene, a divergent *mecA* gene, conferring resistance to methicillin which likely evolved in ruminants before spreading to the human population and the recent emergence of the mobilisable *mcr-1* gene which was detected in intensively reared swine in 2015 and was consequently detected in clinical isolates a few months later (Paterson et al., 2014, Liu, 2015, McGann et al., 2016). Although it has not been conclusively determined if the *mcr-1* gene originated from livestock, zoonotic transmission is likely to occur.

The increase in travel and immigration from the Middle East and Asia has no doubt contributed to the dissemination of ARG (Hawkey, 2015). The study by Wickramasinghe *et al.* demonstrated 22 % of persons from the Middle East and South Asia carried the $bla_{CTX\text{-}M}$ genes whereas only 8.2 % of Europeans carried 3GC

resistance-conferring genes (Wickramasinghe et al., 2012). This relationship was previously reported in Australian travellers where it was recorded that the rate of ARB carriage prior to travelling was 7.8 % and 49 % post-travel with the highest likelihood of carriage arising in travellers who had visited the Middle East. The association between travel and global dissemination of ARG is primarily associated with the ESBL and carbapenemase resistance genes, $bla_{CTX\text{-}M}$ and $bla_{NDM\text{-}1}$ respectively, which have been reported as a direct consequence of food import from, travel to, and poor waste processing and sewage disposal in the Middle East and South Asia (Rizzo et al., 2013, Hawkey, 2015).

Chapter 3 showed a significant difference in the relative abundance of certain genera between water and sediment, however the CGNF remained stable. The aim now was to consider the correlation between culture dependant and culture independent methods to evaluate the variables with a potential to drive resistance in rivers.

AMR within the *Enterobacteriaceae* has been highlighted as a major concern by multiple reports which label them as “serious” threats to human health (WHO, 2014, O'Neill, 2016, Gelband, 2015). The resistome within this specific population of clinically related bacteria is incredibly diverse due to their ability to receive and disseminate resistance containing plasmids (Amador, 2015, Humeniuk et al., 2002, Nordmann, 2014, Nordmann and Poirel, 2014, Poole, 2004, Korzeniewska and Harnisz, 2013, Zhang et al., 2012a, Pai et al., 2001, Coque, 2008, Cremet et al., 2012, Cattoir et al., 2007, Carattoli, 2009). Resistance to 3GCs is of particular concern with an increase resulting in last-resort antibiotics being more frequently used (Gelband, 2015). Notable reports include the dissemination of the $bla_{CTX\text{-}M\text{-}15}$ gene on an IncF plasmid in the human pandemic strain of *E. coli* ST131 which has been frequently reported in clinical isolates, usually from UTI infections (Can et al., 2015).

Many potential pathogens entering the rivers through WWTP effluent may not be readily culturable, but may still be viable and therefore infectious. The study of viable bacteria within the environment is unequivocal as it determines which species likely present risk. Studies investigating the unculturable fraction through total community

analysis fail to identity the risks associated. In the current study the aim was to investigate a specific fraction of the viable bacteria community by investigating the CGNF to evaluate the ARGs within these potentially pathogenic species to determine specific ARG associated with AMR phenotype. The key genes suggested by Berendonk *et al* were considered as potential genes explaining the resistance phenotype and it was evaluated if these genes are responsible or if other genes are involved in dissemination of the AMR phenotype (Berendonk et al., 2015).

4.2 Aims

The focus here was to determine prevalence and abundance of selected ARG in a range of samples to investigate, using culture dependant and culture independent methods, the dissemination of ARG genes in selected river sites.

1. Evaluate if ARG abundance and prevalence is the same in water and sediment communities.
2. Determine if the Thame and Kennet carry different ARG prevalence.
3. Investigate if key marker genes are suitable markers of phenotypic AMR in the selected river environment.
4. Investigate differences in ARG prevalence in water and sediment communities.

4.3 Results

4.3.1 Abundance of bacterial 16S and ARG targets in sediment and water samples

Substantially higher bacterial abundance was found in sediment than in water with an average 16S count of ~6 x 10^8 and 2 x 10^4 respectively. Following, total abundance of all ARG targets was higher in sediment samples than in water (Figure 4.1). To allow direct comparisons between the culturable and total DNA extractions ARG prevalence, relative to 16S, was used for further analysis.

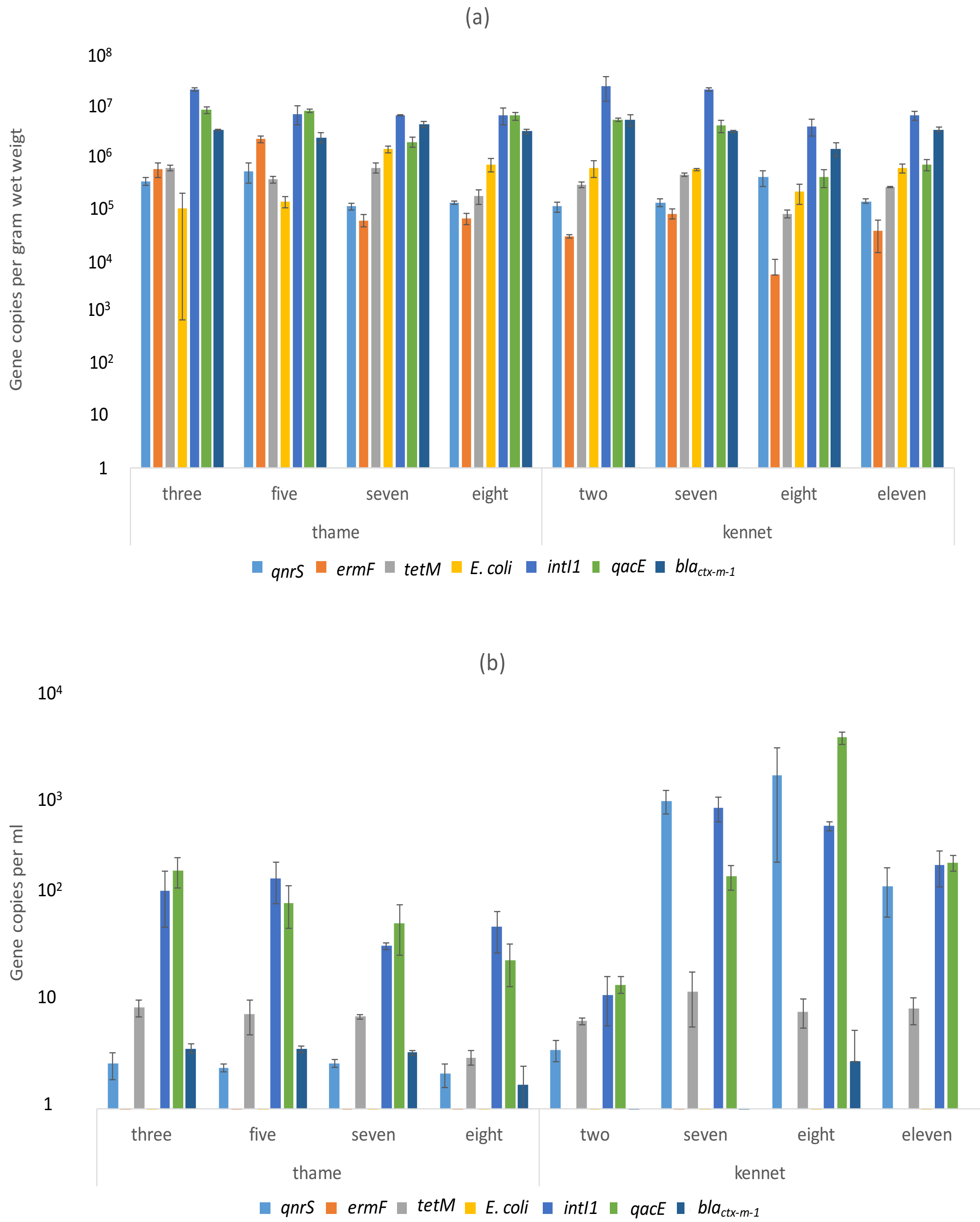


Figure 4.1 Abundance of ARG targets. *bla*$_{CTX-M-1}$ refers to group 1.. (a) Sediment, (b) Water.

4.3.2 ARG prevalence across the rivers Thame and Kennet in both water and sediment samples

The most prevalent genes recorded in this study from total extractions were *intI1* and *qacE* for most sites (Figure 4.2 and Figure 4.3). ARG profiles (Figure 4.2) group

together according to water and sediment sample types suggesting differential prevalence in water and sediment with water samples showing higher prevalence than the sediment samples most likely as a result of lower bacterial abundance.

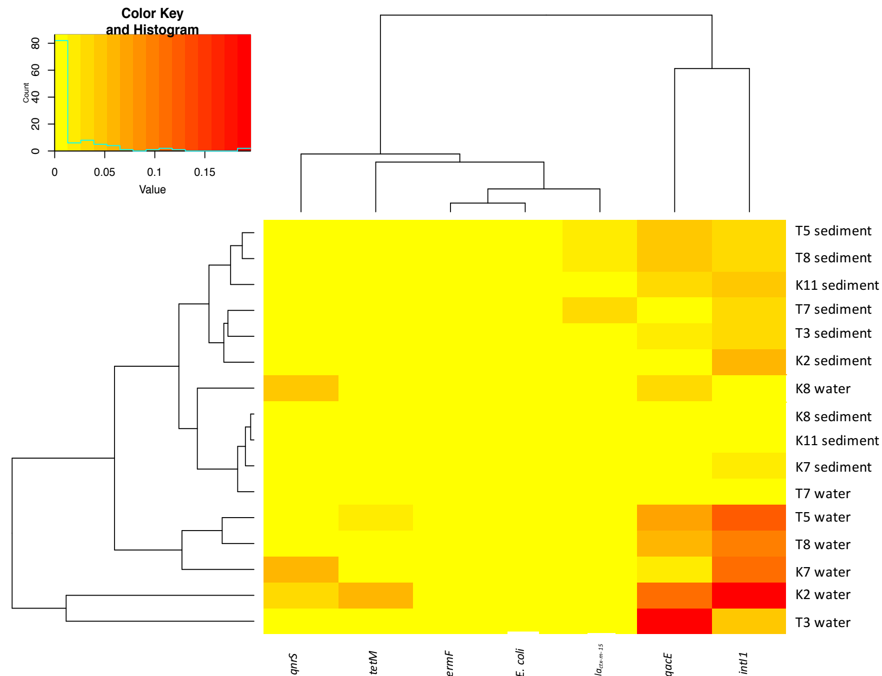


Figure 4.2 *E.* coli and integrase gene prevalence in total DNA extractions from Thame and Kennet water and sediment. Yellow = relatively low prevalence, red = relatively high prevalence.

4.3.3 Gene target prevalence according to river

The Thame and Kennet rivers were chosen based on urban and agricultural impacts. Both rivers run through predominantly rural areas impacted by a variety of animal impacts including livestock and aquaculture. The Thame runs through more densely populated areas which are more urbanised compared to the Kennet. The treatment plants that sit on the Thame all serve more than 10000 people, whereas all Kennet WWTPs serve less than 10000 and therefore sites at the Kennet are less human impacted.

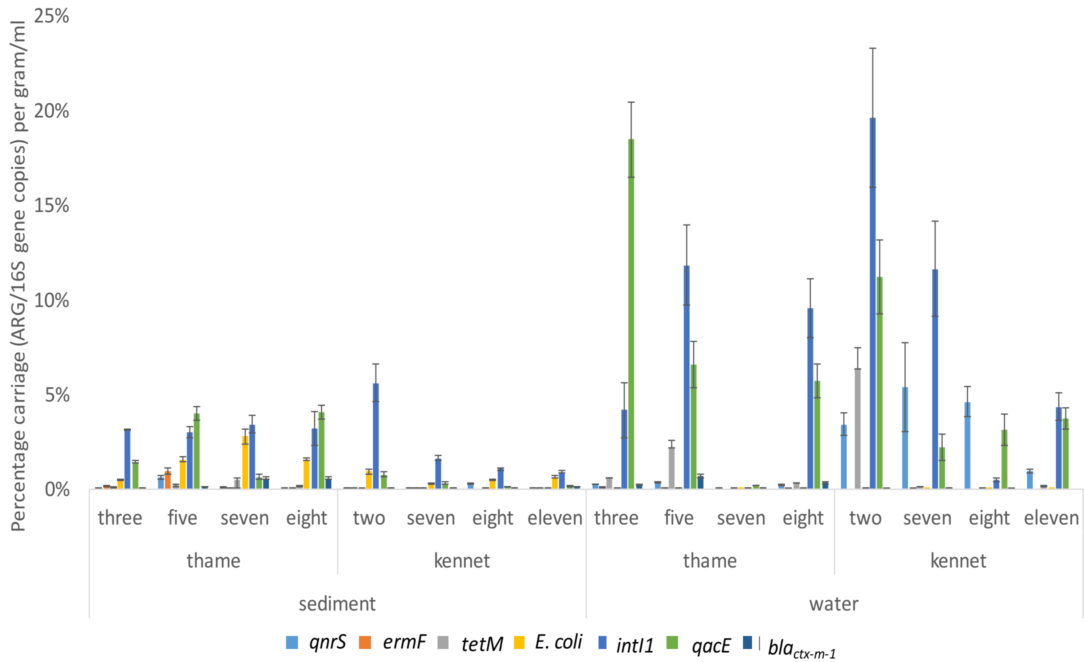


Figure 4.3 ARG prevalence (relative to 16S gene copies) in sediment and water from the Kennet and Thame. *bla*$_{CTX\text{-}M\text{-}1}$ refers to group 1.

4.3.4 Site characteristics

The sampling regime took sediment and water from eight sites, from two rivers. Metadata was collected during the sampling regime to include environmental variables that may be involved in the prevalence of target genes (Table 4.1). The Thame sites were impacted by larger WWTPs compared with the Kennet sites and overall the Kennet sites had a higher agricultural impact (Table 4.1). All sites were downstream of a WWTP effluent outlet and the river source was not tested. The nearest WWTP effluent impact in two cases (Thame 7 and Thame 11) was however over 8 km away. Cattle were the most prevalent agricultural impact and were observed at five of the eight sites.

Although the small sample size allowed thorough characterisation of the CGNF allowing multiple AMR conditions to be tested, it limited the number of samples that could be taken and therefore the number of potential explanatory variables could be analysed.

Table 4.1 Potential explanatory factors involved in ARG prevalence and abundance. Nearest WWTP effluent is defined as the distance from the closest upstream WWTP that may contribute pollution at the site.

River	Nearest WWTP (upstream/ downstream)	Approximate distance to nearest WWTP effluent	Population served by nearest upstream WWTP	Observed Agricultural impact	Overall animal impact (as recorded by CEH)	Canal feeding in to river
Thame 3	Downstream	~ 3 km	104000	Cattle,	High	No
Thame 5	Downstream	< 500 m	11000	Cattle	Low	No
Thame 7	Upstream	~ 10 km	>10000	Cattle	Low	No
Thame 8	Downstream	< 500 m	30000	Cattle	Low	No
Kennet 2	Downstream	< 500 m	<10000	Sheep	High	No
Kennet 7	Downstream	< 500 m	<10000	Sheep, fish farm	High	No
Kennet 8	Downstream	< 500 m	<10000	Swine and fish farm	High	Yes
Kennet 11	Upstream	~ 8 km	<10000	Cattle and fish farm	High	Yes

4.3.5 Investigating target prevalence in bacterial communities

Data was log transformed to overcome the assumption in ANOVA of homogeneity of variation. Two way ANOVAs were fitted to investigate the differences between catchments, sample type, sites, sites within catchments and the differences between these factors.

4.3.5.1 Investigating *qnrS* prevalence

Significant differences were recorded between sediment and water with mean prevalence in water at 2.8×10^{-3} and mean prevalence in sediment at 3.6×10^{-4} (ANOVA $F = 62.72$, $p < 0.001$). The Kennet had a higher overall prevalence of *qnrS* but when investigating sediment and water communities separately the sediment samples from the Thame had a higher prevalence compared to water, however the difference was not significant (Tukey honest significant difference (HSD) $p > 0.05$). There were significant differences (ANOVA $F = 7.71$ $p < 0.001$) across sites at both the Kennet and Thame. Across the Kennet the lowest prevalence was recorded at Kennet 2 and the highest was recorded at Kennet 7. Across the Thame the site with the lowest prevalence was Thame 7 and the site with the highest was Thame 5. The most

impacted site when examining all sites together was Kennet 2, which is directly downstream of a WWTP and has agricultural impacts from sheep and fish farms. The lowest prevalence was recorded at Thame 7 which was the site furthest away from treatment plant effluent impact, with the nearest upstream WWTP approximately 10 km from the sampling site. Thame 7 also had a very small agricultural impact which may suggest, considering Kennet 2 has a high impact, that agriculture is important in determining *qnrS* prevalence.

Individual site analysis showed that all sites in the Kennet presented a higher prevalence of *qnrS* in water communities compared to sediment (Tukey HSD $p < 0.05$) possibly as a result of the high agricultural impact at the Kennet sites. In the Thame the prevalence between sediment and water at different sites was variable but was not significant at any site (Tukey HSD $p > 0.05$). At sites three and eight the prevalence was lower in the sediment, but for sites five and seven the prevalence was lower in water samples.

4.3.4.2 Investigating *ermF* prevalence

The prevalence of *ermF* was low at most sites. Significant differences were observed in prevalence of *ermF* between sediment and water with sediment samples showing a higher mean prevalence than water communities (ANOVA $F = 31.42$, $p < 0.001$). Sediment samples had a mean prevalence of 8.3×10^{-5} and water had a mean prevalence of 2.99×10^{-6}. The river Kennet had a significantly lower prevalence than the Thame (Thame mean = 1.58×10^{-6}, and Kennet mean = 1.58×10^{-4}) ($F = 60.24$, $p < 0.001$) suggesting agriculture does not play a substantial role in determining *ermF* prevalence.

Individual site analysis showed significant differences were recorded across rivers ($F = 6.58$, $p < 0.05$). Kennet eight had the lowest mean prevalence (2.5×10^{-7}) and Kennet seven had the highest (2.2×10^{-5}). Across the Thame sites the site with the highest prevalence was site 5 (1.5×10^{-3}) and site 7 has the lowest (7.4×10^{-6}). Overall, Thame 5 showed the highest prevalence of *ermF* which could be due to large WWTP effluent impact. Although combined differences were significant across catchments

the differences at each site between sediment and water was not. Only Thame 7 showed borderline significant differences between water and sediment (Tukey HSD $p = 0.06$) with a higher prevalence in sediment.

4.3.5.3 Investigating *tetM* prevalence

Significant differences were observed between sediment and water sample *tetM* prevalence (ANOVA $F = 9.28$, $p < 0.05$). Sediment samples presented a lower mean prevalence (5.8×10^{-4}) than water samples 1.4×10^{-3}). Between catchments the Kennet had a lower prevalence than the Thame ($F = 22.17$, $p < 0.05$) suggesting agriculture is not as important as WWTP effluent impacts.

Individual site analysis showed significant differences across the rivers ($F = 6.75$, $p < 0.05$). From the samples collected from the Kennet site 8 had the lowest mean prevalence (1.4×10^{-4}) and site 2 had the highest (1.3×10^{-3}). Across the Thame the site with the lowest prevalence was site 7 (4.8×10^{-4}) and the highest was recorded at site 5 (2.3×10^{-3}). Overall the site with the highest prevalence was Thame 5 and the lowest was Kennet 8, both of which were directly downstream of WWTP effluent releases, however the treatment at Thame 5 was larger and within a more urbanised region.

Sites investigated separately showed that significant differences were observed (ANOVA $F = 9.51$, $p < 0.05$) in the prevalence of *tetM*, however significant differences between sediment and water were only observed at only Kennet 2 and Thame 7. Kennet 2 had a significantly higher prevalence in water (Tukey HSD $p < 0.05$) and Thame seven showed a higher prevalence in sediment ($p < 0.05$).

4.3.5.4 Investigating *E. coli* prevalence

A higher prevalence of *E. coli* was recorded in sediment samples (ANOVA $F = 72.60$, $p < 0.001$) compared to water. The mean prevalence recorded in sediment was 5.01×10^{-4} and the mean prevalence in water was 3.5×10^{-5}. The Kennet had a significantly lower mean prevalence than the Thame ($F = 4.49$, $p < 0.001$). Across the river sampling sites Kennet eight carried the lowest prevalence (2.7×10^{-5}) and Kennet two

presented the highest prevalence (3.0×10^{-4}). The Thame three site had the lowest recorded prevalence across the Thame (4.4×10^{-5}) and Thame eight had the higher prevalence (7.1×10^{-4}). Overall *E. coli* prevalence was greatest at site Thame 8 and lowest at Kennet 8 suggesting urban impacts are more important than agricultural in determining the prevalence of *E. coli.*

Investigating individual site differences between sediment and water showed that significant differences at sites ($F = 6.09$, $p < 0.05$) existed at Kennet seven, Kennet eight, Thame seven (Tukey HSD $p < 0.05$) and a borderline significant difference at Thame three (Tukey HSD $p = 0.06$). At Kennet seven and eight the prevalence of *E. coli* was recorded to be significantly higher than the water collected at these sites. At Thame seven sediment was also significantly higher than water *E. coli* prevalence. At Thame three the water samples had a higher prevalence.

4.3.5.5 Investigating *intI1* prevalence

In contrast to the other ARG targets there were no significant differences observed between sediment and water samples with respect to *intI1* prevalence (ANOVA $F = 0.01$, $p > 0.05$). There was also no significant difference between rivers ($F = 2.12$, $p > 0.05$). Investigating the differences between site prevalence across the river samples showed significant differences ($F = 5.30$, $p < 0.05$) across sites, however post hoc tests investigating pairwise comparisons could not determine significant differences between Kennet sites. Significant differences were observed between Thames sites (Tukey HSD $p < 0.05$) with Thame 7 presenting a significantly lower prevalence compared with sites 3, 8 and 11. Within the Thames river sampling, Thame 7 was the furthest from WWTP effluent impact suggesting effluent does have an important role in determining *intI1* prevalence.

Individual site analysis showed that the only significant difference between water and sediment in *intI1* prevalence was at site 7 in the Thame which showed water had a much lower prevalence (6.5×10^{-4}) than sediment (1.7×10^{-2}) (Tukey HSD $p < 0.05$).

4.3.5.6 Investigating *qacE* prevalence

The prevalence of *qacE* was significantly higher in water samples compared to sediment (ANOVA $F = 27.48$, $p < 0.05$) and was significantly higher in the Thame compared to the Kennet ($p < 0.05$). Sediment samples had a mean prevalence of 5.1×10^{-3} and water samples had a mean prevalence of 1.8×10^{-2}. Thame samples had a mean of 1.6×10^{-2} and Kennet samples had a mean of 6.2×10^{-3}. No significant differences were recorded across the Kennet sites for differences in *qacE* prevalence (Tukey HSD pairwise comparisons $p > 0.05$), but significant differences were observed between Thame sites ($p < 0.05$). Pairwise comparisons between the Thame sites showed significant differences ($p < 0.05$) between prevalence at site 7 compared to sites 3, 5 and 8. The prevalence at site 7 was significantly lower compared to the other Thame sites in the sampling regime.

Kennet sediment and Thame sediment *qacE* prevalence were significantly different (Tukey HSD $p < 0.05$) with Thame sediment presenting a higher prevalence of *qacE* compared to sediment taken from Kennet sites. There was no significant difference between water samples.

Site analysis showed that significant differences between sediment and water samples were observed at Kennet sites 8 and 11 only. No Thame sites showed significant differences between prevalence recorded in sediment and water (ANOVA $F = 4.28$ $p > 0.05$). At Kennet eight and eleven significantly higher prevalence was recorded in water samples (Tukey HSD $p < 0.5$)

4.3.5.7 Investigating $bla_{CTX\text{-}M\text{-}1}$ (Group 1) prevalence

Significant differences in prevalence of $bla_{CTX\text{-}M\text{-}1}$ were found between sediment and water where water samples showed a significantly lower prevalence compared to sediments samples ($F = 59.66$ $p < 0.001$). The mean prevalence in water was reported at 1.73×10^{-5} and the mean prevalence in sediment was 5.7×10^{-3}. The river Kennet had a significantly lower mean prevalence than the Thame samples with a difference in means of 1.9×10^{-3}.

No significant differences between site location was observed in samples taken from the Kennet or the Thame (ANOVA F = 0.45 $p > 0.05$) but differences were observed in the recorded prevalence of *$bla_{CTX\text{-}M\text{-}1}$* between the Kennet sediment and water and Thame sediment and water comparisons with higher prevalence in sediment in both rivers ($p < 0.05$). No significant differences were recorded between sediment samples between Kennet and Thame but significant differences were found between water samples with a much higher prevalence in the Thame water samples compared (mean= 2.2×10^{-3}) to the Kennet (mean = 7.6×10^{-7}).

Investigating individual site differences showed prevalence was significantly different between sediment and water samples at Kennet sites only (Tukey HSD $p < 0.05$). Prevalence was different at Kennet seven, eight and eleven with higher prevalence recorded in sediment samples at each site.

4.3.5.8 Summary of site prevalence

Investigating the prevalence of ARG, *E. coli* and *intI1* targets demonstrated large variation across sample type and river. Each site presented different environmental factors and as a result reported significantly different prevalence's of each target investigated (Table 4.2).

The prevalence of gene targets varied with sample type with some more prevalent in sediment than water, and others more prevalent in water. The only gene target showing no significant differences between sample type was *intI1* which also was reported at the same prevalence in the Kennet and Thame. The prevalence of *intI1* therefore does not appear to be strongly influenced by anthropogenic and other environmental factors.

Thame 7 and Kennet 8 showed the lowest gene prevalence for most targets (Table 4.2). The Thame 7 was the site furthest away from WWTP effluent impact so it was expected that the prevalence would be lower at this site. The low prevalence observed at Kennet 8 however was not expected as it is directly downstream of

effluent release and has high agricultural impact with pig and fish farms near the sampling site.

Overall, target prevalence's was highest in the Thame for most targets with only *qnrS* showing significantly higher prevalence in the Kennet samples. The larger population numbers served by the treatment plants along the river Thame are likely important in determining ARG level.

Table 4.2 Sites with the highest and lowest prevalence of chosen gene target and summarising the river and sample type with highest prevalence. K2 = Kennet 2, K7 = Kennet 7, K8 = Kennet 8, K11 = Kennet 11, T3 = Thame 3, T7 = Thame 7, T8 = Thame 8.

Target	River							Sediment/Water
	Kennet		Thame		Site with highest prevalence	Site with lowest prevalence	Thame/Kennet	
	Highest	Lowest	Highest	Lowest				
qnrS	K7	K2	T5	T7	K7	T7	Kennet*	Water*
tetM	K2	K8	T5	T7	T5	T8	Thame*	Water*
ermF	K7	K8	T5	T7	T5	K8	Thame*	Sediment*
intI1	K7	K8	T5	T7	T5	T7	Thame	Water
E. coli	K2	K8	T8	T3	T8	K8	Thame*	Sediment*
qacE	K2	K11	T5	T7	T5	T7	Thame*	Water*
$bla_{ctx-m-1}$	K2	K11	T3	T7	T3	K11	Thame*	Sediment*

4.3.6 Correlation analysis between ARG targets for water and sediment samples

Correlations between targets were investigated to determine if the prevalence of one determined the prevalence of others. Samples were split by type to determine if water and sediment presented the same patterns. Correlation profiles were different between water and sediment (Figure 4.4) with some positive relationships in sediment samples presenting negative relationships in water (*qnrS* and *intI1, ermF* and *E. coli*) and some negative relationships in sediment showing positive correlations in water (*qnrS* and *ermF*). All relationships showing a switch in correlation were not significant but the change suggests some significant differences

in ARG community structure between water and sediment samples. No significant relationships were significant in both water and sediment samples. The marker gene *inti1* abundance and prevalence was relatively high in all samples (Figure 4.3) particularly high in water samples and didn't correlate with ARG.

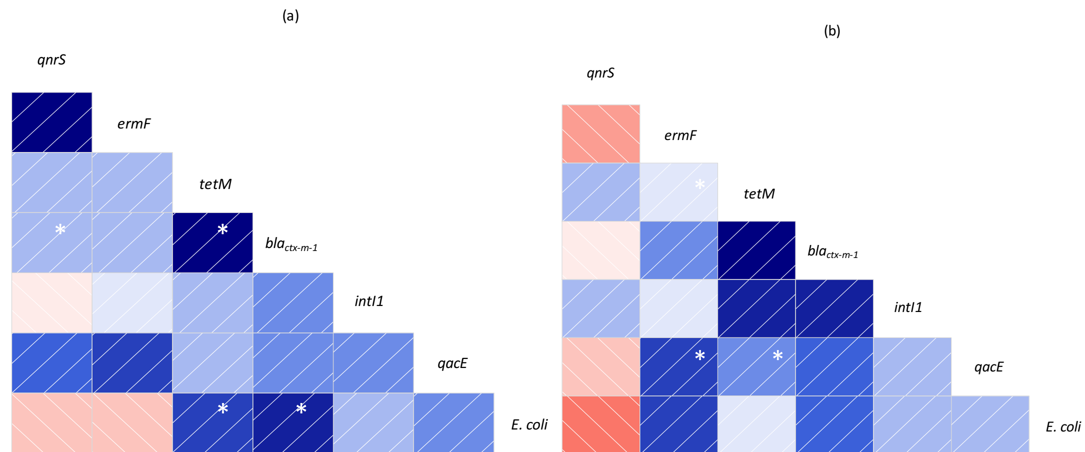


Figure 4.4 Correlations between ARG (*bla*$_{CTX\text{-}M\text{-}1}$ refers to group 1.), *E. coli* and integrase prevalence. (a) sediment (b) water. Correlogram is coloured by the strength of the correlation where dark red= -1, white= 0 and dark blue= 1. Significant correlations were calculated using the Pearson's correlation analysis.

Surprisingly *qnrS* did not correlate with *E. coli,* however *bla*$_{CTX\text{-}M\text{-}1}$ did in sediment. The association between these three genes was expected due to the frequent carriage of these genes on the same MGE within clinical isolates of *E. coli,* however they were not found in the environmental samples presented here (Partridge et al., 2011a, Coque et al., 2008, Can et al., 2015). The prevalence of *qnrS* was significantly higher in the Kennet compared to all other gene targets and was also higher in the water which may be important in determining why these targets did not correlate (Table 4.2). Although in the clinic this association is common, the association in the environment is not significant.

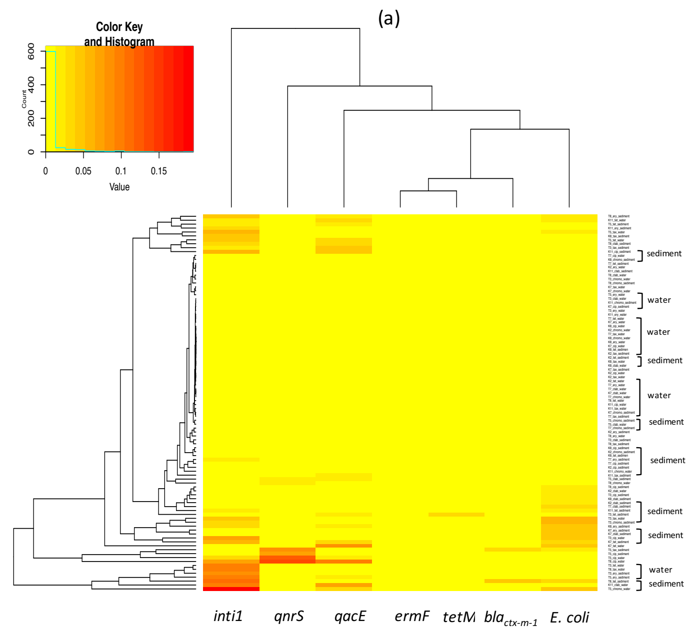
Statistically significant ($p < 0.05$) correlations in water were found between *tetM* and *qacE*, *ermF* and *qacE*, *ermF* and *tetM.*

4.3.7 CGNF population ARG prevalence

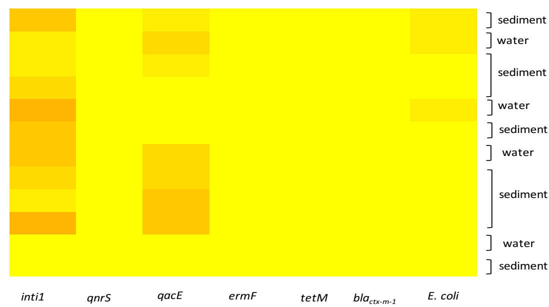
ARG prevalence was investigated in the CGNF to determine if the key ARG selected were present in the phenotypic-resistant subpopulation (Figure 4.5). High prevalence of *qnrs* , *qacE*, *inti1* and *E. coli* was seen in many samples whereas *ermF*, *tetM* and $bla_{CTX\text{-}M\text{-}1}$ didn't show any clear distribution. Only one sample had a high number of *tetM* which as expected came from the CGNF isolated on tetracycline. Interestingly this CGNF displayed a high *qacE* prevalence, but little *intI1* and *E. coli.* The two samples showing high $bla_{CTX\text{-}M\text{-}1}$ prevalence were from the CGNF from cefotaxime and tetracycline isolated communities.

Clearly, the *intI1* gene was the most prevalent gene in the majority of samples, although a high prevalence of *intI1* did not appear to be associated with a high prevalence of *E. coli* as might have been expected (Figure 4.5a). In fact, samples with very high *intI1* generally presented low levels of *E. coli* suggesting this marker gene may not be indicative of potentially important, clinically relevant resistant pathogens in the environment. The water samples did not show as much clustering compared to the sediment samples with only a few clusters of 3 or more (shown by brackets in Figure 4.5a) suggesting more variable prevalence of AMR gene targets within water samples than sediment. The sediment samples clustered towards the bottom of the heat map (Figure 4.5c) with a larger number of sediment samples compared to water samples with greater prevalence's of AMR targets. The majority (64 %) of these samples were from the Thame CGNF. Of the highlighted section (Figure 4.5c), which represents the samples with the highest prevalence of genes, the majority of the samples (63 %) were from the CGNF of sediment suggesting that sediment may contribute more substantially to viable ARB than the water column. The top 12 samples of the heat map (Figure 4.6b) also represent samples with higher prevalence of AMR targets, again the majority (67 %) of these samples were from sediment. The strong associations observed at the top and bottom of the heat map (Figure 4.5a), where prevalence is highest, suggest sediment is likely more important as a reservoirs of AMR than water.

The Kennet and Thame samples showed some clustering, particularly, samples showing little prevalence of any target AMR gene (centre section of Figure 4.5a) were predominantly Kennet sample CGNFs (63 %) and samples displaying the highest prevalence of targets were mainly from the Thame with 60 % of samples in the top clusters (Figure 4.5b) and 61 % in the bottom cluster (Figure 4.5c). This suggests that urbanisation and larger population sizes are likely more important in determining the AMR prevalence in rivers than agricultural impacts.



(b)



(c)

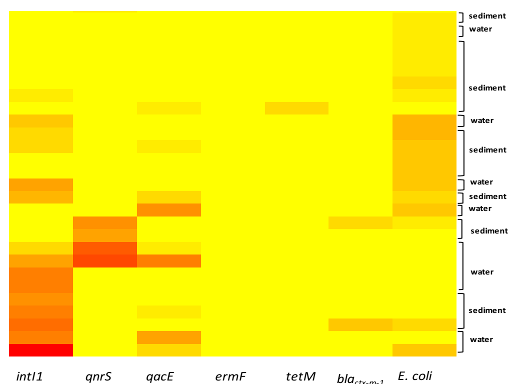


Figure 4.5 AMR target prevalence in CGNF communities. (a) full CGNF samples (b) highlighted top 12 samples showing and (c) bottom 27 samples. Highlighted regions represent the samples with the highest prevalence of AMR target genes Brackets mark clusters, for (a) and (c) brackets represent clusters of more than 3, for (b) brackets do not represent clusters and instead represent different sample type, with minimum 1 sample. ($bla_{CTX\text{-}M\text{-}1}$ refers to group 1.)

The distribution of target prevalence was highly variable across sites within the same rivers (Figures 4.6 and Figure 4.7). The sediment samples showed larger prevalence of most gene targets at Thame sites 3 and 5 with the greatest prevalence of *intI1* observed in phenotypic erythromycin-resistant communities. Surprisingly the prevalence of the ESBL $bla_{CTX\text{-}M\text{-}1}$ did not appear to correlate with the phenotypic cefotaxime communities from sediment and appears highest in the communities presenting phenotypic tetracycline resistance (Thame 8, Figure 4.6). From Chapter 3 it is unclear if competition on the plates may result in resilient communities predominantly of *Pseudomonas* rather than resistant communities which could explain why a higher prevalence of $bla_{CTX\text{-}M\text{-}1}$ was recorded in communities where it would not be expected. The tetracycline-resistant CGNF communities did show a higher number of *Enterobacteriaceae* compared to cefotaxime-resistant CGNF (Chapter 3, Figure 3.16) which may explain in part the unexpected prevalence, particularly at Thame 8.

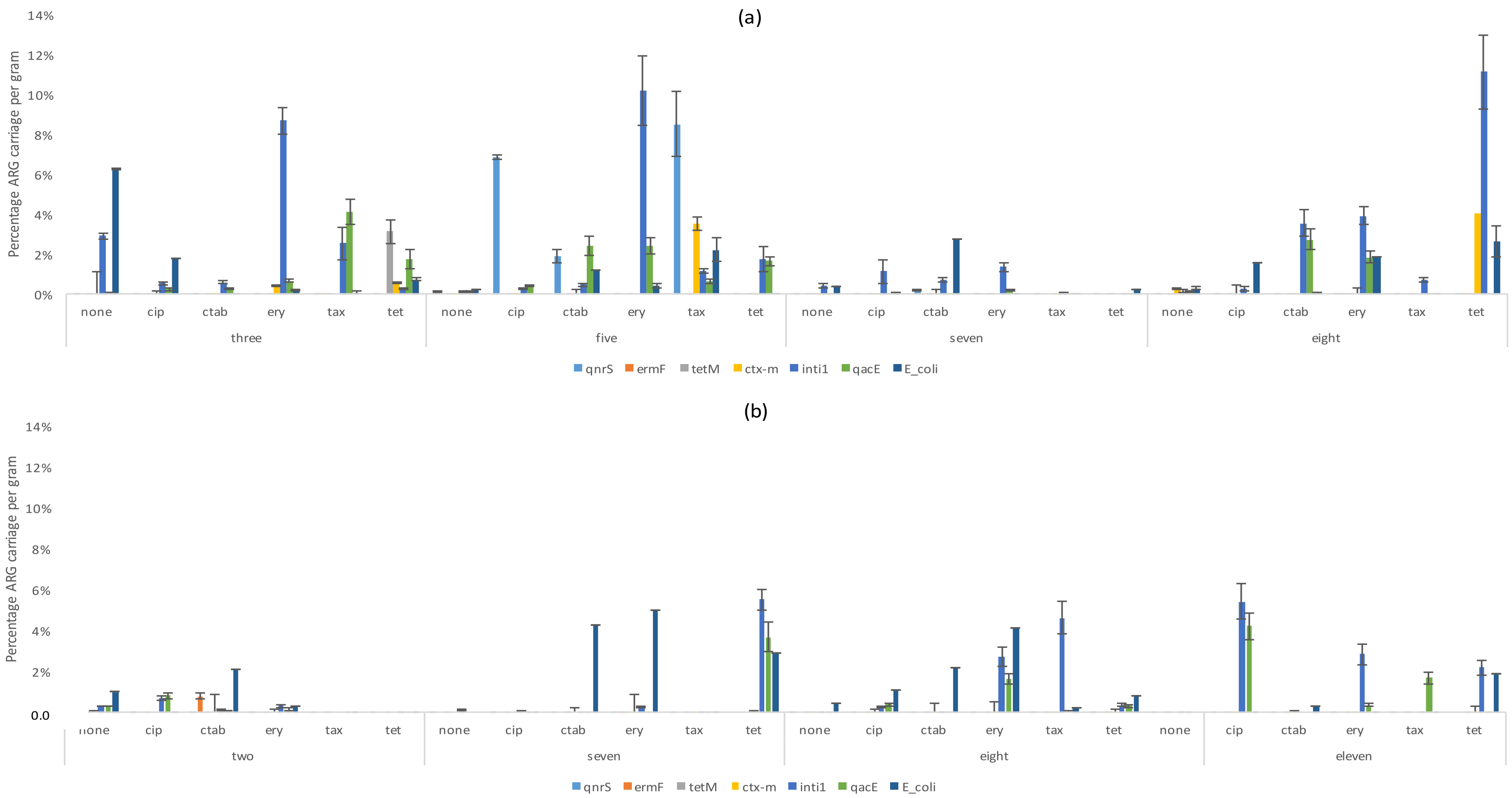


Figure 4.6 Sediment CGNF AMR target prevalence. (a) Thame (b) Kennet. (*bla*$_{CTX\text{-}M\text{-}1}$ refers to group 1.)

The prevalence of target ARG within CGNF appeared lower in water than sediment for the majority of gene targets (Figure 4.6 and Figure 4.7), however the prevalence of *intI1* was substantially higher. In water CGNF communities the prevalence of all genes, except *qacE* and *intI1,* was lower than 5 % for most site and conditions tested. Consistent with prevalence in sediment, the prevalence in water of target genes was greatest at Thame 5.

The prevalence within water CGNF was lower than expected for most targets (excluding *intI1* and *qacE*) and chosen targets were not responsible for phenotypic resistance in most instances. The choice of ARG targets therefore may not be the best choice for monitoring purposes as it suggests other genes are likely to be involved in the environmental dissemination of phenotypic resistance.

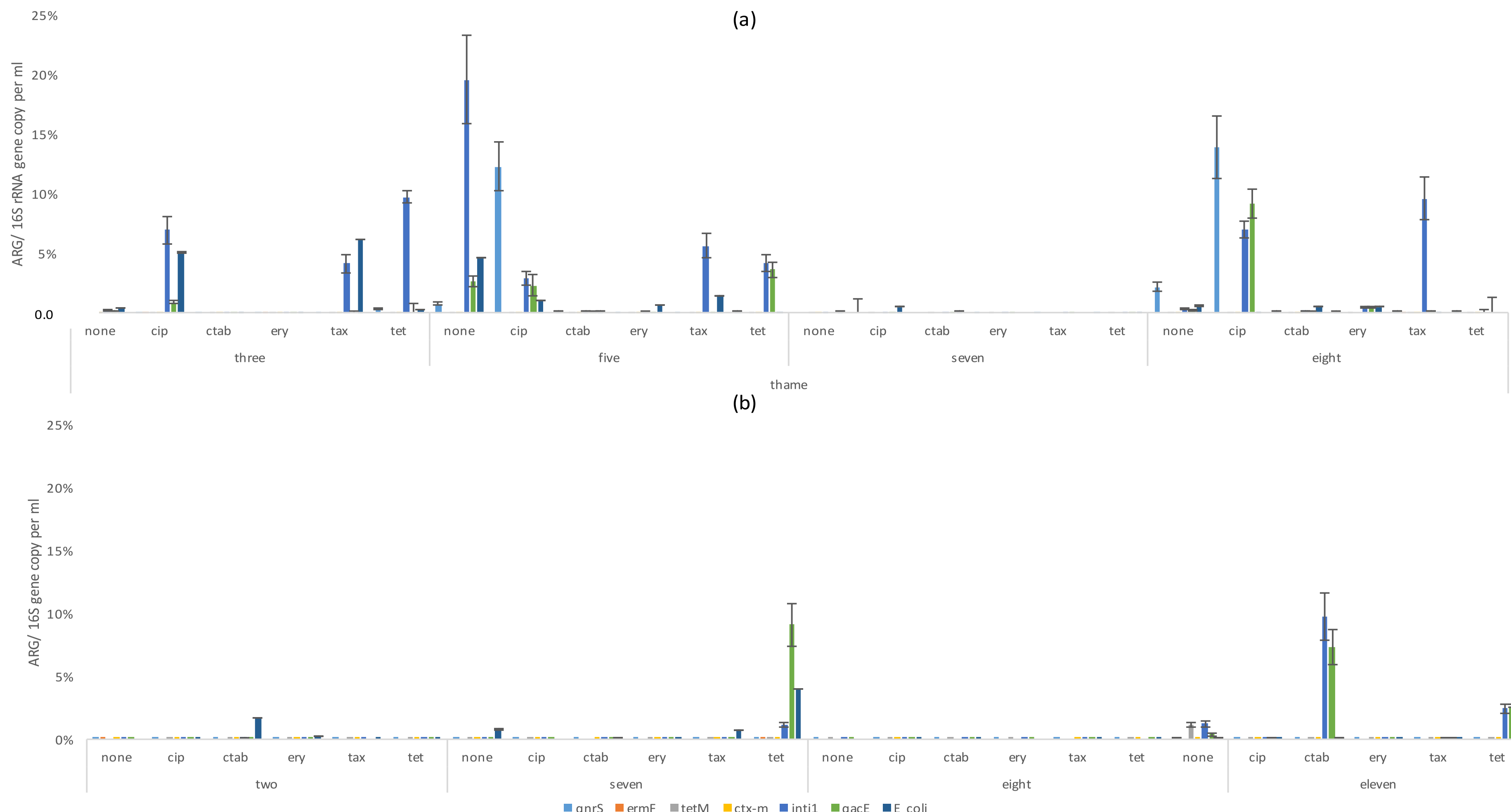


Figure 4.7 Water CGNF AMR target prevalence. (a) Thame and (b) Kennet. ($bla_{CTX-M-1}$ refers to group 1.)

4.3.8 Investigating the prevalence of targets in the CGNF

Six antimicrobial conditions (unamended HiCA, ciprofloxacin, cefotaxime, CTAB, tetracycline and erythromycin) were used to isolate the CGNF. Data was log transformed to overcome the assumption in ANOVA of homogeneity of variation. Two way ANOVAs were fitted to investigate the differences between catchments, sample type, sites, sites within catchments and the differences between these factors.

4.3.8.1 Investigating *qnrS* prevalence in the CGNF isolated on HiCA

Sediment and water samples showed no significant differences in the prevalence of *qnrS* (ANOVA $F = 0.11$, $p > 0.05$) but showed significant differences between the rivers with a higher prevalence at Thame sites compared to Kennet. The Thame mean prevalence was 5.6×10^{-5} and the mean prevalence in the Kennet was 1.4×10^{-6}. Within samples from the same river samples significant differences were observed between Thame sites 5 and 7 and between 7 and 8 where site 7 has significantly lower prevalence in each comparison (Tukey HSD $p < 0.05$). No significant differences were observed between any of the Kennet sites (Tukey HSD $p > 0.05$). Individual site comparisons showed no significant differences in the CGNF prevalence of *qnrS* between sediment and water (ANOVA $F = 2.16$, $p > 0.05$).

4.3.8.2 Investigating *ermF* prevalence in the CGNF isolated on HiCA

No significant differences were found when investigating sediment and water, river or sites suggesting the prevalence of *ermF* is consistent throughout this sampling regime. Most samples did not carry any *ermF* so it unsurprising that no differences were observed.

4.3.8.3 Investigating *tetM* prevalence in the CGNF isolated on HiCA

The sample type was not important in determining the prevalence of *tetM* with no significant differences observed between water and sediment samples. Borderline significance (ANOVA $F = 3.28$, $p = 0.08$) was recorded for prevalence between rivers with the Kennet presenting a higher prevalence than the Thame (mean at Kennet = 1.1×10^{-6} and mean at Thame = 4.4×10^{-7}). Differences in prevalence between

samples taken from the same river showed significant differences in *tetM* between Kennet 7 and 8 only with higher prevalence recorded at 7 seven (Tukey HSD $p < 0.05$). No significant differences were observed between sites in the Thame catchment or between sediment and water samples recorded for each site.

4.3.8.4 Investigating *E. coli* prevalence in the CGNF isolated on HiCA

The prevalence of *E. coli* isolated on HiCA was not significantly different between sediment and water samples. The river from which the sample was taken was borderline significant in determining the prevalence of *E. coli* ($F= 3.25$, $p = 0.08$), the mean prevalence in Kennet samples was lower than the mean prevalence in Thame samples (mean in Kennet samples = 3.2×10^{-4} and the mean in Thame samples = 1.9×10^{-3}).

There were no significant differences recorded in the prevalence of *E. coli* between sites of the same river and no differences between sediment and water at each individual sites were observed.

4.3.8.5 Investigating *intI1* prevalence in the CGNF isolated on HiCA

The prevalence of *intI1* in the CGNF was significantly different between water and sediment samples with a higher prevalence in sediment compared with water samples ($F = 36.47$, $p < 0.05$). In sediment samples the mean prevalence was 1.7×10^{3} and in water the mean prevalence was substantially lower at 6.5×10^{-5}.

The Thame was found to have a significantly higher prevalence of *intI1* than the Kennet (Thame mean = 1.1×10^{-3} and Kennet mean = 1.0×10^{-4}) ($F= 19.31$, $p < 0.05$). There were also substantial differences observed across the sites within each river with significant variation between Kennet 11 and 7, Kennet 11 and 8 (Tukey HSD $p < 0.05$) and borderline significance ($p =0.08$) between Kennet 11 and 2. Comparisons between the prevalence of *intI1* in the sediment and water between rivers was made and found that there were significant differences between water sample prevalence between the Kennet and Thame with a substantially higher prevalence in Thame water samples compared to Kennet (mean in Thame = 2.7×10^{-4}, mean in Kennet =

1.6×10^{-5}). Significant differences were also recorded for prevalence in sediment between rivers with a higher prevalence again found in the Thame samples compared with Kennet (mean Thame = 4.0×10^{-3}, mean Kennet = 6.3×10^{-4}).

Individual site analysis revealed significant differences between sediment and water at Kennet sites 2 and 7 with a higher prevalence recorded in sediment, and at Thame site 7 which had a significantly higher prevalence in water samples (Tukey HSD $p < 0.05$).

4.3.8.6 Investigating *qacE* prevalence in the CGNF isolated on HiCA

The prevalence of *qacE* was found to be significantly higher in the CGNF of sediment than water (ANOVA $F = 12.98$, $p < 0.05$). The mean prevalence of *qacE* in sediment samples was 2.1×10^{-4} and the mean prevalence in water was 3.2×10^{-5}. Significant differences were also recorded for samples taken from the river Kennet and samples from the Thame with a higher mean prevalence in samples from the Thame ($F= 23.58$, $p < 0.05$).

Within the Kennet samples significant differences in *qacE* prevalence were observed between site 11 and all other Kennet sites. The prevalence at site 11 was substantially higher than the prevalence recorded across the other sites with a mean prevalence of 1.3×10^{-3} and an average mean prevalence at sites 2, 7 and 8 of 1.00×10^{-5}.

The only site to have significant differences between sediment and water CGNF prevalence of *qacE* was Kennet 2 which had a higher prevalence in the sediment (Tukey HSD $p < 0.05$) with a mean of 2.2×10^{-3} in sediment and mean of 2.6×10^{-7} in water samples from this site. All other sites showed similar prevalence in sediment and water samples.

4.3.8.7 Investigating *bla_{CTX-M-}* (group 1.) prevalence in the CGNF isolated on HiCA

The prevalence of *$bla_{CTX-M-1}$* was not significantly different between water and sediment CGNFs but was different between rivers with the Thame presenting a significantly higher prevalence than the Kennet (ANOVA, $F= 11.23$, $p < 0.05$). the

mean prevalence at the Thame sites was 7.1×10^{-6} and the mean at the Kennet was 9.7×10^{-7}.

There was no significant difference between sites in the Kennet but there was differences in the Thame, between sites 8 and 5 and also between 8 and 3 (ANOVA $F = 3.89$, $p < 0.005$ and post hoc Tukey HSD < 0.05). Site 8 presented a much higher prevalence of $bla_{CTX\text{-}M\text{-}1}$ than sites 3 and 5 with a mean of 1.1×10^{-4} compared to the means 1.6×10^{-6} and 2.2×10^{-6} of sites 3 and 5 respectively.

Individual site analysis showed that no site displayed significant differences in the prevalence of $bla_{CTX\text{-}M\text{-}1}$ between water and sediment samples. Thame 7 was borderline significant (Tukey HSD $p = 0.059$) with a higher prevalence in water CGNF samples compared with sediment from the same site.

4.3.8.8 Summary of ARG prevalence in site CGNF

In comparison with the total community analysis of ARG prevalence (Table 4.2), fewer significant differences were recorded. In the total community both sample type and river were important in determining differences in prevalence of most ARG targets (excluding *intI1*) but analysis of the CGNF showed river was more important in ARG prevalence with significant differences in sample type recorded for only *intI1* and *qacE* (Table 4.3). This suggests that the resistant CGNF is the same within sediment and water samples (consistent with Chapter 3) and therefore will be affected by the same environmental factors.

Consistent with the observations made for the total community (Table 4.2) the CGNF isolated on unamended HiCA showed that Kennet 8 carried low prevalence of ARG. However, Thame 5 did not present the highest prevalence for most gene targets as with the total community analysis. Instead the sites with the highest prevalence of ARG within the CGNF was variable and was dependant on the specific target investigated suggesting that although the communities are likely similar between water and sediment, the resistance carried in this communities is dependent on the surrounding environment.

The Thame sites generally presented higher carriage of ARG compared to Kennet sites which may be indicative of the urbanisation surrounding the river sampling sites. Consistent with total community analysis this suggests that WWTP effluent impacts are more important in determining the resistant CGFN than agricultural effects.

Table 4.3 Summary of the CGNF communities ARG prevalence. Sites with the highest and lowest prevalence of chosen gene target and summarising the river and sample type with highest prevalence. K2 = Kennet 2, K7 = Kennet 7, K8 = Kennet 8, K11 = Kennet 11, T3 = Thame 3, T7 = Thame 7, T8 = Thame 8. . Significant differences are marked for Thame/Kennet and Sediment/Water comparisons with an asterisk. Borderline significant (0.05 > 0.1) are marked with a tilde.

Target	River							Sediment/Water
	Kennet		Thame		Site with highest prevalence	Site with lowest prevalence	Thame/Kennet	
	Highest	Lowest	Highest	Lowest				
qnrS	K7	K8	T5	T7	T5	K8	Thame*	Water
tetM	K7	K8	T3	T5	K7	T5	Kennet ~	Water
ermF	K2	K7/K8/K11	T3	T5/T7	K2	K7/K8/K11/T5/T7	Kennet	Water
intI1	K11	K8	T3	T6	T3	K8	Thame*	Sediment*
E. coli	K2	K7	T8	T97	T8	T7	Thame ~	Sediment
qacE	K11	K7	T5	T7	K11	K7	Thame*	Sediment*
$bla_{ctx\text{-}m\text{-}1}$	K7	K8	T8	T5	T8	K8	Thame*	Sediment

4.3.9 Correlation analysis of ARG and *E. coli* in the CGNF of sediment and water

Correlation analysis was carried out for all combinations of ARG targets and *E. coli* for cultured DNA extractions from both water and sediment (Figure 4.8). Significant correlations in water and sediment culturable fraction were different with the only pair of ARG targets correlating in both water and sediment being *intI1* and *qacE*.

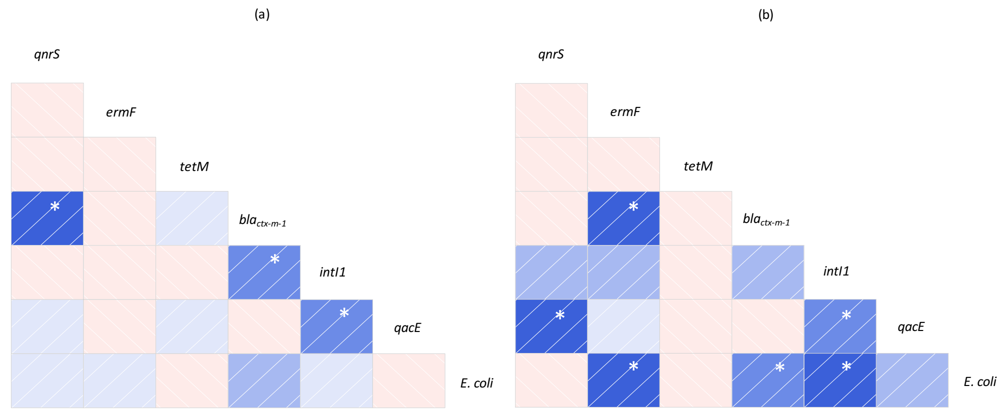


Figure 4.8 Correlations between AMR target prevalence (*$bla_{CTX-M-1}$* refers to group 1) in CGNF from planktonic and sediment communities. (a) Sediment (b) Water. Correlogram is coloured by the strength of the correlation where dark red= -1, white=0 and dark blue=1. Significant relationships are marked with an asterisk.

Significant correlations, calculated using the Pearson's correlation analysis, showed that statistically significant ($p < 0.05$) correlations in sediment CGNF existed between *qnrS* and *$bla_{CTX-M-1}$, init1* and *qacE* and *intI1* and *$bla_{CTX-M-1}$* and significant correlations in water existed between *qnrS* and *qacE*, *ermF* and *E. coli*, *ermF* and *$bla_{CTX-M-1}$*, *E. coli* and *intI1, E. coli* and *$bla_{CTX-M-1}$*, *intI1* and *qacE.* This provides a complex picture of the large number of variables that may be affecting individual sites and demonstrates the clear association of *E. coli* and ESBLs and integrons with *qacE* and suggests that *E. coli* entering the environment through anthropogenic contamination is likely to carry to clinically relevant ARG genes which would therefore make treatment of infection difficult if this *E. coli* were to be ingested.

4.3.10 Investigating the prevalence of phenotypic resistant *E. coli* within the CGNF

Investigating the prevalence of *E. coli* within each resistant culturable community was carried out to determine if different rivers and different sites carried the same phenotypic number of resistant *E. coli*.

4.3.10.1 Prevalence of phenotypic ciprofloxacin-resistant *E. coli*

Ciprofloxacin-resistant *E. coli* were equally prevalent in samples taken from sediment and water (ANOVA $F = 1.81$, $p > 0.05$) but were different between rivers. The river Thame had a significantly higher prevalence than the river Kennet ($F = 5.00$, $p < 0.05$).

The mean prevalence in the Thame was 4.7×10^{-4} and the mean in Kennet CGNF was 4.2×10^{-5}. Across sites, there were no significant difference in prevalence recorded in either river suggesting although prevalence differs between rivers it does not differ within the same river. There were no significant differences recorded for individual site analysis.

4.3.10.2 Prevalence of phenotypic cefotaxime-resistant *E. coli*

There were borderline significant differences between the prevalence of phenotypic cefotaxime-resistant *E. coli* detected in sediment and water (ANOVA $F = 3.07$, $p = 0.089$) with a mean prevalence of 1.44×10^{-4} in sediment CGNF and 4.9×10^{-5} in water. Significant differences were recorded between rivers with the Thame carrying a significantly larger prevalence in the CGNF than the Kennet ($F = 16.55$, $p < 0.05$) (Thame mean = 3.0×10^{-4} and Kennet mean = 2.3×10^{-5}). The site prevalence across the Kennet was consistent with no significant variation recorded. Differences were observed between sites in the Thame between Thame 3 and 7, 3 and 8, and 5 and 7. Within the Thame samples site 7 had the lowest prevalence at 9.2×10^{-6} and site 3 had the highest at 5.2×10^{-3}. Sediment sample prevalence was borderline significantly (Tukey HSD $p = 0.071$) higher in the Thame. Water samples were significantly different ($p < 0.05$) and again showed higher prevalence in the Thame CGNF. Therefore, the variation was found to be greatest in the Thame but also on average was reported to have a higher prevalence than Kennet samples.

When individual site analysis was performed only Kennet site 8 showed significant differences in the prevalence of cefotaxime-resistant *E. coli* in water and sediment of the CGNF (Tukey HSD $p < 0.05$). Prevalence was higher in sediment than the water (mean sediment = 1.4×10^{-3} and mean of water = 1.1×10^{-6}.

4.3.10.3 Prevalence of phenotypic erythromycin-resistant *E. coli*

Phenotypic erythromycin resistant *E. coli* was significantly higher in sediment CGNF than water CGNF (ANOVA $F = 13.39$ $p < 0.05$), but there were no significant differences in prevalence between the Kennet and the Thame and no individual site variation.

4.3.10.4 Prevalence of phenotypic tetracycline-resistant *E. coli*

Significant differences in the prevalence of phenotypic tetracycline-resistant *E. coli* were observed between sediment and water CGNF communities (ANOVA $F = 9.70$, $p < 0.05$). with sediment presenting a significantly higher prevalence compared to water. The mean prevalence of *E. coli* in sediment samples cultured on tetracycline was 1.5×10^{-3} and 1.0×10^{-4} in communities cultured from water. No difference was recorded between river with similar overall prevalence, however within each river site variation was significant for some sites ($F = 5.50$, $p < 0.05$). Pairwise comparisons between sites showed that prevalence was significantly different between Kennet sites only with differences between sites 2 and 7, 2 and 11, 7 and 8 and 8 and 11 (Tukey HSD $p < 0.05$). Within the Kennet samples site 11 reported the highest prevalence of tetracycline-resistant *E. coli* which was comparable to site 7. The lowest prevalence was at site 8 and was similar to the prevalence at site 2 (mean prevalence at site two = 1.5×10^{-5}, site seven = 9.5×10^{-3}, site eight = 1.5×10^{-5} and site eleven = 1.00×10^{-2}.) No individual site comparisons between water and sediment were significant.

4.3.10.5 Prevalence of phenotypic CTAB-resistant *E. coli*

The prevalence of CTAB-resistant *E. coli* within water and sediment CGNF was significantly different (ANOVA, $F = 11.84$, $p <0.05$). A higher prevalence was found in sediment samples (mean = 3.0×10^{-3}) compared to water sample (mean = 3.5×10^{-4}). There was borderline significance ($F= 3.77$, $p = 0.061$) reported for differences between rivers with the Kennet carrying a higher prevalence of *E. coli* compared with the Thame.

No significant differences were recorded for differences in prevalence of *E. coli* within the same rivers and no differences between sediment and water existed between the river Thame and Kennet.

4.3.10.6 Summary of *E. coli* prevalence in site CGNF

Phenotypic resistant *E. coli* prevalence was different between sites with a varying level of *E. coli* found in resistant communities at each site. There are no obvious

determinants of resistant *E. coli* prevalence with some communities displaying significant differences between sediment and water and some dependent upon river. Interestingly, site prevalence varied according to the condition used to isolate on with CTAB-resistant *E. coli* isolates most prevalent at Thame seven and least prevalent at Thame 3 contrasting to ciprofloxacin-resistant *E. coli* and cefotaxime-resistant *E. coli* which were most prevalent at Thame 3 and least prevalent at Thame 7. Lower prevalence at Thame 7 is consistent with the total community analysis (Table 4.2). Overall, CTAB-resistant *E. coli* were more prevalent at Kennet sites than Thame, with borderline significance, suggesting agricultural impact is potentially more important in determining the prevalence of CTAB-resistance in river communities than WWTP effluent impact.

The large variation observed between phenotypic resistance of the same bacterial strain suggests massive environmental diversity which is not simply dependant on sample type or location. Investigating the prevalence of phenotypic *E. coli* from the CGNF has highlighted the importance of determining host in relation to determining the effects of environmental factors and has shown that factors affecting gene prevalence in total communities do not necessarily correlate with differences in important human pathogens.

Table 4.4 Summary of phenotypic resistant *E. coli* from CGNF communities. Sites with the highest and lowest prevalence of chosen gene target and summarising the river and sample type with highest prevalence. K2 = Kennet 2, K7 = Kennet 7, K8 = Kennet 8, K11 = Kennet 11, T3 = Thame 3, T7 = Thame 7, T8 = Thame 8. Significant differences are marked for Thame/Kennet and Sediment/Water comparisons with an asterisk. Borderline significant (0.05 > 0.1) are marked with a tilde.

Phenotypic resistance	River							Sediment/Water
	Kennet		Thame		Site with highest prevalence	Site with lowest prevalence	Thame/Kennet	
	Highest	Lowest	Highest	Lowest				
HiCA	K2	K7	T8	T7	T8	T7	Thame ~	Sediment
Ciprofloxacin	K7	K11	T3	T7	T3	K11	Thame*	Sediment ~
Cefotaxime	K8	K2	T3	T7	T3	K2	Thame*	Sediment*
CTAB	K2	K8	T7	T3	K2	T3	Kennet ~	Sediment*
Erythromycin	K7	K2	T8	T7	T8	T7	Thame	Sediment*
Tetracycline	K7	K2	T3	T5	K7	K2	Kennet	Sediment*

4.4 Discussion

Studies investigating ARG prevalence in the environment have previously focussed on either water or sediment samples (Amos et al., 2014, Caucci, 2016b). In this study, both water and sediment samples were taken to evaluate the relative ARG prevalence in each sample type. ARG abundance was found to be higher in sediment than water and consistent with the results in Chapter 3, the work here suggests sediment presents a greater reservoir of ARB than water. There was no significant variation in gene prevalence between the CGNF from water or sediment suggesting that prevalence in this population does not vary with sample type and that in accordance with findings in Chapter 3, population similarity results in similar prevalence of ARG.

The prevalence of selected AMR targets was significantly different in all samples collected, with variation between river and sample type. There were fewer significant differences in water samples which may be due to dilution effects resulting in similar prevalence observed in water samples suggesting that sediment is a more suitable method of monitoring resistance in rivers. This contrasts to the majority of studies which have sampled WWTP effluent impacts on the surrounding rivers water rather than sediment aiming to investigate the direct effects of the treatment plant effluent on the river (Tang et al., 2016, Drury et al., 2013) .The cumulative effects of the treatment plant on the environment are likely to be more notable in river sediment however due to floc formation and settlement that may allow bacteria to persist in the sediment longer compared to water communities which are likely diluted with river flow. The sediment samples show large numbers of viable Gram-negative coliforms within the sediment community which would normally not be present in the indigenous population (Munck et al., 2015).

The most prevalent gene was *intI1* which was detected at levels consistent with previous studies (Berglund et al., 2014, Zhang et al., 2009b). There were no differences in prevalence between water and sediment samples but there were significant differences in abundance between these sample types with sediment carrying a higher number of *intI1* genes than the water (comparison between per

gram wet weight sediment and per ml water). Significant differences were not recorded between rivers however the Thame did present higher prevalence which might be related to the larger urban impact. Previous studies have demonstrated the association with integrons and anthropogenic impacts; one study showed enrichment of integron-associated genes during the WWTP processes reporting high levels of the *intI1* gene and other studies have demonstrated the association of this gene with *E. coli* isolates from WWTP effluent impacted rivers (Bengtsson-Palme, 2016, Kotlarska et al., 2015). The association of class 1 integrons at sites with anthropogenic impacts has led to the assumption that the integrase gene may provide a suitable marker gene to evaluate the extent of human pollution (Gillings et al., 2015). Although this gene is commonly detected in environmental samples, the detection of the *intI1* gene does not necessarily infer detection of clinical class 1 integrons (which are characterised by the possession of the *sul1* and *qacEΔ1* genes)(Deng et al., 2015). In the current study, although the *qacE* gene (primers amplify both *qacEΔ1* and *qacE*) was shown to correlate with *intI1* gene (suggesting some clinical class 1 integrons are present in the environment), the prevalence and abundance of this gene was different, often with the *intI1* gene at much higher levels than the *qacE* gene. The higher levels of the *intI1* gene suggest that integrons without the *qacEΔ1* are likely to be present in the environment. If the clinical integrons are to be used as a measure of anthropogenic pollution, measuring *intI1* alone is not sufficient to evaluate pollution and therefore alternative primer sets should be used that capture the 3' conserved region, measuring *sul1* and *qacEΔ1* genes to ensure measurements reflect clinical integrons and not environment-associated ones. The *qacEΔ1* gene has only been found on clinical class 1 integrons and therefore primers designed to capture this gene in association with the *sul1* gene would provide a more accurate measure of contamination from these MGEs (Paulsen, 1993). Correlation analysis showed that the *intI1* gene did not correlate with any other ARG target in water or sediment. Combined with the higher prevalence of *intI1*, this work suggests that, in contrast to previous work that *intI1* is not a suitable predictor of environmental ARG (a point which is further discussed in Chapter 5) (Amos et al., 2015).

Correlations between *qnrS* and $bla_{CTX\text{-}M\text{-}1}$, and $bla_{CTX\text{-}M\text{-}1}$ and *E. coli* were observed. The association between *qnrS* and $bla_{CTX\text{-}M\text{-}1}$has previously been observed in the emerging human pandemic strain of *E. coli* ST131 which has been detected both the clinic and environment (Matsumura et al., 2013, Coque et al., 2008, Can et al., 2015, Dhanji, 2011). Although it cannot be concluded that the correlation between *qnrS* and $bla_{CTX\text{-}M\text{-}1}$ is caused by *E. coli* strains carrying these genes, it is notable that this correlation does occur in the environment and is an association that is further investigated in Chapter 6.

The prevalence of ARG did not correlate with the antimicrobial used to isolate. The chosen ARG targets were picked based on the likelihood that they would be important environmental markers of phenotypic resistance as suggested by Berendonk *et al.,* however low prevalence of each expected resistance gene target was observed under each selective condition (Berendonk et al., 2015). The *ermF* gene was not detected in many samples and when it was detected it was at considerably low levels, suggesting this gene is unlikely to play an important role in environmental dissemination of erythromycin resistance. Further work investigating key genes must be carried out to determine if they are suitable markers for environmental resistance levels and if other sites display higher prevalence. Many environmental factors are important in determining prevalence of ARG therefore it is important to investigate another catchment to elucidate if *ermF* and other ARG are prevalent (and therefore suitable markers) in different rivers. It was recently found by Forsberg *et al.* that soil resistomes are distinct suggesting that monitoring resistance in another river would likely give a different resistance profile (Forsberg et al., 2014).

Investigating the differences in prevalence of ARG targets between sites showed that Thame 7 was often the least impacted site with respect to ARG pollution which was unsurprising considering it was the furthest away from WWTP effluent impact at approximately ~10 km downstream of effluent release. This suggests that it is not the size of the population served by the WWTP plant that contributes to the dissemination but the location, with sampling sites less than 500m from the effluent

release, presenting, often significantly, higher prevalence of ARG compared to samples taken further downstream from the effluent release site. Although, dilution factor therefore plays a role in prevalence of ARG in water and sediment, all ARG (except *ermF*) were found at all sampling points in both rivers in both sediment and water. Dilution of effluent clearly does play a role in reducing the prevalence of ARG further away from the release point (Jalliffier-Verne et al., 2015), however it is not sufficient to reduce it below the detection limit.

The combination of WWTP and agricultural impact contributes to ARG prevalence in both sediment and water. Although the effects of WWTP may be more important, with Thame sites often presenting higher prevalence compared to Kennet sites (due to the impact of larger populations served by WWTPs). The presence of different animals near sites clearly plays a role, however it is not possible from the current study to determine which agricultural impacts were most important in determining prevalence. The presence of fish farms at Kennet 7, 8 and 11 however is likely involved in the higher prevalence of resistance genes at these sites compared to Kennet 2 (which was also recorded to have high animal impact but did not have the direct impact of fish farms). Fish farm effluents are released directly in to the river, therefore any antimicrobial that is used in the treatment of disease in fish is consequently released in to the surrounding river creating large selection pressures in water and sediment (Schmidt, 2000). Studies have demonstrated the association of AMR with aquaculture with ARB isolated from the surrounding ecosystem even when there has been no recent antimicrobial use (Huang et al., 2015). Schmidt *et al.* investigated rainbow trout farms and the surrounding environmental effects with respect to ARB recovered from the streams impacted by fish farms (Schmidt, 2000). Consistent with the current study they discovered high levels of resistance among the culturable fraction of bacteria in water and sediment samples (Schmidt, 2000).

Many studies have investigated agriculture as a route of ARG dissemination in the environment showing different factors, such as antimicrobial usage in treatment, and in some countries, as growth promoters, contribute to ARG levels in manure which is often spread to land (Gantzhorn et al., 2014, Chantziaras et al., 2013, Byrne-Bailey

et al., 2011, VMD, 2016). The use of manure as fertilizer is primarily responsible for ARG and ARB dissemination in to the environment due to run-off resulting in river contamination. The application of manure to land in the surrounding area was not known in the current study so it cannot be determined if this did contribute to the prevalence of ARB in the environment however, previous studies have demonstrated that this is likely to occur in agricultural regions (Udikovic-Kolic et al., 2014, Chee-Sanford, 2009).

ARG prevalence in the total community and the CGNF were not significantly determined by the same factors. Within the total communities both the sample type (water or sediment) and the river, carried significantly different prevalence whereas in the CGNF the prevalence of ARG was not reliant on sample type and in only a few cases was significant regarding river. The resistant CGNF therefore appears to be determined by other environmental factors that were not evaluated in the current study. For example, river flow (which will reduce prevalence with faster rate), climate and seasonal impacts, and type of treatment plant which should all be investigated in future studies attempting to evaluate environmental ARG prevalence.

Chemistry data was provided by Centre the Centre of Ecology and Hydrology (Table 4.5) showing some significant differences in chemistry between the two rivers which may be important in determining the ARG prevalence at sites. Significantly higher concentrations ($p < 0.05$) of soluble reactive phosphorus (SRP), total dissolved phosphorus (TDP), total phosphorus (TP), dissolved fluorine and chlorine, nitrate, sulphate, total dissolved nitrogen, dissolved organic carbon, sodium, potassium, magnesium and boron were all observed in the Thame samples compared to the Kennet. The largest differences were recorded for SRP and TDP which were both on average 11 times higher at the Thame sites than the Kennet sites. High phosphorus levels can be indicative of sewage and agricultural pollution and each of the Thame sites was considered ‘poor’ with respect to phosphorus levels, whereas all Kennet sites were classed as ‘high’ or ‘good’ (DEFRA, 2014). The high level of phosphorus may correlate with high ARB levels as a direct consequence of sewage and manure entering the river and may explain why Thame sites showed higher ARG levels than

Kennet sites. The significant differences in chemistry between the Thame and Kennet highlights diversity between these two rivers which may be important in explaining differences of ARG prevalence.

Table 4.5 Chemistry data from the Thames catchment sites. Data was provided by Centre of Ecology and Hydrology. SRP = Soluble reactive phosphorus, TDP = Total dissolved phosphorus, TP = Total phosphorus, NH4 = Dissolved ammonium (NH_4), F = Dissolved fluoride (F), Cl = Dissolved chloride (Cl), NO2 = Dissolved nitrite (NO_2), NO3 = Dissolved nitrate (NO_3), SO4 = Dissolved sulphate (SO_4), TDN = Total dissolved nitrogen, DOC = Dissolved organic carbon, detection limit of Na and K is 0.01 mg/L and detection limit of Boron is 0.5ug/L. Other detection limits were not provided.

Site name	Measured element/compound																				
	SRP (µg/L-P)	TDP (µg/L-P)	TP (µg/L-P)	NH4 (mg/L)	F (mg/L)	Cl (mg/L)	NO2 (mg/L)	NO3 (mg/L)	SO4 (mg/L)	TDN (mg/l-N)	DOC (mg/L-C)	Na (mg/l)	K (mg/l)	Ca (mg/l)	Mg (mg/l)	B (ug/l)	Fe (ug/l)	Mn (ug/l)	Zn (ug/l)	Cu (ug/l)	Al (ug/l)
Thame3	466	483	519	0.083	0.14	82.34	0.05	68.24	66.17	17.96	6.55	66.06	14.17	94.71	4.77	68.24	27.62	10.58	9.46	7.20	3.14
Thame5	295	339	380	0.083	0.19	56.58	0.11	49.62	82.17	12.06	6.13	38.13	8.19	135.50	5.62	68.70	10.78	6.97	5.34	-0.35	-3.70
Thame7	812	812	864	0.051	0.17	76.35	0.00	45.39	67.17	12.4	5.4	55.59	12.42	110.04	4.73	77.68	18.48	3.76	5.34	7.06	3.65
Thame8	816	914	952	0.067	0.17	74.98	0.00	45.21	66.39	11.48	5.74	56.20	12.67	111.50	4.74	77.76	15.21	3.12	6.90	5.21	7.81
Kennet2	75	73	104	0.136	0.11	21.76	0.04	38.64	27.13	10.04	3.89	3.92	1.94	38.02	0.99	11.42	655.56	85.31	61.56	13.24	424.15
Kennet7	77	75	83	0.103	0.11	20.55	0.09	33.40	16.94	8.68	2.35	10.65	2.07	114.82	1.76	16.91	33.18	4.04	3.70	0.28	8.69
Kennet8	36	46	47	0.004	0.12	17.83	0.05	24.62	13.23	6.5	1.93	9.24	1.63	115.47	1.79	16.05	37.10	5.16	5.95	-0.06	19.24
Kennet11	16	23	125	0.021	0.11	18.72	0.03	8.80	20.91	2.8	4.63	8.70	2.37	90.09	2.08	23.85	151.02	13.53	5.82	-0.47	44.32

The presence of ARG in the environment is no doubt a consequence of human impact which has consequently accelerated the rate of dissemination from the clinic to the environment through misuse and overuse of antibiotics both in clinical and agricultural settings. It has been established in the current study that faecal coliforms isolated from both water and sediment present substantial source of AMR genes which are carried in species likely able to colonise the human gut. Many of the families identified from the CGNF (Chapter 3) are associated with human pathogenesis and likely originated from the human gut. The characterisation of these viable potential pathogens demonstrates that these bacteria are able to persist in

the environment, potentially disseminating ARG through HGT. Although the review by Forsberg *et al.* suggested HGT is not likely to occur in the environment the CNCF of river communities is likely able to transfer genes due to the high level of *intI1* genes carried in this population, which are likely to carry resistance gene cassettes (Forsberg et al., 2014, Roe et al., 2003).

The work presented here suggests that one of the most important factors in determining ARG prevalence in the environment is the influence of WWTP effluent suggesting that distance from effluent release reduces ARG. However, although it can be concluded that WWTP impact and agriculture are involved in environmental ARG, it is obvious that there are other environmental factors involved that have not been considered in this study. For example, seasonal effects may be involved in the prevalence of genes as well as cumulative effects of WWTPs along a river. It was not possible to evaluate the cumulative effects in this study but it can be concluded that, despite some dilution, WWTPs do contribute to ARG in river communities at least 10 km away from the nearest effluent release contrasting to the study by Munck *et al.* which suggested the WWTP effluent impact is not significant on the receiving river but consistent with many other studies which have recovered AMR *E. coli* from effluent receiving rivers (Munck et al., 2015, Korzeniewska and Harnisz, 2013, Olayemi, 1987, Dhanji, 2011, Amos et al., 2014).

Chapter 5

The impact of seasonal wastewater release from WWTPs including treated effluent and CSO release events.

5.1 Introduction

In times of heavy rainfall WWTPs are unable to cope with very large volumes of surface runoff therefore CSOs collect this runoff from both industrial and domestic sources and bypass treatment discharging directly in the river with the assumption that dilution effects will result in minimal disruption and pollution of the environment (Jalliffier-Verne et al., 2015, Jalliffier-Verne, 2016, DEFRA, 2012). The volume of untreated water that can be released via CSO spills is defined by the EA to be any quantity over the "pass forward flow", which is the required volume of wastewater that must flow to the treatment plant for full treatment. The pass forward flow is defined by litres/day = (PG+I+E) + 1360 P + 2E where P = population served G = water consumption per head per day I = infiltration (maximum) and E = trade effluent flow to sewer. Alternatively, operators can model discharge effects and if they can prove the spill will not result in 'significant deterioration' release can occur (EA, 2014). The number of spills is monitored by the Government

environmental agency (EA), however no monitoring took place at the CSO in the current study (Robert Huxham, SevernTrent Water, personal correspondence, 13th July 2016). We hypothesise flocs are likely to form allowing biomass to sink and persist in sediment accounting for higher levels of ARB and ARG at CSO event impacted sites.

The impact of CSO release events on rivers has primarily focussed on examining water samples (Jalliffier-Verne, 2016, Wang, 2014). The release of faecal indicator bacteria was determined reporting *E. coli* concentrations varied by several orders of magnitude during overflow events (Madoux-Humery, 2015). Chapters 3 and 4 reported that river sediment poses a significant reservoir of ARB and ARG with higher numbers in sediment compared with water. In the current study river sediment was investigated to determine WWTP effluent release and CSO release event impact. It was previously recorded that samples upstream of WWTPs show little ARG and ARB impact, therefore it was the aim of the current study to evaluate the impacts of different plant releases (Amos et al., 2014).

The Finham WWTP was selected for seasonal monitoring because previous work identified it as a source of *intI1* and ARB (Gaze et al., 2011, Amos et al., 2014). Sediment was sampled at two sites downstream of a CSO; one site was impacted by CSO release events only and the other was downstream of treated WWTP effluent release and was therefore impacted by both diluted CSO release and treated effluent. The aim was to see if sites are similarly affected and determine if they were equally affected by season. The hypothesis was that higher numbers of ARB would be present in Winter/Autumn compared to Spring/Summer. The Finham WWTP serves 450000 people with an average intake of 120 million litres per day of sewage from two inlets, the Sowe and Sherbourne (Figure 5.1). There are two detritors at the inlets which function to remove large objects and rubbish before treatment with three storm tanks which are used when necessary (the Sherbourne storm tank alone can hold up to 28 million litres). The treatment plant is of the tertiary type, consisting of six primary settlement tanks, three activated sludge plants and tertiary treatment involving sand filter to further remove suspended solids (Figure 5.1).

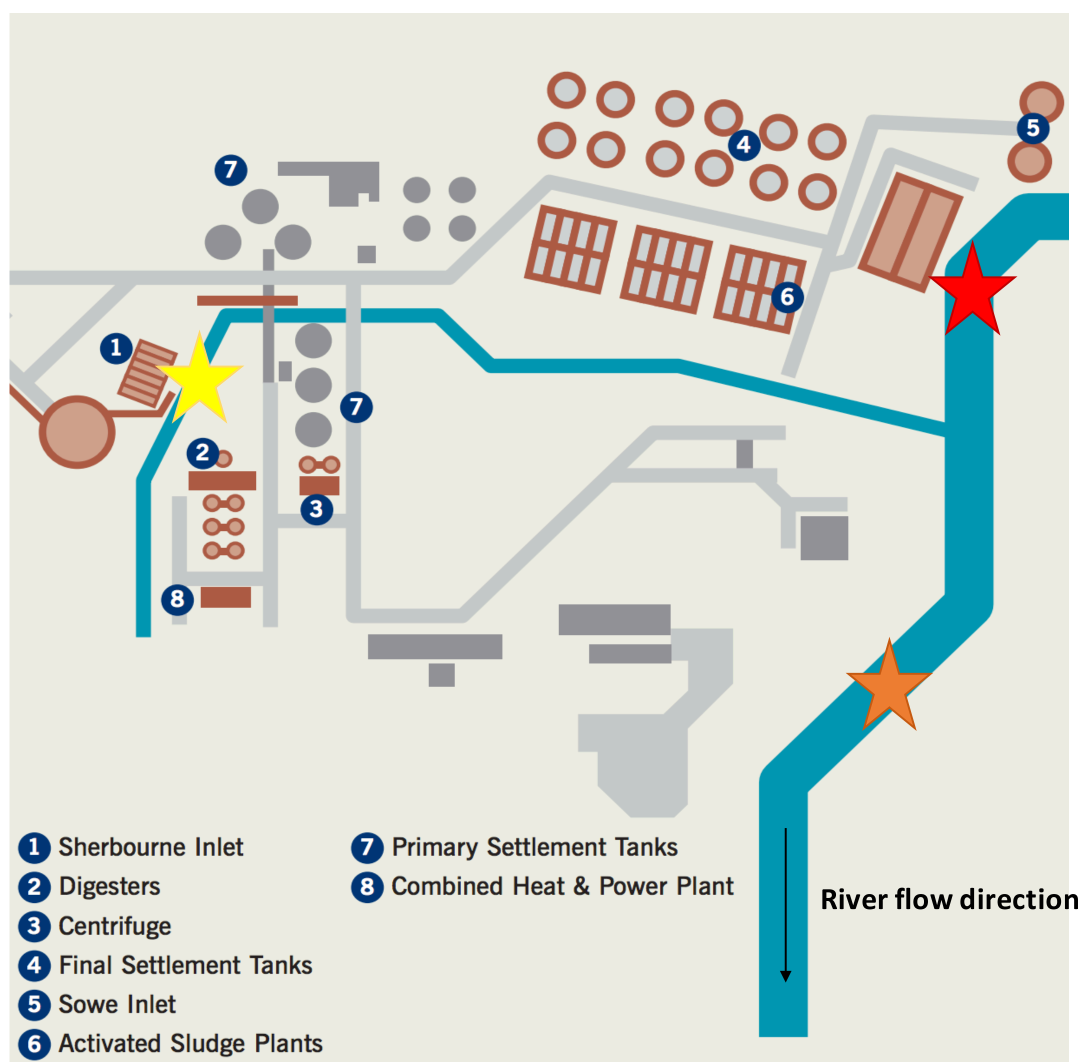


Figure 5.1 Finham WWTP layout, modified from B2682 Sewage pack from Severn Trent (SevernTrent). Red star shows CSO outlet (Site 1) sampled in this study which is permitted to spill in to the River Sowe, Yellow star indicates CSO permitted to spill in to Finham Brook and Orange star represents main WWTP effluent outlet (Site 2).

There are currently no regulations on the release of biocides, antibiotics or metals (except mercurial compounds) to rivers (Agency, 2014). Co-selection of ARG has previously been suggested due to co-carriage of ARG, BRG and MRG on plasmids (Popowska and Krawczyk-Balska, 2013, Pal et al., 2015). It was found that BRB often carried more ARG than biocide-susceptible bacteria (Pal et al., 2015). The class 1 integron integrase gene, *intI1,* may be an accurate marker for determining ARG contamination with models generated to predict the resistome based on *intI1* prevalence (Gillings et al., 2015, Amos et al., 2015). In the current study the aim was to investigate the seasonal variation of ARG and *intI1* to further elucidate if the class

1 integrase gene is a suitable proxy for determining antibiotic resistance pollution and consider if shifts in ARG correlate with changes in *intI1* abundance. The gene targets chosen for monitoring were used previously in Chapter 4 and were chosen based on proposals outlined by Berendonk *et al.* who listed ARGs believed to be important in both clinical and environmental settings (Berendonk et al., 2015).

Culture dependant methods were used to record 3GC resistant, biocide resistant and fluoroquinolone resistant *E. coli* and PCE as well as total counts on plates without amendment. Conditions selected in this study were based upon the risk posed by 3GC and fluoroquinolone antibiotic resistance which has been previously detected in river sediment samples and has been considered a threat by several reports regarding *Enterobacteriaceae* with acquired resistances (WHO, 2014, England, 2014, The Center for Disease Dynamics, 2009).

Prescription increases during the Winter months resulting in increased clinical ARB (Achermann et al., 2011, Caucci, 2016b, EXASOL, 2015, Suda et al., 2014, Sun et al., 2012, Lopez-Lozano, 2000, Lepper et al., 2002, Hay et al., 2005, Gottesman et al., 2009). The extent of ARG selection in the human gut as a result of antibiotic use is unknown but studies have demonstrated HGT events occur in the gut resulting in excretion of ARB and non-metabolized antibiotics which accumulate in sewage entering WWTPs (Sun et al., 2012, Lester et al., 2006, Trobos et al., 2009, Cremet et al., 2012, Karami et al., 2007, Goren et al., 2010, Marx et al., 2015, Coutu et al., 2013). The accumulation of antibiotics, ARB, metallic compounds and biocides in influent, from domestic, agricultural and industrial wastes, provide optimal conditions for ARG selection and dissemination (Zhang et al., 2011).

A range of studies in different countries have investigated WWTP effluent impact on the environment but less than five studies have explored seasonal effects on effluent and CSO release events (Kotlarska et al., 2015, Li et al., 2015a, Korzeniewska and Harnisz, 2013, Atashgahi et al., 2015, Munck et al., 2015, Amos et al., 2014, Garcia-Armisen et al., 2014, Knapp et al., 2012, Caucci, 2016a). Investigations of seasonal effects have primarily focussed on treated and untreated wastewater through

monitoring changes in ARG and BRG over the period of a year. Work investigating CSO impact is very limited and studies have focussed on changes in the number faecal indicator bacteria recovered from water (Jalliffier-Verne et al., 2015, Scheurer et al., 2015) .

5.2 Aims

The current study hypothesised that seasonal changes resulting in increased rainfall might impact sediment ARG and ARB numbers with higher counts in Winter months compared to Summer. The aim was therefore to take samples over a year from two sites on the Sowe; one impacted by WWTP effluent and CSO, and one CSO only, to evaluate impacts of one CSO and compare the cumulative effects of WWTP effluent and CSO.

1. Determine if there are significant changes in bacterial composition between sites.
2. Conduct a culture based study of CGNF to determine viability and phenotype of resistant strains between season and site.
3. Investigate the variation in ARG prevalence and abundance with site and season.

5.3 Results

5.3.1 Culture dependant variation in AMR *E. coli* and PCE with season

Monthly rainfall data was recorded (Figure 5.2), (taken from the Met Office; http://www.metoffice.gov.uk/climate/uk/summaries) to investigate changes in precipitation with respective changes in count data which was recorded at seasonal intervals between Winter 2015 and Winter 2016. Sampling took place at two locations near the Finham WWTP: Site 1 was defined as the CSO release site and site 2 was defined by combined CSO and WWTP effluent release (defined in Figure 5.1 by the red and orange stars respectively). Sediment samples were plated on HiCA plates with and without antimicrobial selection and counts for each site and antimicrobial condition were recorded (Figure 5.3 and 5.4) The antibiotic meropenem was also used HiCA plates but no bacterial growth was recorded at any point in the sampling regime.

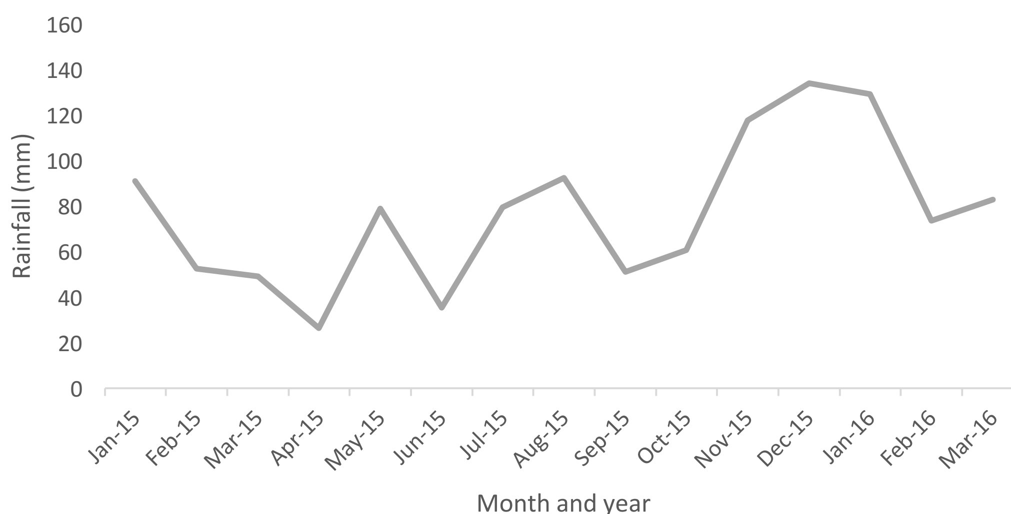


Figure 5.2 Monthly rainfall between January 2015 – March 2016. Data taken from Met office http://www.metoffice.gov.uk/climate/uk/summaries

5.3.1.1 Seasonal variation of phenotypic resistant *E. coli* from site 1 and site 2

Site 1 and Site 2 counts were not significantly different for any antimicrobial tested (Mann-Whitney; HiCA $p = 0.7491$, ciprofloxacin $p = 0.3749$, CTAB $p = 0.1142$ and cefotaxime $p = 0.2039$). The highest *E. coli* counts were in Winter 2016 for both sites (Figure 5.3) correlating with the highest rainfall (Figure 5.2). The lowest counts were

recorded in Spring which correlates with the lowest rainfall in the sampling regime (Figure 5.3) but contrastingly reported the highest number of phenotypic ciprofloxacin resistant *E. coli* at both sites (Figure 5.3). In Spring, Site 1 had higher *E. coli* counts on ciprofloxacin amended HiCA than on unamended HiCA (most likely as a result of competition on the plate). Differences in counts between Winter 2016 and Spring 2015 were significant for HiCA alone, CTAB and cefotaxime plate counts (Dunn's test $p < 0.05$) at both sites (Table 5.1).

Both Sites 1 and 2 presented a significant increase in *E. coli* counts over the period of the year (Table 5.1) with significant differences between Spring 2015 and Winter 2016 ($p < 0.05$). The counts of *E. coli* at Site 1 were higher in Spring 2015, Summer 2015, Autumn 2015 and Winter 2016 compared with Site 2 (Site 1 Winter 2015-time point was missed because Site was inaccessible). Phenotypically cefotaxime-resistant *E. coli* counts were highest in Winter 2016 at both Sites 1 and 2 and CTAB counts were consistently high throughout the year. Surprisingly, Summer presented the highest count at Site 1 and Winter (2016) presented the highest at Site 2. The levels of phenotypic CTAB resistant *E. coli* was consistently higher at the treated effluent Site 2 over the year with the exception of the Summer time point which may be a direct result of coselective effects of biocide accumulation in the WWTP.

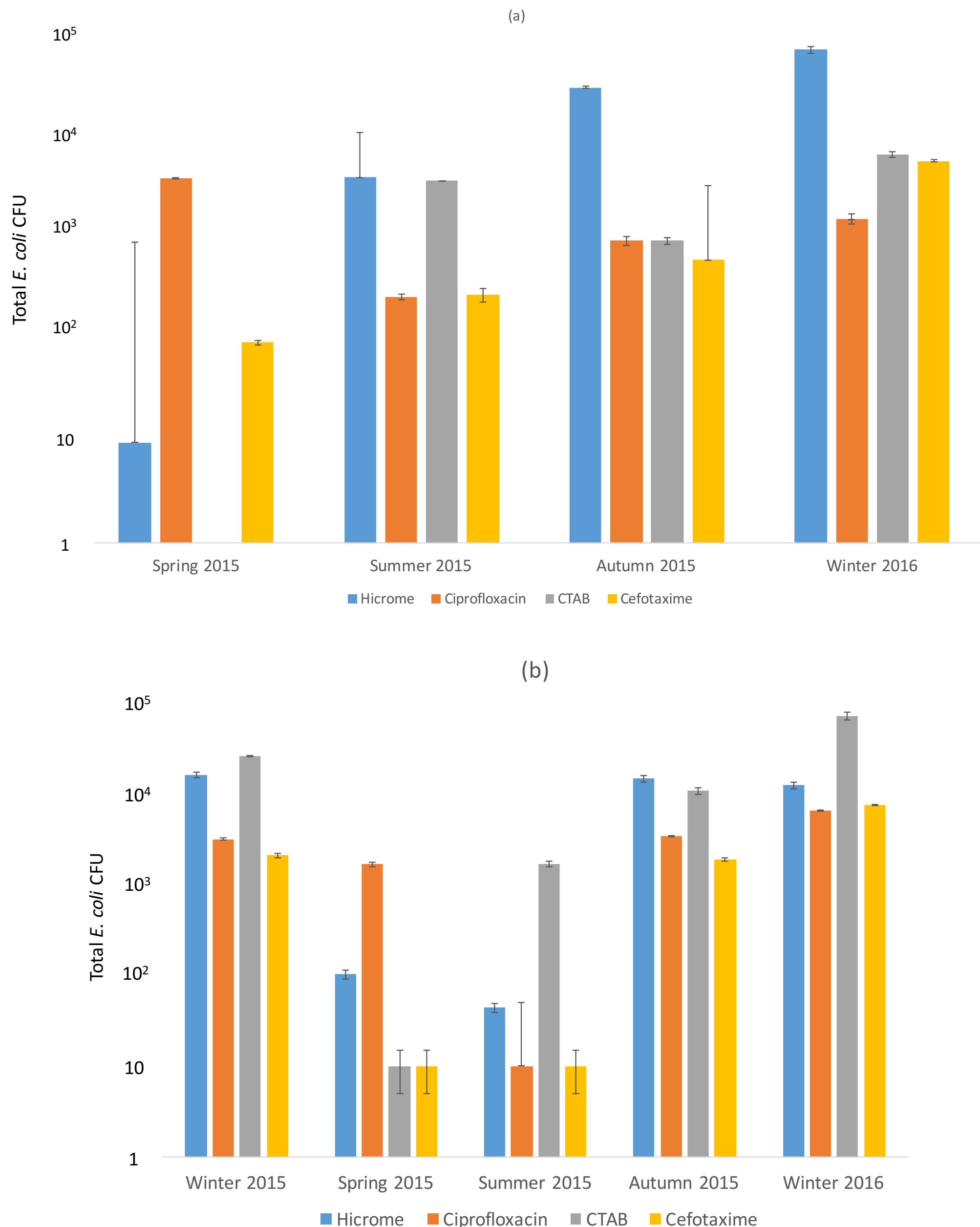


Figure 5.3 Viable *E. coli* counts from seasonal sampling between Winter 2015 – Winter 2016. (a) Site 1, (b) Site 2

Non-parametric statistical tests determined season significantly affected counts of *E. coli* at Site 1 for HiCA (Kruskall-Wallis p = 0.00034), ciprofloxacin (p = 0.0025), CTAB (p = 0.00021) and cefotaxime (p = 0.00032). Individual comparisons can be seen in Table 5.1. At Site 2, season significantly affected *E. coli* HiCA counts (p = 0.00031), ciprofloxacin (p =0.00015), CTAB (p = 5.925 x 10^{-5}) and cefotaxime (5.382 x 10^{-5}).

5.3.1.2 Seasonal variation of phenotypic resistant PCE from CSO and WWTP impacted sites

PCE counts were not significantly different between sites when isolating on HiCA media (Mann-Whitney $p = 0.5771$), but showed significant differences for all antibiotic and biocide conditions used ($p < 0.05$). The phenotypic fluoroquinolone and biocide resistant PCE population was significantly higher at Site 2 but the phenotypic cefotaxime resistant PCE population was higher at Site 1.

Significant differences between season for culture dependant PCE counts were determined using non-parametric tests. PCEs isolated from Site 1 and 2 presented significant differences between Season (for all antimicrobial conditions tested) (Kruskall-Wallis $p < 0.05$, posthoc Dunn's test shown in Table 5.1).

Table 5.1 Dunn's test to show significant pairwise comparisons of count data sampled seasonally. Z values are shown with * signifying significant correlations

Count	Site	Condition	Winter 2015 - Spring 2015	Winter 2015 - Summer 2015	Winter 2015 - Autumn 2015	Winter 2015 - Winter 2016	Spring 2015 - Summer 2015	Spring 2015 - Autumn 2015	Spring 2015 - Winter 2016	Summer 2015 - Autumn 2015	Summer 2015 - Winter 2016	Autumn 2015 - Winter 2016
E. coli	**CSO**	HiCrome	N/A	N/A	N/A	N/A	0.041*	4.075*	-3.03*	2.33*	-1.29*	1.04
		Ciprofloxacin	N/A	N/A	N/A	N/A	3.34*	-1.21	0.12	2.13*	-3.22*	-1.08
		CTAB	N/A	N/A	N/A	N/A	-3.29*	1.56	-4.04*	-1.73*	-0.74	-2.47*
		Cefotaxime	N/A	N/A	N/A	N/A	-1/-2	2.45*	-4.05*	1.43	-3.03*	-1.60
	WWTP	HiCrome	-3.5*	-3.06*	-0.26	-0.18	-0.098	2.89*	-2.97*	2.79*	-2.88*	-0.082
		Ciprofloxacin	-2.89*	-4.30*	-0.99	-1.51	1.41	1.91*	-1.38	3.32*	-2.79*	0.53
		CTAB	-4.09*	-2.89*	-1.44	-0.20	-1.20	2.64*	-3.89*	1.44	-2.69*	-1.25
		Cefotaxime	-3.99*	-4.15*	-1.75*	-1.57	0.16	2.24*	-2.42*	2.41*	-2.59*	-0.18
PCE	**CSO**	HiCrome	N/A	N/A	N/A	N/A	-1.06	3.94*	-3.00*	2.88*	-1.94*	0.94
		Ciprofloxacin	N/A	N/A	N/A	N/A	-2.19*	3.71*	-0.08	1.52	2.11*	3.63*
		CTAB	N/A	N/A	N/A	N/A	-3.15*	4.01*	-1.51	0.86	1.64	2.50*
		Cefotaxime	N/A	N/A	N/A	N/A	-1.84*	4.41*	-2.58*	2.58*	-0.74	1.84*
	WWTP	HiCrome	-3.62*	-2.24*	-2.24*	0.20	-1.38	1.38	-3.82*	0.00	-2.44*	-2.43*
		Ciprofloxacin	-1.18	-2.96*	-2.96*	1.18	1.77*	-1.77*	-2.37*	0.00	-4.14*	-4.14*
		CTAB	-2.96*	-1.18	-2.96*	1.18	1.77*	-1.77*	-2.36*	0.00	-4.14*	-4.14*
		Cefotaxime	-3.67*	-1/89*	-1.89*	0.95	-1.78*	1.78*	-4.63*	0.00	-2.85*	-2.85*

PCE counts displayed variable numbers at both sites (Figure 5.4). Counts at Site 1 were greatest in Autumn and demonstrated significantly higher counts compared with Winter 2015, Spring 2015 and Winter 2016 for all conditions except HiCA (Table 5.1, Dunns test $p = <0.05$). Equally high counts at Site 2 were recorded in Winter 2015 and 2016 (no significant difference, as determined by Dunn's test was observed between the two Winter samplings, see Table 5.1). Antibiotic and biocide resistant PCE counts were higher at Site 1 than Site 2 consistent with *E.* coli count data, however rather than showing a gradual accumulation as observed for *E. coli,* counts of PCEs were highly variable (Figure 5.4) suggesting this subpopulation is not as stable as *E. coli.*

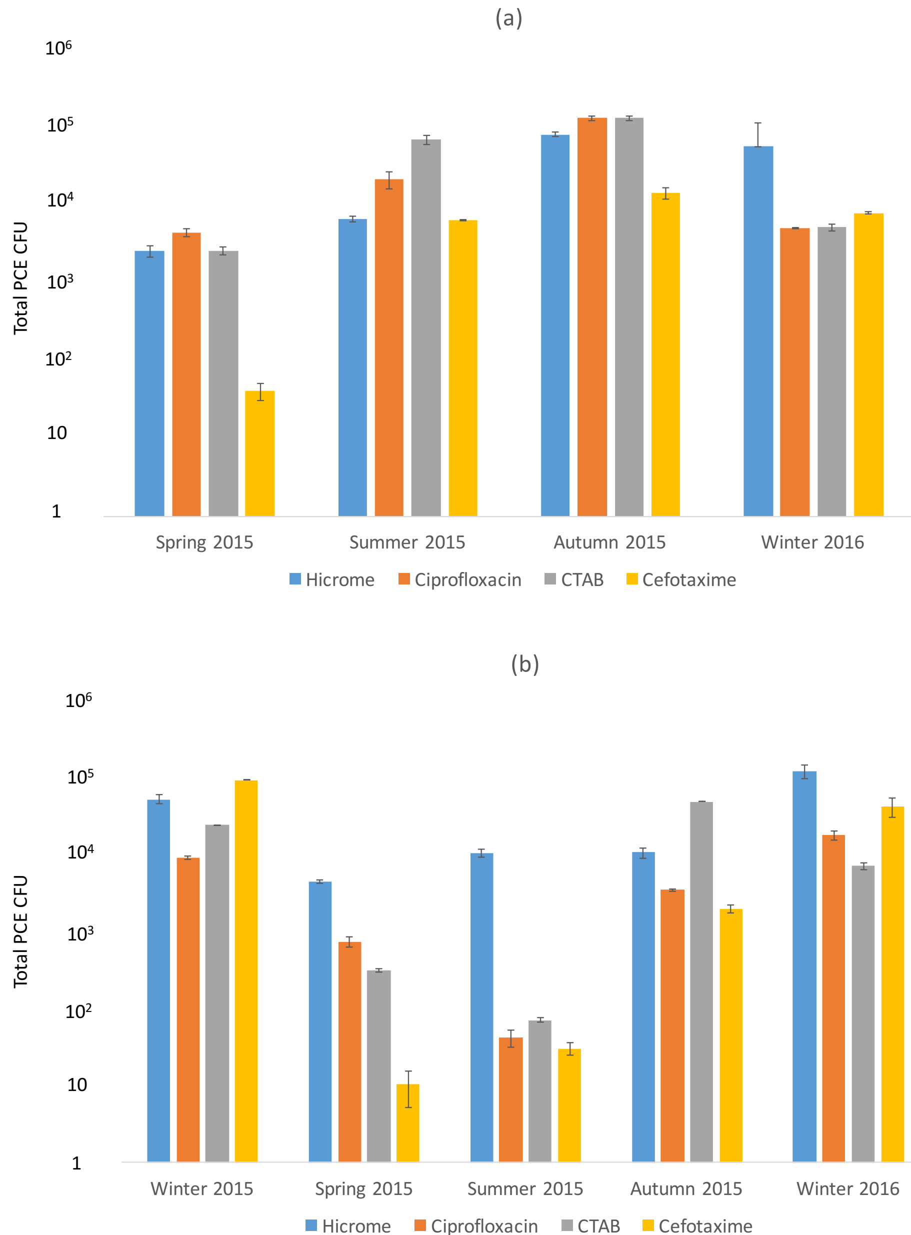


Figure 5.4 PCE count data from seasonal sampling between Winter 2015 – Winter 2016. (a) Site 1, (b) Site 2

5.3.2 Culture independent study to investigate ARG at Site 1 and Site 2

The total sediment bacterial load was consistent over the year (Table 5.2). Some change was observed at Site 1 with an increase from Spring to Autumn of ~ 1.8 times but an overall decrease of ~2.9 times from Spring 2015 to Winter 2016. Site 2 exhibited a gradual increase in bacterial number over the period of the year with an approximate increase in 16S copies of ~3.8 times that of Winter 2015 in Winter 2016.

Increase in 16S copies at Site 2 number may be reflective of accretion within the river from WWTP effluent release.

Table 5.2 16S copy number per gram wet weight sediment

Season	Sample	16S copies
Winter	WWTP	1.75×10^8
Spring	CSO	3.73×10^8
	WWTP	2.00×10^8
Summer	CSO	4.66×10^8
	WWTP	4.41×10^8
Autumn	CSO	6.66×10^8
	WWTP	5.23×10^8
Winter	CSO	1.30×10^8
	WWTP	6.70×10^8

Culture independent methods were used (Methods 2.12) to determine if ARG, *intI1* and *E. coli* levels varied according to seasonal change at both Site 1 and Site 2. qPCR data was generated (Primers can be found in Methods Table 2.4) for each site and season over the year to evaluate if site or season was more important in determining resistance gene and potential pathogen prevalence and evaluate if *intI1* provides a suitable marker of anthropogenic pollution by investigating ARG prevalence.

The relative prevalence's of ARG, biocide resistance gene (*qacE*) and *E. coli* results showed clustering of samples collected at the same sites (Figure 5.6) suggesting sample site is more important than season. The only season showing clustering according to ARG prevalence was Winter 2016 which showed separate clustering of samples from all others. Interestingly, the Winter 2016 sampling time point was taken in January 2016 which had the largest level of precipitation recorded over the sampling regime (Figure 5.2). ARG prevalence of $bla_{CTX\text{-}M\text{-}1}$ and *E. coli* was highest at Site 1 in the Winter 2016 suggesting CSO release events may have contributed to the high number of faecal coliforms entering the environment. ARG prevalence was lowest in the Spring/Summer months (Figure 5.6) but clustering was based primarily on site of sampling with site clusters clearly showing differences between sites impacted by CSO release events and sites impacted by both CSO release events and continual release of WWTP effluent.

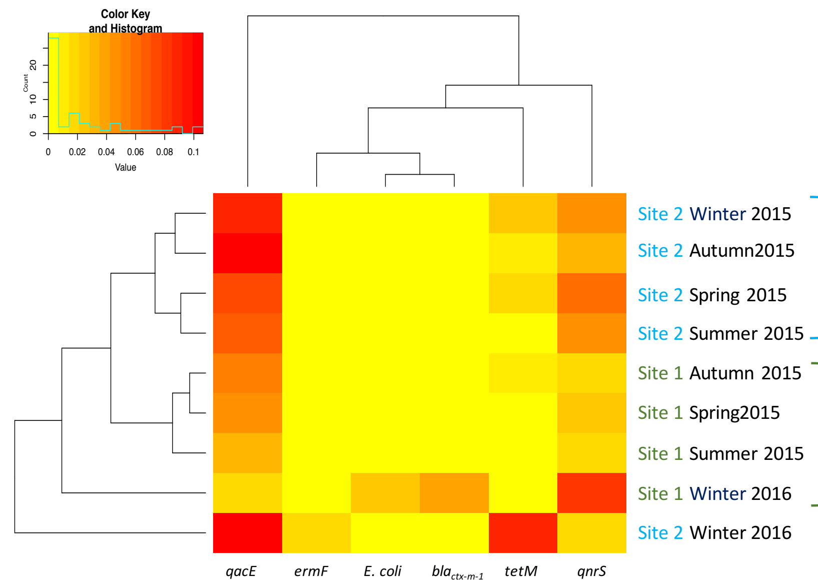


Figure. 5.6 Prevalence (relative to 16S gene copies) of AMR targets and *E. coli* at Site 1 and Site 2 over the year. Yellow = low, Red = high

The prevalence of *intI1* was significantly higher than all other gene targets ($p < 0.05$) investigated. Site 2 presented higher proportions of resistance genes consistently throughout the sampling period compared with Site 1 and showed substantially higher *intI1* prevalence with some samples presenting 45 % carriage compared to 15 % at Site 1. This high level of carriage is likely due to accretion of clinical class 1 integrons in the environment combined with environmental background of class 1 integrons. There are clear differences exhibited between Site 1 and Site 2 in Winter 2016 where *tetM* prevalence at Site 2 is greater and *E. coli* and $bla_{CTX\text{-}M\text{-}1}$ are greater at Site 1, which may be a direct result of raw human sewage entering Site 1 through CSO release events. *ermF* was detected at very low prevalence at all sampling time points except for Winter 2016 where Site 2 showed a larger increase in prevalence compared to all other samples. *E. coli* prevalence was constant over the year with a slight increase presented in Winter 2016 at Site 1. The prevalence of *qacE, qnrS* and *tetM* was typically higher compared to *E. coli* 23S, *ermF* and $bla_{CTX\text{-}M\text{-}1}$ (Figure 5.6).

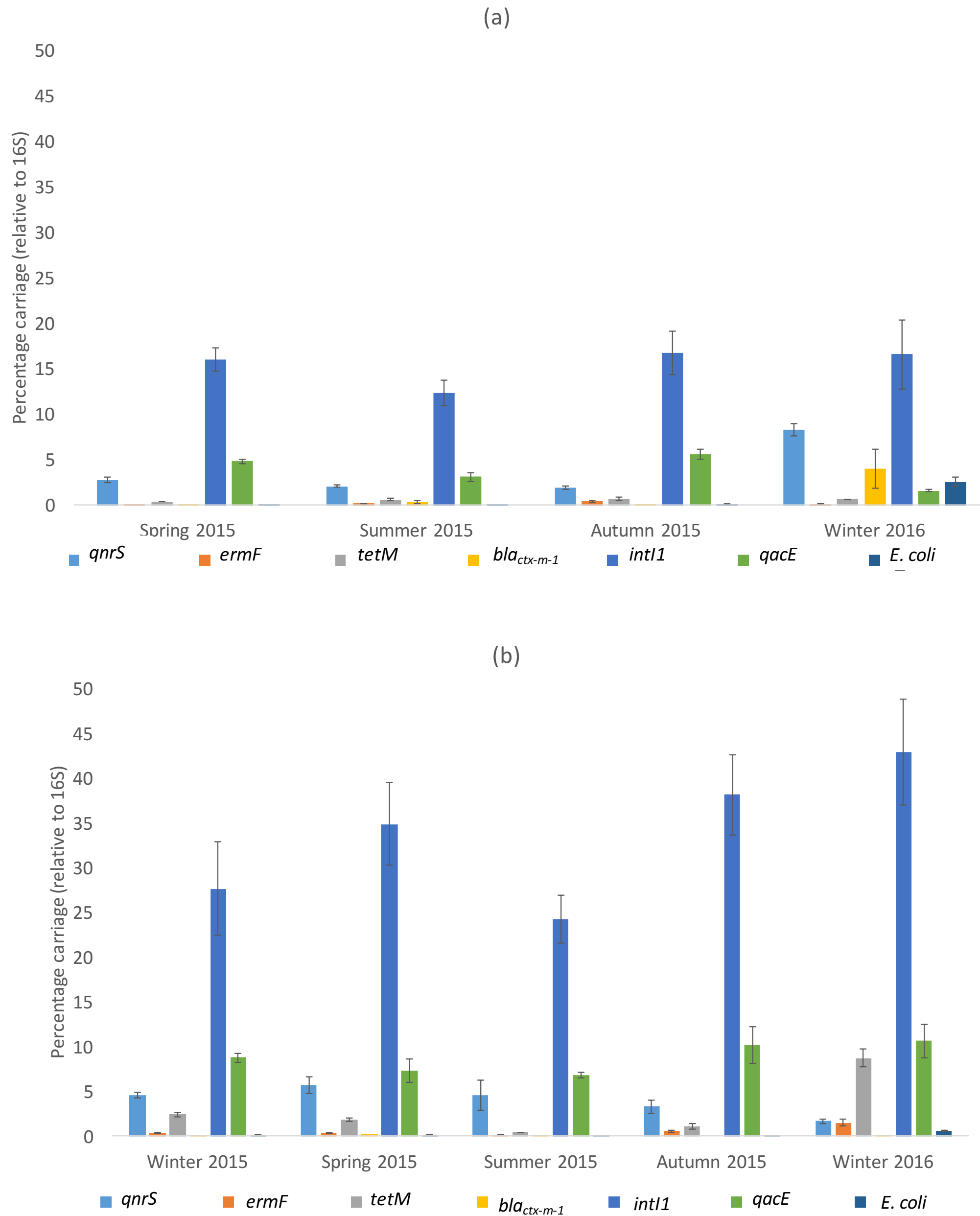


Figure 5.7 AMR, *intI1* and *E. coli* percentage carriage (relative to 16S). (a) Site 1, (b) Site 2

ARG prevalence data was not normally distributed (Shapiro-Wilk $p < 0.05$) for any target and followed an over-dispersed Poisson distribution. Non-parametric tests were conducted to determine if ARG populations were significantly different. All ARG were found to be independent (Mann-Whitney-Wilcoxon $p < 0.05$) for each comparison made. ARGs levels were not significantly different between season (Adonis $p = 0.324$, $R^2 = 0.14$), but when separated in to individual ARG targets

significant differences were observed between season suggesting that some target prevalence's can be explained by seasonal change.

No significant differences in *qnrS* prevalence were observed between site (Kruskal-Wallis p > 0.05, prevalence at Site 1 = 3.74 % and Site 2 = 3.98 %), however, when samples were split according to site significant differences were recorded between seasons at Site 1 (Spring 2015 and Autumn 2015 (Dunn's test p = 0.045), Spring 2015 and Winter 2015 (p = 0.0016) and Summer 2015 and Winter 2015 (p = 0.0271)). Seasonal variation did not account for variation in *qnrS* prevalence at Site 2 (Kruskall-Wallis p = 0.36). The seasonal release from overflow drains may be responsible for the shifts in *qnrS* abundance observed only at Site 1.

The prevalence of *ermF* was significantly different between Site 1 and Site 2 (Kruskall-Wallis p = 0.013, Site 1 prevalence = 0.18 % and Site 2 prevalence = 0.60 %). When samples were separated according to site, seasonal variation was significant (Kruskall-Wallis p = 0.03) and significant differences were observed between Spring 2015 and Autumn 2015 (Dunn's test p = 0.045), Winter 2015 and Spring 2015 (p = 0.0016) and between Winter 2015 and Summer 2015 (p = 0.027) at Site 1. Seasonal variation was not significant at explaining the prevalence of *ermF* at Site 2 (Kruskall-Wallis p = 0.07).

The prevalence of *tetM* was significantly different (Kruskall-Wallis p = 0.0497) between sites with a higher prevalence recorded at site 2 (2.91 %) compared to site 1 (0.59 %). When samples were separated by site, no overall difference was recorded for *tetM* prevalence according to season at site 1 (Kruskall-Wallis p = 0.35), however significant differences in seasonal prevalence were observed at site 2 (Kruskall-Wallis p = 0.02). Site 2 significant differences were observed between Winter 2015 and Summer 2015 (Dunn's test p = 0.02), Winter 2016 and Autumn 2016 (p = 0.0053), Winter 2016 and Summer 2015 (p = 0.0013).

Site 1 and site 2 *intI1* prevalence was significantly different (Kruskall-Wallis p = 0.00034) with a greater prevalence of *intI1* recorded at site 2 compared with site 1

(33.55 % and 15.3 % respectively). Seasonal variation did not affect *intI1* prevalence at site 1 (p = 0.74) or Site 2 (p = 0.35) suggesting seasonal variation does not account for variation of environmental levels *intiI1*.

The proportion of *qacE* was significantly (Kruskall-Wallis p = 0.0018) greater at site 2 than site 1 with gene carriage at 8.72 % at site 2 and 3.7 % at site 1. Seasonal variation was borderline significant at p = 0.05 with significant differences observed between prevalences recorded at Winter 2015 and Autumn 2015 (Dunn's test p = 0.0064) and Winter 2015 and Spring 2015 (p = 0.0118). There was no significant difference between seasonal prevalence of *qacE* at site 2 suggesting that seasonal variation doesn't contribute to relative abundance of *qacE.*

Prevalence of $bla_{CTX\text{-}M\text{-}1}$ was significantly different between site (Kruskall-Wallis p = 0.45) with higher prevalence at site 1 (1.09 %) than at site 2 (0.09 %). Seasonal variation did not significantly explain changes in prevalence at either site (p > 0.05) suggesting other factors are involved in determining the higher prevalence at site 1.

The prevalence of *E. coli* was not significantly different between sites (Kruskall-Wallis p = 0.45, 0.68 % at Site 1 and 0.20 % at Site 2). Seasonal variation was important in significantly determining the prevalence of *E. coli* (Kruskall-Wallis p = 0.03 for Site 1 and p = 0.04 for Site 2). Significant differences at site 1 were observed between Spring 2015 and Autumn 2015 (Dunn's test p = 0.045), Winter 2015 and Spring 2015 (p = 0.0016) and Winter 2015 and Summer 2015 (p = 0.271). Seasonal variation at Site 2 was significant for Winter 2016 and Autumn 2015 (p = 0.0053), Winter 2016 – Spring 2015 (p = 0.0276) and Winter 2016 – Summer 2016 (p = 0.0041).

5.3.2.1 Correlation analysis of ARG in sediment

A key element of investigating ARG prevalence was to determine correlations to elucidate if the presence of one can predict the presence of another (Figure 5.8). The genes *qnrS* and $bla_{CTX\text{-}M\text{-}1}$ are frequently detected in *E. coli* isolates therefore it was expected that significant positive correlations would be detected between *qnrS* and $bla_{CTX\text{-}M\text{-}1}$, *qnrS* and *E. coli* and $bla_{CTX\text{-}M\text{-}1}$ and *E. coli*. The strongest correlation was

observed between *E. coli* and $bla_{CTX-M-1}$, which had a cor value of 0.97 ($p = 1.096 \times 10^{-5}$). Significant correlations were identified between *ermF* and *intI1, ermF* and *qacE, tetM* and *init1, tetM* and *qacE* and *init1* and *qacE* (Figure 5.8). No negative correlations were significant.

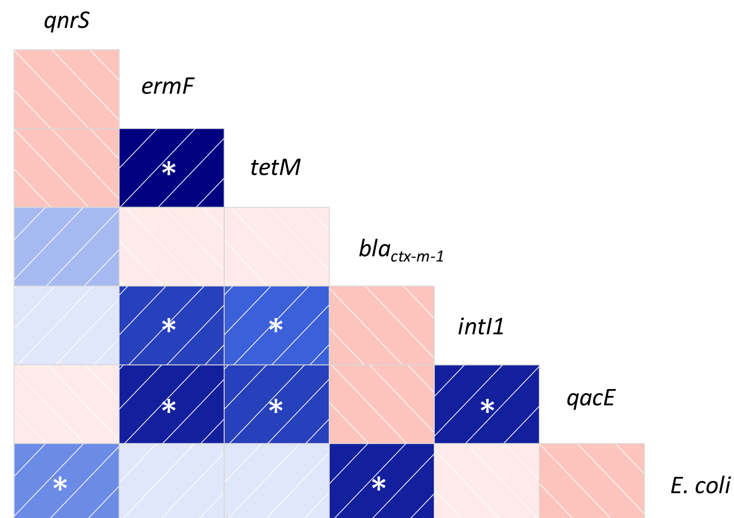


Figure 5.8 Correlations between AMR, *intI1* and *E. coli.* Correlogram is coloured by the strength of the correlation where dark red= -1, white=0 and dark blue=1. Significant correlations were calculated using the Pearson's correlation analysis showed that statistically significant ($p < 0.05$) correlations existed between *qnrS* and $bla_{CTX-M-1}$, *qnrS* and *E. coli* (23S), *ermF* and *tetM*, *ermF* and *intI1, ermF* and *qacE, tetM* and *init1, tetM* and *qacE, E. coli (23S)* and $bla_{CTX-M-1}$, *init1* and *qacE.* Significant correlations are marked with a white asterisk.

Correlation analysis between the *intI1* gene and the number of 3GC-resistant *E. coli* and PCE (count data) were carried out to investigate if the results from this study supported the results from Amos *et al* who similarly investigated the relationship between this integrase gene and 3GC resistance in river isolates. In contrast to the study by Amos *et al*, the current study did not find any significant correlations between the number of 3GC-resistant PCEs or 3GC-resistant *E. coli* at either site ($p > 0.05$) suggesting that *initI1* is not a suitable predictor for monitoring 3GC-resistant coliforms.

The current study investigated the correlation of the *intI1* gene with the other ARG targets to investigate if this gene may be a more suitable predictor of other targets

in the river. Significant correlations were observed between the *intI1* gene and *ermF, tetM* and *qacE* suggesting this integrase gene may play some role in disseminating these genes, however the abundance of *intI1* was significantly higher than any other gene target and would therefore overestimate the abundance of these genes if used as a predictor.

5.3.3 Community analysis of sites 1 and 2 over the period of a year

16S amplicon sequencing was carried out to determine if there were significant changes in the bacterial diversity at the sampling sites over the year. Samples were split according to season, site, temperature, sunshine hours and rainfall to evaluate the importance of these factors in determining community diversity.

The medium and mean number of sequences per sample was 28172 and 189418 respectively. Rarefaction was chosen at 7775 because the smallest sample sequence number was 203 was too low to retain significant species diversity (Figure 5.9a). The Simpsons reciprocal diversity measure in non-rarefied communities ranged from 2.52-421.38. Communities rarefied to 203 Simpsons reciprocal diversity ranged from 2.52 to 166.84 and for communities rarefied to 7775 Simpsons reciprocal diversity ranged from 111.97 to 411.97, therefore justifying the rarefaction at 7775 with very little loss in diversity according to Simpsons reciprocal diversity measure.

Chao1 species richness measure was used to confirm 7775 as a suitable number to rarefy at (Figure 5.9b). Chao1 determines species richness based on the number of rare species. In the current study, one of the aims was to investigate abundance of *Enterobacteriaceae* in sediment communities, many of which are not indigenous sediment bacteria. Rarefying at 7775 did result in some species diversity loss but the implications of increasing sequence number would have resulted in omission of a high number of samples from the analysis (Figure 5.9).

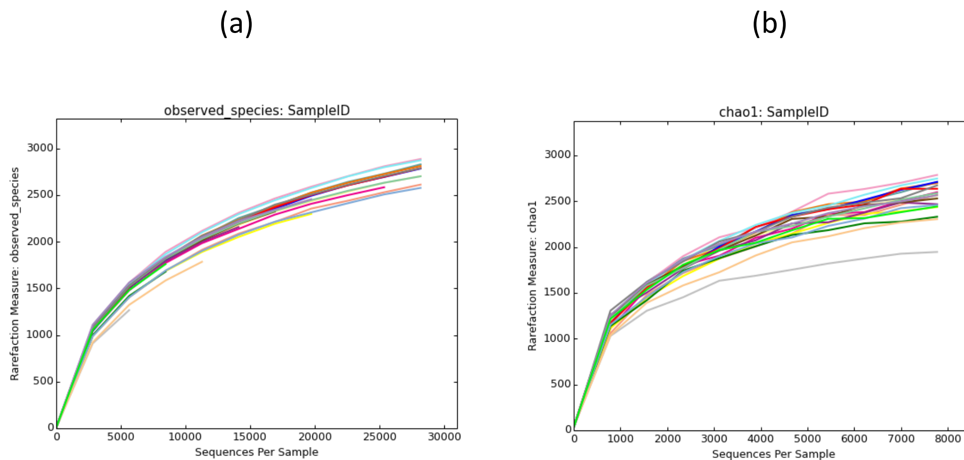


Figure 5.9 Number of species observed per sample. (a) observed species in non-rarefied communities and (b) number of rare species (as determined by Chao1 species richness measure) of communities rarefied at 7775.

5.3.3.1 Investigating alpha diversity of site 1 and site 2

The dominant phyla at both Site 1 and Site 2 was Proteobacteria (47.99 % at Site 1 and 45.01 % at Site 2). Other dominant phyla included Bacteroidetes (11.83 % at CSO and 13.50 % at Site 2), Actinobacteria (8.82 % at CSO and 9.85 % at Site 2), Chloroflexi (7.70 % at CSO and 8.24 % at WWTP) and Acidobacteria (4.76 % at CSO and 2.75 %). The largest difference in phyla was observed between abundance of the Firmicutes (3.1 % at Site 1 and 7.3 % at Site 2). Higher prevalence of all dominant phyla (except Proteobacteria) was observed at Site 2. Small differences were observed between site with respect to the prevalence of Gammaproteobacteria (11.14 % at Site 1 and 10.11 % at Site 2) (Figure 5.11).

There was no significant difference in alpha diversity in rarefied communities between site ($t = -0.76$, $p = 0.49$). *Enterobacteriaceae* populations were not significantly different between sites ($p > 0.05$).

The core microbiome of sites was determined to investigate if core diversity was consistent between the two sites. 90 % of samples had a core microbiome of 787 OTUs at Site 1 and 752 OTUs at Site 2 the majority of which were present in both sample types suggesting highly similar communities at both sites.

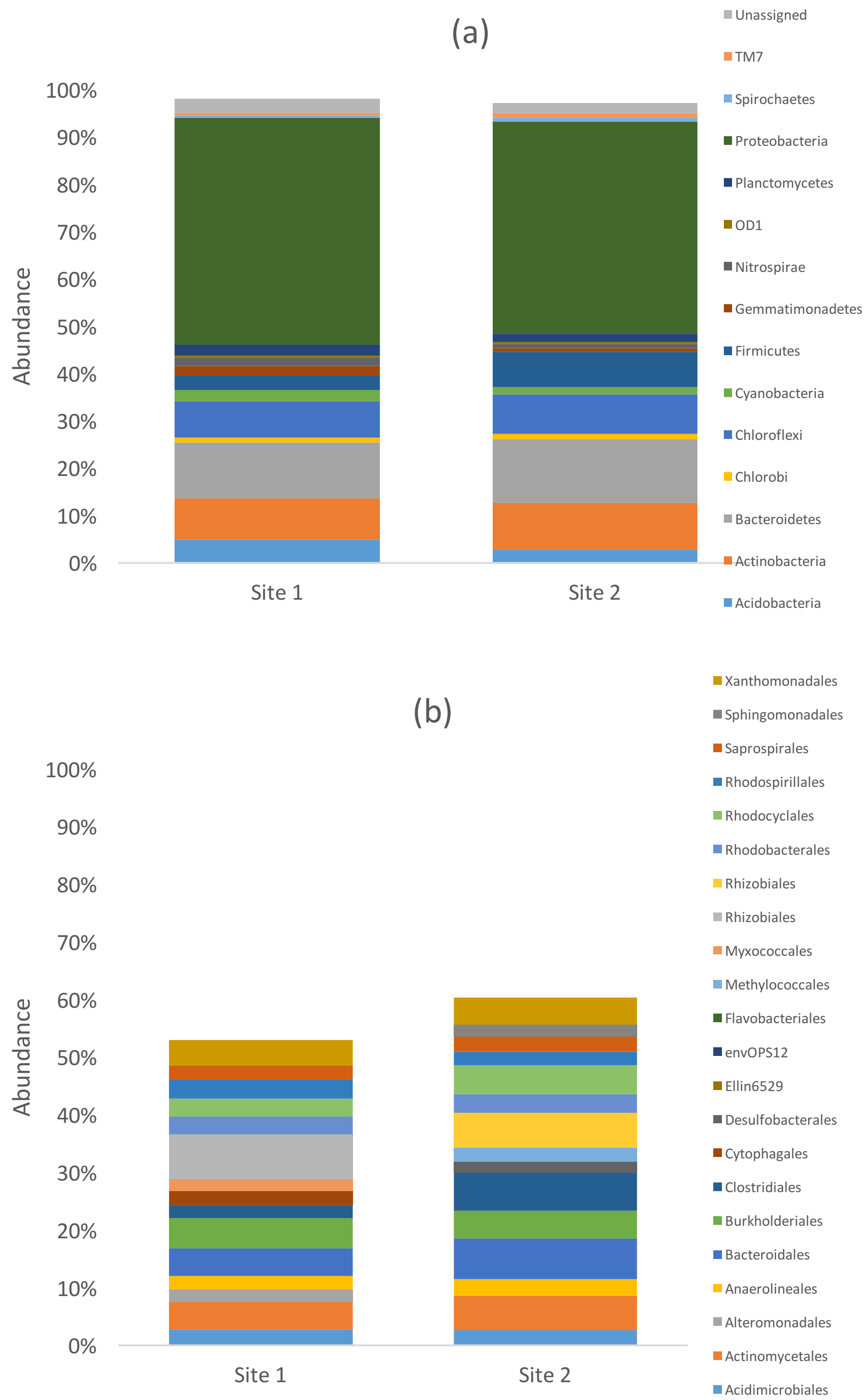


Figure 5.10 Relative abundance of the top 15 most abundant OTUs from sites 1 and 2. (a) phyla and (b) order.

5.3.3.2 Investigating alpha diversity to compare seasonal effects on community structure

The dominant phyla recorded for each season was Proteobacteria (Spring 43.43 %, Summer 48.98 %, Autumn 48.56 % and Winter 46.87 %). Other Phyla with relatively high prevalence's were Bacteroidetes (Spring 13.02 %, Summer 11.33 %, Autumn 14.31 %, Winter 14.00 %), Actinobacteria (Spring 9.65 %, Summer 9.09 %), Autumn 8.01 %, Winter 11.32 %), Chloroflexi (Spring 9.10 %, Summer 8.73 %, Autumn 6.28 %, Winter 6.08 %) and Acidobacteria (Spring 2.61 %, Summer 4.43 %, Autumn 4.6 %, Winter 2.90 %). The most significant difference in Phyla when investigating seasonal change was for Firmicutes prevalence with Spring showing over double the prevalence compared to Autumn (Spring 7.78%, Summer 3.83 %, Winter 6.89 %, Autumn 3.00 %). The prevalence of the dominant Phyla showed no obvious pattern with some increasing in Winter and some greatest in Summer. Within the Proteobacteria, the Gammaproteobacteria did not vary greatly (Spring 9.8 %, Summer 11.7 % Autumn 10.6 % and Winter 10.5 %) (Figure 5.12).

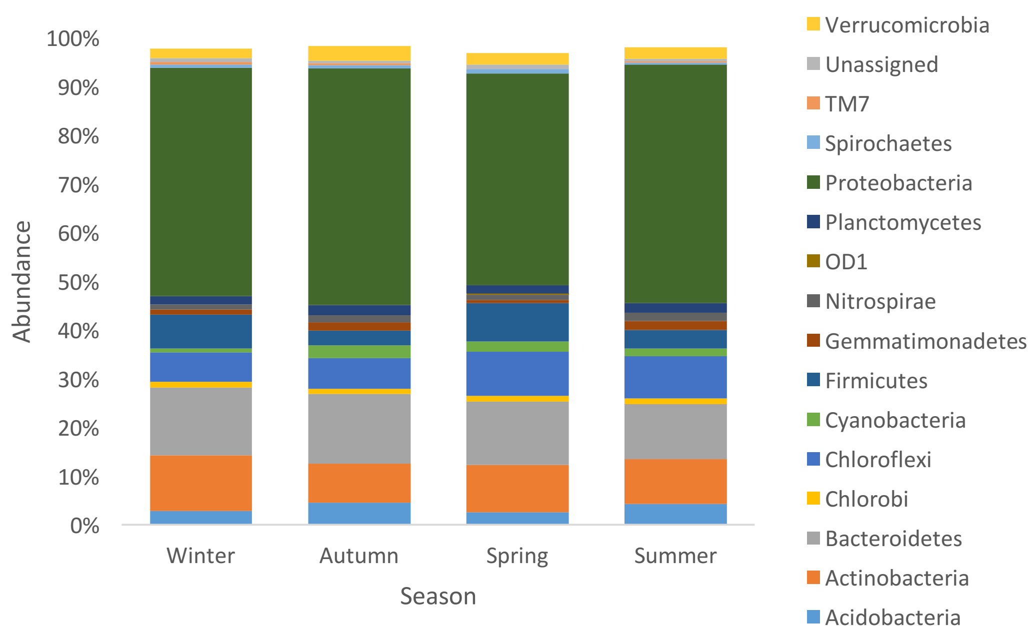


Figure 5.11 Relative abundance of the top 15 most abundant phyla recorded at each season.

Rarefied communities displayed no significant difference between alpha diversity with season, rainfall, sunshine hours or temperature for each pair of samples ($p > 0.05$).

5.3.3.3 Investigating changes in beta diversity with site, season, temperature, rainfall and sunshine hours

Unweighted PCoA plots, generated using rarefied (at 7775 sequences) communities, showed samples clustered based on sample site only, suggesting sample differences are likely due to differences in taxa between sites (Figure 5.13).

Weighted communities showed clustering based on season and rainfall suggesting differences in samples are related to taxa abundance than different taxa (Figure 5.14) prevalence. Site also showed separation in weighted communities suggesting that both prevalence of taxa and taxa abundance are important factors between site 1 and site 2.

ANOSIM analysis of unweighted communities showed that in rarefied communities there were significant differences between site communities ($R = 0.17$, $p = 0.016$), although difference is considerably small. For samples separated according to season ($R = 0.076$, $p = 0.132$) and temperature ($R = -0.104$, $p = 0.776$), rainfall ($R = 0.077$, $p = 0.137$) and sunshine hours ($R = 0.077$, $p = 0.162$) there was no significant variation in community structure as determine by ANOSIM analysis.

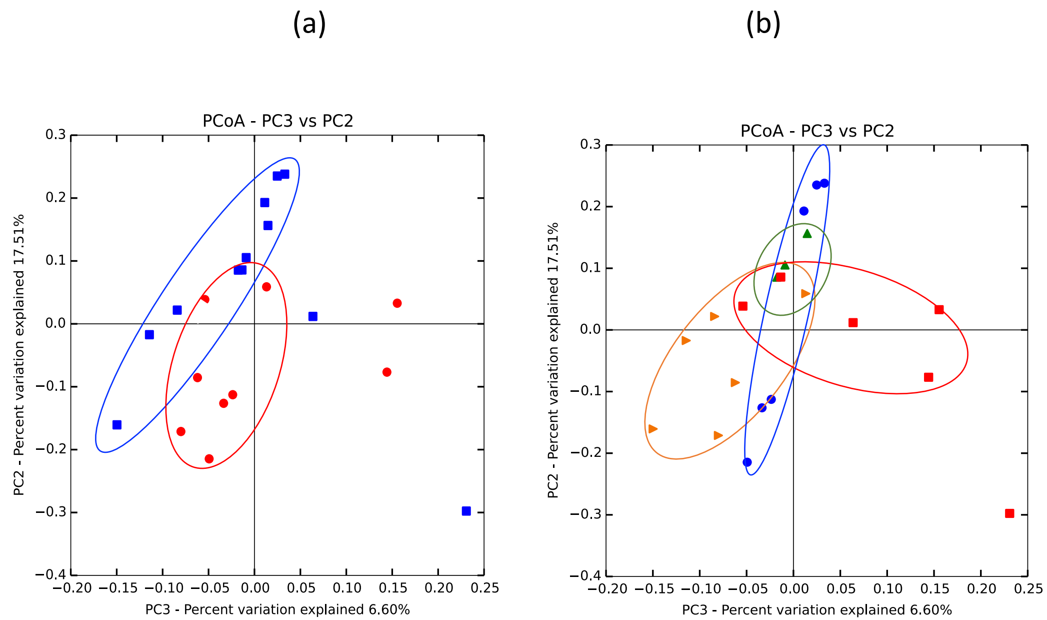


Figure 5.12 Unweighted PCoA plots for samples separated according to site and season. (a), red = Site 1, blue = Site 2; and season (b) blue = Spring, Orange = Summer, Red = Autumn and Green = Winter

Investigating the weighted community difference due to changes in the relative taxon abundance using ANOSIM revealed significant difference between site (R = 0.3444, p = 0.001), season (R = 0.1646, p = 0.028), rainfall (R = 0.1646, p = 0.035) and sunshine hours (R = 0.1646, p = 0.023), however only site clustered most substantially with only a small level of clustering according to different factors. No significant difference was found for temperature (R = -0.133, p = 0.8777).

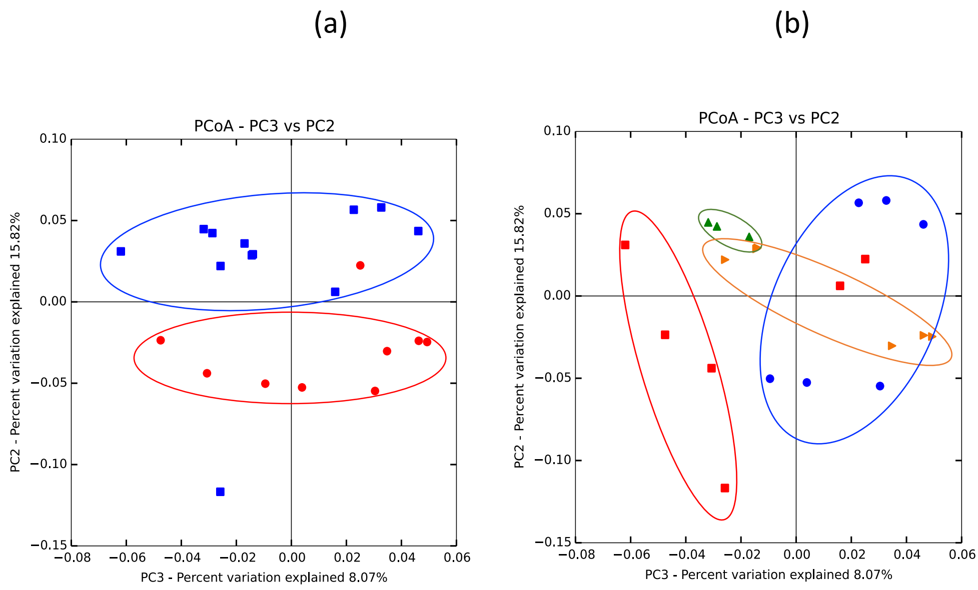


Figure 5.13 Weighted PCoA plots for samples separated according to site and season. (a) blue = Site 2, red =Site 1; season (b) blue = Spring, Orange = Summer, Red = Autumn and Green = Winter; Temperature

5.4 Discussion

Studies investigating seasonal impacts on ARB and ARG, resulting from increased precipitation and consequent CSO spills, are underrepresented in the literature (Caucci, 2016b, Dealtry et al., 2014, Knapp et al., 2012, Sui et al., 2015). Most of the published work investigating WWTPs as a source of ARG and ARB pollution has focussed on the prevalence in bio-solids from digesters and effluents, usually overlooking CSOs as a reservoir (Amos et al., 2014). In the current study sediment was investigated to examine the prevalence of ARG, the AMR CGNF and the total bacterial community to evaluate differences between two sites; one impacted by raw sewage release from a CSO (site 1) and the other impacted by treated effluent release (site 2). The current study hypothesised that site 1 would have a higher ARG and ARB prevalence than site 2 and that prevalence would be influenced by increased precipitation in Winter months.

The only gene target found at significantly higher prevalence at site 1 than site 2 was $bla_{CTX\text{-}M\text{-}1}$ which correlated with the prevalence of *E. coli* and *qnrS* however no significant differences between site were recorded for these two targets. Although site was not an important factor in determining prevalence, season was important with significantly higher numbers of *E. coli* and *qnrS* in Autumn/Winter months compared to Spring/Summer. The number of fluoroquinolone antibiotics detected in sludge has previously been shown to correlate with season presenting higher numbers in Winter months than Summer which will select for *qnrS* (Baquero et al., 2008, Sun et al., 2012, Suda et al., 2014, EXASOL, 2015, Caucci, 2016b). The increased selection pressures may indirectly select $bla_{CTX\text{-}M\text{-}1}$ in strains of *E. coli*. The association of *E. coli* carrying *qnrS* and $bla_{CTX\text{-}M\text{-}1}$ has been increasingly prevalent in the clinic due to carriage of fluoroquinolone and 3GC resistance conferring genes on MGEs, particularly in the human pandemic strain ST131 O25:H4 (discussed further in Chapter 6) (Ewers et al., 2010, Dhanji, 2011, Can et al., 2015, Zhang et al., 2011).

Detecting *E. coli* as a result of CSO spills and WWTP effluent release has previously been recorded with spikes of *E. coli* after CSO release events and frequent detection of *E. coli* occurring downstream of WWTP effluent release (Madoux-Humery, 2015,

Kotlarska et al., 2015, Amos et al., 2014). Season significantly influenced abundance of *E. coli,* both in plate counts and qPCR for sites 1 and 2 which further reinforces the impact of WWTP discharges on the river environment suggesting new measures must be reinforced to prevent further contamination and consequent dissemination of clinically important ARGs.

MGE often carry additional accessory genes enabling long-term persistence which may in part explain why seasonal variation was not responsible for variations in gene abundance of some gene targets (Cohen et al., 2013, Marcusson et al., 2005, Fricke et al., 2009, Gelens et al., 2013, EXASOL, 2015). Investigating MGE carried by *E. coli* isolated from river sediment will ultimately aid the understanding of the potential mechanisms involved in the possible long-term persistence of non-indigenous multidrug-resistant species in the environment and is further investigated in Chapter 6.

The number of ARB as a result of accretion and constant inflow in the environment must be considered when investigating the river as a potential reservoir of AMR. The current study demonstrated that seasonal effects were more important at site 1 than site 2 suggesting that the constant flow of treated effluent in to the river maintains a stable population of ARB whereas the CSO release events resulting from seasonal spikes of high rainfall result in changes at site 1.

It is unknown to what extent sediment taken directly downstream of effluent release is actually attributable to this release or if the diluted effects from CSO release events are in fact more important. WWTPs do not monitor the release of antibiotics and biocides in to the rivers, consequently they are frequently detected in receiving rivers and will induce selective effects (Bengtsson-Palme, 2016, DEFRA, 2012). Further to this, the EA specifies that CSO release events are monitored (Robert Huxham, ,SevernTrent Water, personal correspondence, 13th July 2016) yet the WWTP in the current study has no records of the number of release events occurring within the sampling regime so it cannot be concluded from this current study which factors are most important in determining ARG and ARB numbers.

Significantly greater prevalence of *ermF* and *tetM* was recorded at site 2 than site 1 with correlations between these two genes also reported. Erythromycin and tetracycline resistance genes are often carried on broad host range conjugative elements however previous correlations between erythromycin and tetracycline resistance genes are typically between *ermF* and *tetQ* and *ermB* and *tetM* and no studies investigating wastewater have previously found an association between *ermF* and *tetM* (Roberts, 1999a, Sui et al., 2015, Zhou et al., 2009).

Seasonal variation was important in explaining prevalence of *ermF* at site 1 but not site 2 suggesting CSO release events are likely involved in contributing to the prevalence. Consistent with Chapter 4, the prevalence of *ermF* was low throughout the sampling regime further supporting the suggestion made in Chapter 4 that *ermF* may not be primarily responsible for the dissemination of erythromycin resistance.

Seasonal changes explained prevalence of *tetM* at site 2. Tetracyclines are highly stable and not readily metabolised resulting in up to 60 % excreted and consequently found in WWTPs (Agwuh and MacGowan, 2006). The stability of these antibiotics results in detection in effluent which may increase *tetM* prevalence from constant selective pressures (Agwuh and MacGowan, 2006, Gao, 2012).

It has previously been suggested that the class 1 integron integrase gene, *intI1,* may be a suitable marker for anthropogenic pollution and might predict the environmental resistome (Gillings et al., 2015, Amos et al., 2015). Consistent with previous studies a high prevalence of *intI* was reported at both sites in the current study with significantly higher prevalence of *intI1* than all other gene targets (Table 5.3). No significant differences were identified in *intI1* prevalence or abundance in river sediments from site 1 and site 2 and no correlations between *intI1* prevalence and 3GC-resistant coliforms were identified inconsistent with findings by Amos *et al* who predicted 3GC-resistant coliforms based on the prevalence of *intI1* (Amos et al., 2015). The only significant correlation with *intI1* was *qacE* which was expected considering clinical class 1 integrons are characterised by carriage of *qacE*Δ*1* (Barraud

and Ploy, 2015). The current study suggests that the class 1 integrase gene is not a suitable marker for anthropogenic pollution or a predictor of the resistome as it will overestimate contamination. The same sites tested by Amos *et al* were tested in the current study so it is surprising that correlations that were previously detected were not detected presently. However, the results presented in the current chapter are consistent with Chapter 4 and suggest that *intI1* cannot be used as a predictor within the three rivers tested to determine AMR.

Table 5.3 Prevalence of *intI1* in water and sediment samples taken WWTP effluent impacted rivers

Source	Copies	Reference
Downstream WWTP	~10^7 per litre	(Stalder et al., 2014)
WWTP Effluent	~10^9 per litre	(Stalder et al., 2014)
WWTP effluent	~10^6 per ml	(Ma et al., 2013)
WWTP effluent	~10^7 per ml	(Du et al., 2014)
Sediment	~10^7 per ml	(Zhang et al., 2009a)
Tertiary treated Water sample	~10^4 per ml	(LaPara et al., 2011)
River sediment	3.8 x 10^4 copies/10^6 16S rRNA gene copies	(Berglund et al., 2014)
Water samples	10^{6-}/10^6 16S rRNA gene copies	(Khan et al., 2013)
WWTP sludge	$10^{6.95}$ -$10^{7.85}$ copies per gram	(Wen, 2016)
River sediment	10^{-3} - 10^{-2} /16S rRNA gene copies	(Wright et al., 2008)
CSO impacted river sediment	~10^6 copies per gram ~10^{-2} copies/16S rRNA gene copies	This study
WWTP impacted river sediment	~10^7 copies per gram ~10^{-2} copies/16S rRNA gene copies	This study

There have been contrasting reports of changes in ARG prevalence with season with some reporting prevalence is greatest in the dry season and some in the wetter months (Su et al., 2014a, Knapp et al., 2012). Seasonal variation of AMR *E. coli* has

been observed to increase in Autumn/Winter months compared to Spring/Summer consistent with results presented in the current study (Sun et al., 2012, Wertheim et al., 2009). Increased prescription in the Winter months is likely to be involved in the increased AMR *E. coli* (December 2014 saw a 59 % increase in prescription compared to July 2014 in the UK) due to increasing levels of ARG from direct selection in the human gut (EXASOL, 2015, Achermann et al., 2011, Caucci, 2016b, Elseviers et al., 2007, Suda et al., 2014, Sun et al., 2012, Lester et al., 2006, Trobos et al., 2009, Cremet et al., 2012, Karami et al., 2007, Goren et al., 2010, Hu et al., 2016). The combination of increased ARG and precipitation in the Winter months (resulting in CSO release of raw sewage) will result in higher environmental ARB (Jalliffier-Verne, 2016). Closer stewardship of prescription and monitoring of storm drains release should therefore be performed to reduce environmental resistance.

Community structure was remarkably similar in both sites and not significantly impacted with exception of the elevated Proteobacteria prevalence at site 1. The most dramatic difference was observed in the Firmicutes, with the greatest difference within the class Clostridia with a 3.4 % increase at site 2 compared to site 1 which may be attributable to the process of anaerobic digestion in the wastewater treatment process selecting for spore forming bacteria (Yutin and Galperin, 2013).

This study shows that community structure, presence of resistant coliforms and ARG levels in river sediment varies with season and site. Seasonal variation therefore must be considered in future sampling regimes which may currently under or overestimate resistant pathogens depending on sampling time. Previous studies monitoring seasonal change due to WWTP impact have primarily focussed on the water column which presents a measureable risk but work here suggests that exposure and risk assessments should take in to account the river sediment as it may provide a more substantial risk (Leonard et al., 2015, Giannoulis et al., 2005, Coffey et al., 2007, Yillia et al., 2009, Jacob et al., 2015). Current estimates predict 8 % of *E. coli* within river samples are pathogenic sequence types and that resuspension of sediment in to river water increases total *E. coli* count by up to 2 log measures (Garcia-Armisen et al., 2014, Muirhead et al., 2004). The shifts in abundance of ARB

and ARG observed between season are important in future predictions of anthropogenic effects of environmental contamination induced by CSO and WWTP effluent impact.

To summarise, this study provided insight in to seasonal variation of ARG and ARB and highlighted the importance of monitoring the release of untreated wastewater from CSOs. It provides useful understanding of the importance of seasonal release from CSOs and suggests further work in this area is required to understand full implications of release events.

Chapter 6

Diversity within genomes of selected *E. coli* isolates

6.1 Introduction

E. coli is a commensal inhabitant of the gastrointestinal tract, however there are several pathotypes responsible for one of the most important nosocomial-acquired and community-acquired human infections of the 21st century (Kaper et al., 2004, Prevention, 2013). The acquisition of the IncF type plasmids has allowed *E. coli* to develop resistance to an extensive range of antibiotics and due to their highly promiscuous nature had aided the rapid dissemination of clinically important resistance genes resulting in untreatable infections (McGann et al., 2016, Yang et al., 2015, Phan et al., 2015, Partridge et al., 2011b). The rise of ESBL producing *E. coli* as a direct result of the global dissemination of the $bla_{CTX\text{-}M\text{-}15}$ gene on the IncF plasmids has led to an accelerated level of resistance within this species also acquiring the recently reported mobilisable *mcr-1* gene conferring resistance to the last-resort colistin antibiotics (Prevention, 2013, McGann et al., 2016).

Virulence between *E. coli* strains is highly variable with some strains able to cause infection with a dose as low as 10 and some requiring 10^8 (Kaper et al., 2004). There are an extensive number of virulence genes found in *E. coli,* some associated with

specific pathotypes (such as Shiga-toxin producing EHEC strains) and some are found commonly in most *E. coli,* for example siderophore encoding genes. One of the most important human pathotypes of *E. coli* are the opportunistic UPEC strains which cause UTI infections and have become increasingly difficult to treat due to plasmid acquired AMR. The acquisition of AMR and increased virulence factors within *E. coli,* allowed widespread, difficult-to-treat infections in the clinic. The acquisition of virulence factors in *E. coli* has arisen from pathogenicity islands, phage and plasmids. Virulence factors that have been acquired through these MGE include intimin (*eae*) which is encoded on a ~35 kb pathogenicity island, Shiga toxins (*stx1* and *stx2*) which are carried on phage, and enterohaemolysin (*ehly*) and catalase *katP* which are carried on a ~90 kb plasmid (Reid, 2000). The carriage of virulence encoding plasmids is a typical characteristic of APEC strains which typically carries increased serum resistance, temperature-sensitive haemagglutination, adhesins and iron scavenging and transport mechanisms (Tivendale et al., 2009).

Environmental AMR-*E. coli* has previously been reported in water samples and at the sampling sites investigated in the current study (Anastasi et al., 2012, Kotlarska et al., 2015, Roe et al., 2003, Koczura et al., 2013, Bonetta et al., 2016, Dhanji, 2011, Franz et al., 2015, Amos et al., 2014). Despite the many reports of *E. coli* prevalence, the pathogenic potential of these environmental resistant *E. coli* has not been well studied (Franz et al., 2015). One study however did reported 60 % of ESBL-producing *E. coli* isolated from a river were potential pathogens highlighting the potential threat the environment may pose to human health (Jang et al., 2013).

The aim was to investigate resistance and virulence profiles from potentially pathogenic *E. coli* isolated from the river sediment taken seasonally. Samples were taken at the same time points specified in Chapter 5 from the river Sowe with the aim to determine if population increased from seasonal variation resulted in different ST types of *E. coli* in sediment. 77 *E. coli* isolates were purified on HiCA media by picking blue colonies and streaking to purity. Whole genome sequencing was carried out and strains were investigated for ARG, plasmid carriage, virulence

genes and associated persistence genes to attempt to evaluate the possible impacts of these *E. coli* in sediment.

6. 2 Aims

The aim of the current study was to investigate AMR profiles and virulence profiles of 77 *E. coli* isolates from river sediment to evaluate the potential risk associated with ingestion. Investigating the extent of AMR within potentially clinically important pathogens will improve the understanding of WWTP effluent impact on the river and elucidate if disruption of sediment could lead to difficult-to-treat infections. The transmissibility of resistance genes was also evaluated by investigating MGE carriage to determine the potential dissemination that could occur within the sediment.

1. Evaluate if *E. coli* isolates are of the same sequence type at each sampling time point and whether strains are more associated with human or animal infection.
2. Identify ARG and virulence factors within each strain.
3. Determine which plasmids are present within strains and other MGE including transposons and integrons.

6.3 Results

6.3.1 Phenotypic testing of 77 *E. coli isolates*

Each isolate was tested for phenotypic antibiotic resistance against 6 different classes of antibiotics; β-lactam (ampicillin, cefotaxime and imipenem), tetracycline, macrolide (erythromycin), chloramphenicol, sulphonamide (sulphafurazole) and quinolone (nalidixic acid) using disc diffusion assays.

Of the 77 isolates 91 % were phenotypically resistant to ampicillin, 77 % resistant to cefotaxime, 81 % tetracycline, 63 % chloramphenicol, 97 % erythromycin, 91 % sulphafurazole, 81 % resistant to nalidixic acid and none were resistant to imipenem. 51 % of isolates were resistant to all antibiotics tested except imipenem. Individual strain phenotypic profiles can be found in Appendix 1.

There were 22 isolates that were isolated with no antimicrobial selective agent. Only one isolate showed no phenotypic resistance to any of the antibiotics tested. 67 % of these isolates were phenotypically resistant to at least six of the antibiotics tested.

6.3.2 Illumina sequencing, summary statistics for initial sequencing

Genome size of the 77 *E. coli* isolates ranged from 4400792 to 6098924 with an average of 5096399 bp. Rough assemblies were generated using the A5-miseq pipeline and were carried out at UTS. The number of contigs for each rough assembly ranged from 55 to 1199 with N50 ranging from 10025 to 566858. The average median coverage was 48.5. Summary statistics for each strains sequences are found in Appendix 2.

6.3.3 Phylogenetic analysis of Sowe *E. coli* isolates

The A5-pipeline was used to provide rough assemblies for each genome from the raw Illumina data. The pipeline involves 5 steps: 1) read cleaning involving removal of sequence adapters and low-quality regions by Trimmomatic and correction of errors using SGA's algorithm; 2) contig assembly involving paired and unpaired reads for assembly using the IDBA-UD algorithm, 3) crude scaffolding; 4) missassembly correction to detect read pairs that do not map with expected distance and 5) final

scaffolding to repair any previously broken contigs from step 4 (Coil, 2015). Using the assemblies generated by the A5-pipline, phylogenetic analysis using the phylotyping method updated by Clermont *et al.* was performed to investigate evolutionally relationships based on phylogenetic inferences of strains within the River Sowe (Clermont et al., 2013). Phylogrouping is based on the screening of 4 genes; *arpA* (encodes a Ankyrin-like regulatory protein), *chuA* (encoding an outer membrane haemoglobin receptor), *yjaA* (conserved protein with unknown function) and TspE4.C2 (encodes a putative lipase esterase gene), differential presence/absence of these genes defines the phylogroup to which *E. coli* strains belong to (Clermont et al., 2013). MLST was also carried out to determine the sequence type of *E. coli* isolate. MLST of *E. coli* is based on seven housekeeping genes: *adk, fumC, gyrB, icd, mdh, recA* and *purA*. Different polymorphisms in these seven genes determine the sequence type (Wirth et al., 2006). Serotyping of strains was conducted through BLAST searches for O and H antigens. Best hit was recorded as true antigen type. These combined methods of identification were used the *E. coli* isolates to ensure best characterisation of species was made.

32 different ST types were isolated over the year. The most prevalent ST types were ST940, ST3202 and ST744 (14, 14 and 11 isolates respectively). Other ST types isolates included ST46, ST69, ST1421, ST73. The most common phylogroups were A and B1 (25 and 22 isolates respectively).

To investigate relatedness of strains, PhyloSift analysis was performed to generate phylogenetic relationships (Darling et al., 2014). PhyloSift combines LAST, HMMER, and pplacer to investigate phylogenetic relationships through analysis of protein coding and RNA sequences. It used 37 "elite" (nearly) universal single-copy gene families representing approximately 1 % of the average bacterial genome (Darling et al., 2014). In addition to these 37, 16S and 18S rRNA genes, mitochondrial gene families, Eukaryote-specific gene families and viral gene families are included in the Phylosift database (Darling et al., 2014). FastTree was used to generate the phylogenetic tree (Price, 2010)(Figure 6.1). FastTree generates trees based on

minimum-evolution subtree-pruning-regrafting and maximum likelihood (Price, 2010).

Four main clusters of *E. coli* were observed (Figure 6.1 designated A, B, C and D). Strains carrying the $bla_{CTX\text{-}M\text{-}1}$ clustered together in two groups (Figure 6.1, groups A and C), with clusters predominantly consisting of ST940 and ST131. Predominantly strains carrying $bla_{CTX\text{-}M\text{-}1}$ were isolated from the February 2015 and May 2015 sampling (14 and 10 isolates respectively) with 2 isolates from July 2015 and 1 from December 2015. No $bla_{CTX\text{-}M\text{-}1}$ carrying strains were isolated from the February 2016 sampling time point. Cluster A, which consisted of the ST940 strains clustered separately from all other strains in the current study and did not show any relationship with the reference strains (Figure 6.1 and Table 2.7).

Cluster B consisted of human related opportunistic strains such as ST46, ST1421 and ST10 (also associated with animal infections). This cluster was diverse in ST types but showed sequences of the same ST generally clustered together. Some strains were identified to carry the ESBL $bla_{CTX\text{-}M\text{-}15}$ gene but prevalence of this gene was not as high in isolates compared to Cluster A.

No isolates in Cluster D were characterised to carry the $bla_{CTX\text{-}M\text{-}15}$ gene and many of the ST types in this cluster were unknown. Interestingly this cluster did contain an ST46 which was clustered away from the other ST46 identified in this study, which was grouped in cluster B with other pathogenic strains. Cluster D did not contain any other known pathogenic ST types demonstrating the relationship between pathogenic ST types and the carriage of $bla_{CTX\text{-}M\text{-}15}$.

2.0

101* ST940 WWTP May-15
109* ST940 WWTP May-15
51 * ST940 WWTP Feb-15
75 *ST940 WWTP May-15
77* ST940 WWTP May-15
79 * ST940 WWTP May-15
81 ST940 WWTP May-15
89 * ST940 WWTP May-15
92 * ST940 WWTP May-15
95 * ST940 WWTP May-15
96* ST940 WWTP May-15
99 * ST940 WWTP Feb-15
72 * ST940 WWTP May-15
67 * ST940 WWTP Feb-15
42* ST455 WWTP Jul-09
30 ST212 WWTP Dec-15
33 ST3576 CSO Dec-15
34 ST295 WWTO Dec-15
104 ST155 WWTP Jul-15
76 ST58 CSO Jul-15
26 ST453 WWTP Dec-15
E24377A
32 ST162 CSO Dec-15
70 ST154 WWTP Jul-15
35 ST1431 CSO Dec-15
O26:H11
O111:H-
O103:H2
125 ST3574 CSO Feb-16
129 ST3202 CSO Feb-16
S. boydii
S. sonnei
S. flexneri
S. flexneri

A

60 ST4105 WWTP Dec-15
41* ST1421 WWTP Feb-16
62* ST1421 WWTP Feb-16
63* ST1421 WWTP Feb-16
78* ST1421 WWTP Jul-15
80 ST1421 WWTP Feb-15
65 ST1421 WWTP Feb-15
98* ST1421 WWTP Jul-15
121 ST46 WWTP Feb-16
122 ST46 WWTP Feb-15
123 ST46 WWTP Feb-15
50* ST3202 WWTP Feb-15
64 ST3202 WWTP Feb-15
87* ST3202 WWTP Feb-15
103 ST3202 CSO Jul-15
140* ST46 WWTP Feb-16
124 ST399 WWTP Feb-16
38 ST399 CSO Dec-15
39 ST399 WWTP Dec-15
36 ST607 WWTP Dec-15
59 ST607 WWTP Dec-15
134 (Unknown ST) WWTP Feb-16
29* ST46 WWTP Dec-15
119 ST10 WWTP Feb-16
K-12 MG1655
K-12 W3110
4 ST1286 WWTP Dec-15
131 ST744 WWTP Dec-15
UMNK88
143 (unknown ST) WWTP Feb-16
71 ST716 CSO Jul-15
O157:H7
O157:H7 Sakai
O55:H7 EPEC
O55:H7 EPEC
127 ST69 WWTP Feb-16
37 ST69 WWTP Dec-15
042

B

47* ST38 WWTP Feb-15
40 ST131 CSO Dec-15
ST131 CD306
ST131 EC958
ST131 JJ1886
ST31 NA114
61* ST131 CSO Dec-15
ST131 ZH063
ST131 JJ1897
102 ST131 WWTP Jul-15
48* ST131 WWTP Feb-15
66* ST131 WWTP Feb-15
128 ST582 CSO Feb-16
E2348
142* ST73 WWTP Feb-16
CFT073
115 ST80 WWTP Feb-16
UTI89
536
F82

C

85 ST69 WWTP Jul-15
130 ST3574 WWTP Feb-16
28 (unknown ST) CSO Dec-15
117 ST3747 WWTP Feb-16
31 ST4105 CSO Dec-15
27 ST3727 CSO Dec-15
25 (unknown ST) CSO Dec-15
86 ST3568 WWTP Jul-15
106 (unknown ST) WWTP Jul-15
120 ST5128 WWTP Feb-16
112 (unknown ST) WWTP Feb-15
135 ST46 WWTP Feb-16

D

Figure 6.1 Midpoint rooted phylogentic analysis of the 77 *E. coli* strains isolated from the river Sowe with site and date isolated. Generated using Phylosift (Darling et al., 2014)and FastTree (Price, 2010). The scale is number of substitutions per site. Strains marked with an asterisk represent $bla_{CTX-M-15}$ positive. Strains in red are reference strains listed in Table 2.7

6.3.4 Plasmid carriage

To determine if *E. coli* strains had the potential to transfer ARG, the plasmid Inc groups were investigated to determine carriage in strains. The IncF type plasmids were found in ~75% of isolates. Other plasmids detected included IncI, IncH and unexpectedly IncN, IncP and IncU (Figure 6.2).

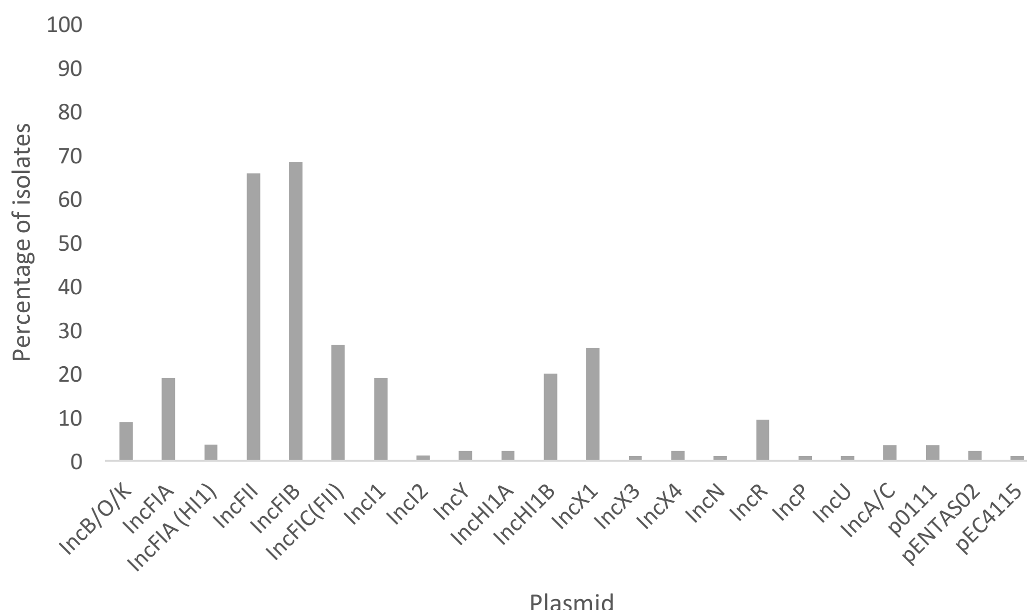


Figure 6.2 Prevalence of plasmids identified in the 77 *E. coli* isolates

6.3.5 Investigating *bla*$_{\text{CTX-M}}$ carrying *E. coli* isolates

35 % of the *E. coli* strain sequences possessed the 3GC resistance conferring genes *bla*$_{CTX\text{-}M\text{-}14}$ (1 isolate) and *bla*$_{CTX\text{-}M\text{-}15}$ (27 isolates). These 28 strains were further investigated to evaluate what resistance genes, virulence genes and persistence-associated genes were carried in these strains to evaluate the potential of these ESBL producing strains. The most common phylogroup detected was B1 (Table 6.1).

From the 77 isolates, six of them were typed as ST131. Three of these isolates carried *bla*$_{CTX\text{-}M}$ genes, two of which carried *bla*$_{\text{CTX-M-15}}$ (strains 48 and 66) and one carried both *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-99}}$ (strain 61). The ST type most commonly associated

with $bla_{CTX-M-15}$ was ST940. From the 77 isolates fourteen of these were ST940 and thirteen of these carried the $bla_{CTX-M-15}$ gene.

Table 6.1 MLST identification, serotyping and phylogrouping of bla_{CTX-M} positive *E. coli* strains

Strain identifier	Sequence type	O type	H type	Phylogroup
29	ST46	O8	H4	A
41	ST1421	O9	H4	A
42	ST940	O9	H5	B1
47	ST38	O1	H15	D OR E
48	ST131	O25	H4	B2
50	ST3202	O8	H21	A
51	ST940	NO HIT	H5	B1
61	ST131	O25	H4	B2
62	ST1421	O9	H4	A
63	ST1421	O9	H4	A
66	ST131	O25	H4	B2
67	ST940	NO HIT	H5	B1
72	ST940	NO HIT	H5	UNKNOWN
75	ST940	NO HIT	H5	B1
77	ST940	NO HIT	H5	B1
78	ST1421	O9	H4	A
79	ST940	NO HIT	H5	B1
87	ST3202	O8	H21	A
89	ST940	NO HIT	H5	B1
92	ST940	NA	H5	B1
95	ST940	NO HIT	H5	B1
96	ST940	NO HIT	H5	B1
98	ST1421	O68	H4	A
99	ST940	NO HIT	H5	B1
101	ST940	NO HIT	H5	B1
109	ST940	NO HIT	H5	B1
140	ST46	O9	H4	A
142	ST73	O6	H1	B2

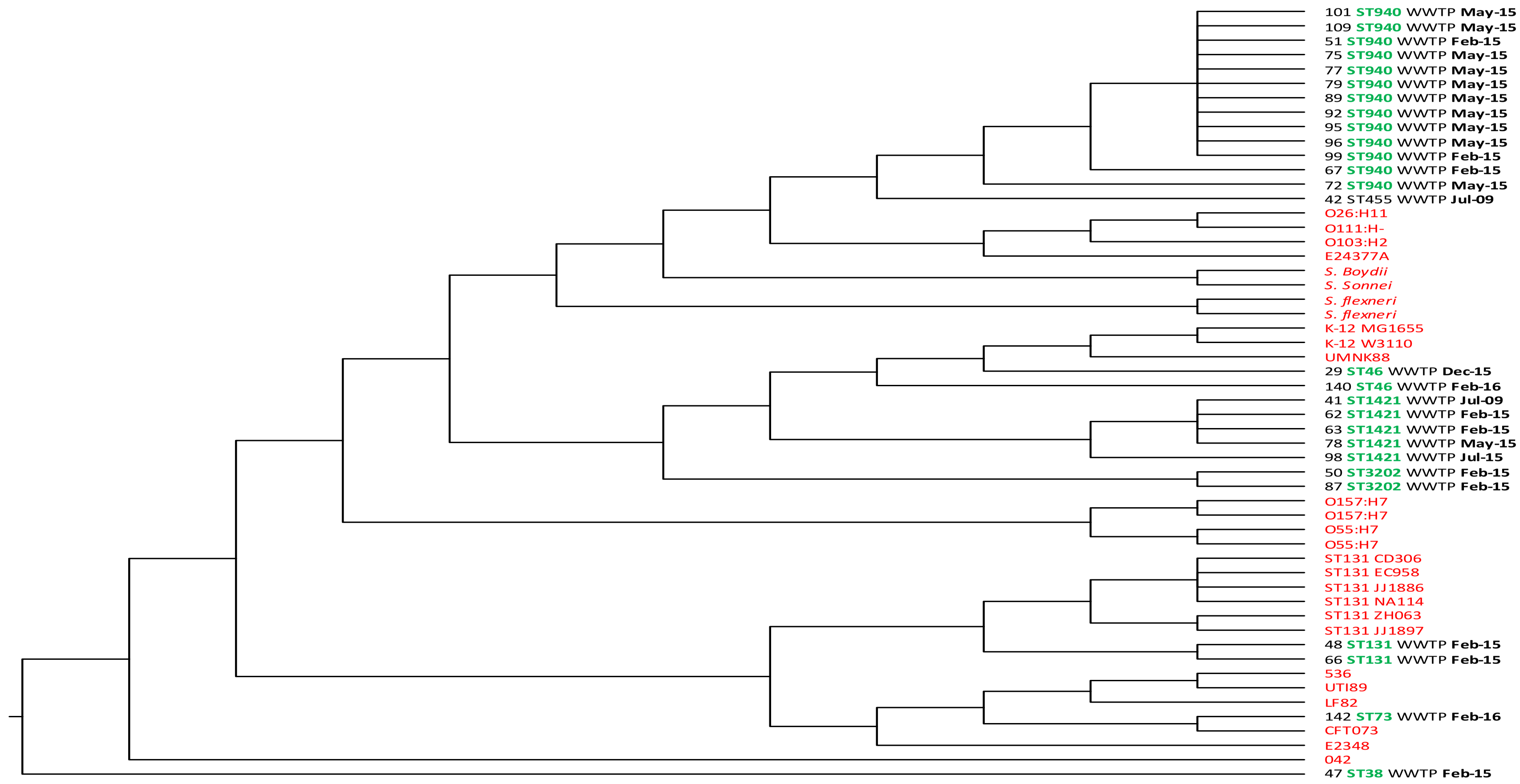


Figure 6.3 Midpoint phylogenetic analysis of *E. coli* strains possessing the $bla_{CTX\text{-}M\text{-}15}$ gene and reference strains. Generated using using Phylosift (Darling et al., 2014)and FastTree (Price, 2010) . Reference strains can be found in Table 2.7 and shown here in red. The scale is

6.3.6 MGE, including plasmid, transposon and integron, carriage in ESBL producing *E. coli*

The most abundant plasmids within strains carrying *bla*$_{CTX\text{-}M\text{-}15}$ and *bla*$_{CTX\text{-}M\text{-}14}$ were IncF type plasmids. 100 % of ESBL producing strains possessed IncFII and IncFIB (Figure 6.4). Other plasmids frequently detected were IncHI1B and IncX. IncI plasmids were less commonly detected than expected in the ESBL producing strains. A summary of MGEs carried by each strain can be found in Appendix 3. BLAST searches were performed to identify MGE within isolates. 100 % of isolates carried IS26 and the *intI1* and *intI2* genes were found in 50 % of isolate (Figure 6.4). All strains carried one integrase gene which was either the *intI1* or the *intI2* with no strains identified to carry both types of integron. ST940 and the closely related ST455 were responsible for the high prevalence of the *intI2* gene in the current study. Genes associated with the Tn6029 (*strA, strB, sul2* and *aphA1*) were detected in two isolates carrying ESBL genes (Table 6.2 strains 63 and 98), however due to the repetitive sequences of the IS26 which result in sequence breaks, it could not be determined if these strains did carry this transposon.

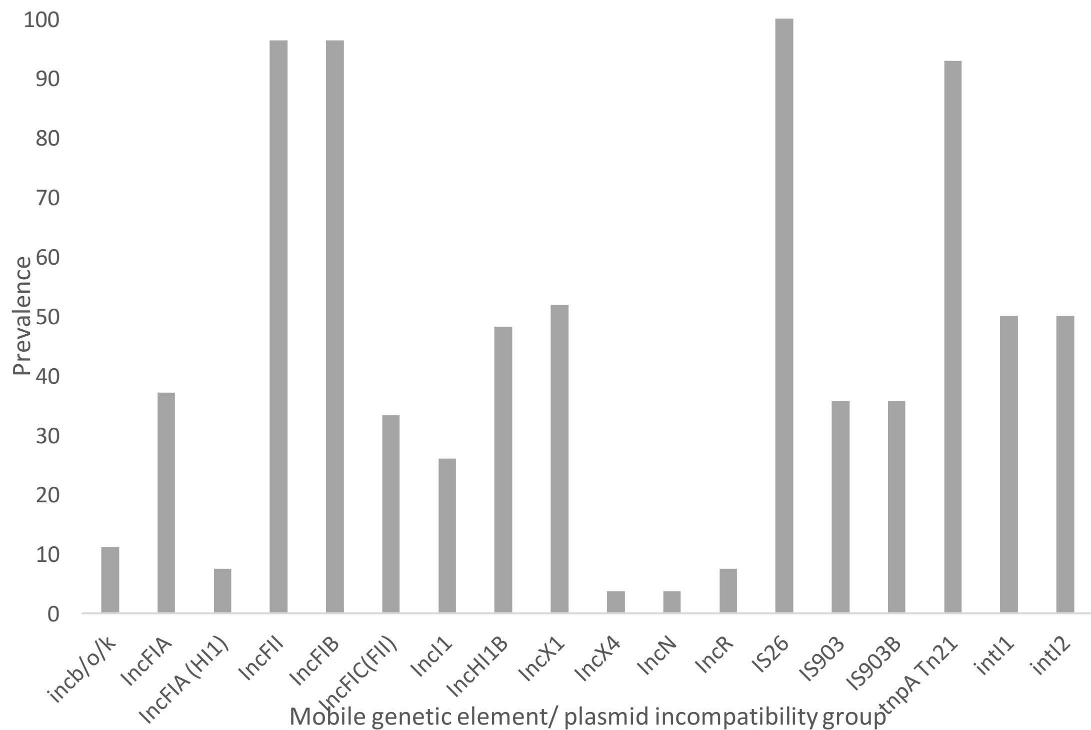


Figure 6.4 Plasmid Inc group and other MGE prevalence in the 28 isolates carrying the ESBL *bla_{CTX-M}* genes

6.3.7 Additional ARG in *bla_{CTX-M}* positive isolates

ARG were investigated in the 28 strains carrying ESBL genes to determine ARG profiles for each strain sequenced. All strains carried multiple resistance genes to a variety antimicrobial classes including quinolone, macrolide, tetracycline, sulphonamide, chloramphenicol, vancomycin, aminoglycoside, polymyxin and various multidrug efflux pumps. Antibiotic resistance genes investigated are shown in Appendix 4 and strain summaries for each gene identified are found in Appendix 5.

6.3.7.1 Quinolone and fluoroquinolone resistance

All 28 *$bla_{CTX-M-15}$* carrying strains, except isolate 42, were phenotypically resistant to nalidixic acid. 100 % carried the mutated *gyrA* gene conferring resistance to fluoroquinolones and 50 % also carried *parC.* Six strains carried additional quinolone resistance genes. *qnrS, qepA, qnrB* (strains 47, 48, 50, 61, 87, 140 and 142).

6.3.7.2 Macrolide resistance

All isolates were phenotypically resistant to erythromycin (8 μg/ml) however the only macrolide conferring resistant gene identified in this study was the *macB* gene which was identified in 21 of the 28 isolates. *macB* encodes an ATP binding cassette transporter which forms a complex with MacA and TolC to export 14- and 15-membered lactones from the cell. The marker gene *ermF* was not identified in any of the isolates analysed in the current study.

6.3.7.3 Tetracycline resistance

Tetracycline genes detected included *tetA, tetB, tetG, tetC* and *tetD* (found in 75 %, 71 %, 25 %, 4 % ad 54 % of isolates respectively). All 28 isolates carried at least one *tet* gene explaining phenotypic resistance to tetracycline (10 μg/ml) which was recorded for all strains. The suggested marker gene *tetM* gene was not carried in any of the ESBL producing strains.

6.3.7.4 Sulphonamide resistance

The 28 isolates showed phenotypic resistance to sulphafurazole (300 μg/ml). The *sul2* gene was the most prevalent sulphonamide resistance gene with 26 of the 28 strains carrying this gene. Three strains carried the *sul1* gene. All isolates carried at least one of the *sul* genes, explaining the phenotypic resistance.

6.3.7.5 Beta-lactam resistance

Isolate 92 showed resistance to ampicillin (25 μg/ml) but did not present phenotypic resistance to cefotaxime(5 μg/ml). The remaining 27 isolates displayed phenotypic resistance to ampicillin and cefotaxime. Strain 61 was the only isolate to carry $bla_{CTX\text{-}M\text{-}14}$, the other 27 carried $bla_{CTX\text{-}M\text{-}15}$. Additionally, bla_{oxa} bla_{tem} bla_{DHA2} bla_{CRP} $bla_{ACT\text{-}7}$ $bla_{CMY\text{-}63}$ and $bla_{CTX\text{-}M\text{-}99}$ genes were identified. Within the bla_{oxa} genes the predominant gene was $bla_{oxa\text{-}1}$, a narrow spectrum D-class beta-lactamase and $bla_{oxa\text{-}31}$. A diverse range of bla_{tem} *genes* were found including $bla_{tem\text{-}1}$, $bla_{tem\text{-}22}$, $bla_{tem\text{-}116}$, $bla_{tem\text{-}154}$ and $bla_{tem\text{-}190}$. All isolates carried at least 3 beta-lactamase genes with some

strains carrying 6: bla_{oxa} bla_{tem} bla_{DHA2} bla_{CRP} bla_{ACT-7} and $bla_{CTX-M-15.}$ The median number of beta-lactamase genes carried in one strain was 5.

6.3.7.6 Chloramphenicol resistance

Eighteen isolates were phenotypically resistant to chloramphenicol (30μg/ml). Of the ten that did not show phenotypic resistance, eight carried genes associated with chloramphenicol resistance. Overall 21 isolates carried genotypic resistance to chloramphenicols. The most prevalent of the chloramphenicol resistance genes was *cat* which was carried in thirteen of the 28 strains in the current study.

6.3.7.7 Polymyxin resistance

22 isolates carried polymyxin resistance genes. The mobilisable *mcr-1* gene was not detected in any strain but the chromosomal *arnA, pmr* and *phoPQ* genes were found in 64 %, 68 % and 4 % of isolates respectively.

6.3.7.8 Vancomycin resistance

Most Gram-negatives are intrinsically resistant to glycopeptides due to the inability to permeate their outer membrane, however the *vanG* gene was detected in ten of the 28 isolates.

6.3.7.9 Aminoglycoside resistance

Sixteen aminolgycoside resistance genes were identified in the 28 strains. The most commonly detected amininoglycoside resistance genes were *strA* and *strB* which were identified in 27 isolates (Figure 6.6). Isolates carried at least three different aminoglycoside resistance genes with a median value of eight aminoglycoside conferring resistance genes. Four strains carried ten genes. The carriage of the *aac (6')-lb-cr* gene is notable as it confers resistance to both aminoglycosides and fluoroquinolones.

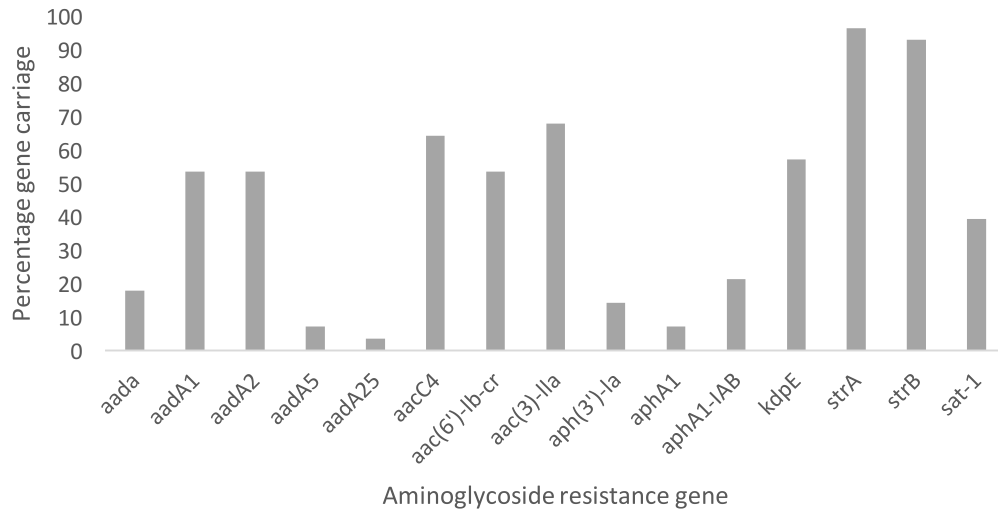


Figure 6.5 Percentage of isolates carrying aminoglycoside resistance genes

6.3.7.10 Trimethoprim resistance

Trimethoprim resistance genes were found in 27 of the 28 isolates. The most prevalent was the *dfrA5* gene which was found in 50 % of isolates followed by *dfrA1* which was present in 46 % of isolates. Additionally, *dfrA14* and *dfrA17* was also present in isolates (in 25 % and 4 % respectively). The only strain not to carry trimethoprim resistance-associated genes was strain 61 which was also the only strain to carry the $bla_{CTX-M-14.}$ The association of the *dfr* genes and the $bla_{CTX-M-15}$ gene may be indicative of co-carriage of resistance on the same MGE.

6.3.7.11 Additional AMR and MDR

Bacitracin resistance was identified in 27 of the 28 *E. coli* isolates and novobiocin resistance in 26 isolates. All strains carried multidrug efflux pumps alongside all the other resistance mechanisms (Figure 6.7). The *tolC* pump was identified in all 28 strains as was *mdtD* and *emrA.*

Figure 6.6 Multidrug efflux pumps identified in strains carrying *bla*$_{CTX\text{-}M\text{-}15}$

Ten key strains were investigated for carriage of ARG and MGEs (Table 6.2) and showed extensive carriage of ARG to a diverse range antibiotic classes. These strains were chosen based on the resistance and virulence profiles exhibited. It is not possible to determine which plasmids were involved in the carriage of ARG, however the presence of the IncF plasmids in all but one strain (142, which displayed no plasmid carriage) suggests, consistent with previous observations, that this group of plasmids is likely responsible for dissemination of the *bla*$_{CTX\text{-}M\text{-}15}$ gene. Numerous ARG were detected in strains conferring resistance to the same antibiotic class, for example, up to six different genes associated with aminoglycoside resistance were identified in individual strains suggesting a possible cumulative role in conferring resistance to this class of antibiotics.

Table 6.2 Resistance profiles from 10 *E. coli* isolates

Isolate	Sequence Type	Mobile genetic elements (plasmid Inc groups, IS, Tn and integron associated genes)	Antibiotic and associated resistance genes														
			Beta-lactam	Bacitracin	Novobiocin	Macrolide	Quinolone and fluoroquinolone	Polymixin	Vancomycin	Streptothricin	Chloramphenicol	Trimethoprim	Aminoglycoside	Tetracycline	Sulphonamide	Efflux pumps	Metal resistance
29	ST46	IncFIA, IncFII, IncFIC, IS26, IS903, IS903B, *intI1*	bla_{OXA}, bla_{TEM}, $bla_{ctx-m-15}$,	*bacA*	*baeRS*	*marA, macB*	*parC, arnA*	*pmrCAB, phoPQ*	*vanG*			*dfrA17*	*aadA5, aacC4, aac(6')-Ibm aac(3')-IIa,, strA, strB*	*tetA, tetG*	*sul2*	*emr* operon, *mdt* operon, *tolC, marR, robA*	*cusA, merA, terA*
42	ST455	IncFII, IncFIB, IncI1, IncHI1B, IncX1, IS26, Tn21, *intI2*	bla_{OXA}, bla_{TEM}, $bla_{ctx-m-15}$,	*bacA*	*baeRA*	*marA, macAB, mphA*		*pmrCAB*	*vanG*	*sat-1*	*cat1*	*drfA1*	*aadA1, aad2, aacC4, aac(6')-Ib-cr, aac(3')-IIa, strA, strB*	*tetA, tetB, tetD*	*sul2*	*emr* operon, *mdt* operon, *tolC, marR, robA*	*cusA, merA*
47	ST38	IncFIA, IncFIB IncFII, IncFIC, IncX4, IS26, Tn21, *intI1*	$bla_{ctx-m-15}$	*bacA*	*baeRS*	*marA, mphA*	*qepA, parC*	*pmcCAB*	*vanG*		*cat1, catA1*	*dfrA5*	*aadA1, aadA2, aadA25, aad(3)-L, strA, strB*	*tetA, tetB*	*sul1*	*emr* operon, *mdt* operon, *tolC, marR, robA*	*cusA*
48	ST131	IncB/O/K, IncFIA, IncFIB, IncFII, IncFI1, IS26, Tn21, *intI1*	bla_{OXA}, bla_{TEM}, $bla_{ctx-m-15}$,	*bacA*	*baeRS*	*marA, macAB,*	*qepA, parC, arnA*		*vanG*			*dfrA5, dfrA14*	*aac(6')-Ib-cr, aac(3')-IIa, aac(3)-I, strA, strB*	*tetA, tetG*	*sul1*	*emr* operon, *mdt* operon, *tol,C, marR, robA*	*cusA*
50	ST3202	IncFII, IncFIB, IncR, IS26, IS903, IS903B, Tn21, *intI2*	bla_{OXA}, bla_{TEM}, $bla_{ctx-m-15}$,	*bacA*	*baeRS*	*marA, macAB*	*qnrB, arnA*	*pmrCAB*			*cat1*	*drfA5, dfrA14*	*aac(6')-Ib-cr, aac(3')-IIa, aac(3)-I, strA, strB*	*tetA, tetG*	*sul2*	*emr* operon, *mdt* operon, *tolC robA,*	*cusA*
61	ST131	IncFIA, IncFIB IncFII, IncFIC, IS26, IS903, IS903B, Tn21, *intI1*	$bla_{ctx-m-99}$, $bla_{ctx-m-14}$,	*bacA*	*baeRS*	*marA, macA, mphA*			*vanG*				*aac(3)-I,, strA, strB*	*tetA, tetG*	*sul2*	*emr* operon, *mdt* operon, *tolC marR , robA*	*cusA, merA, terA*
66	ST131	IncB/O/K, IncFIA, IncFII, IncFIB, IS26, Tn21, *intI1*	bla_{OXA}, bla_{TEM}, $bla_{ctx-m-15}$,	*bacA*	*baeRS*	*marA, macAB*	*parC, arnA*	*pmrCAB*			*cat1*	*dfrA5*	*aacC4, aac(6')-Ib-cr, aac(3')-IIa aac(3)-I, strA, strB*	*tetA, tetG*	*sul1, sul2*	*emr* operon, *mdt* operon, *tolC, marR, robA*	*cusA*
79	ST940	IncFII, IncFIB,, IncFIC, IncHI1B, IncX1, IS26, Tn21, *intI2*	bla_{OXA}, bla_{TEM}, $bla_{ctx-m-15}$,	*bacA*	*baeRS*	*mphA*			*vanG*		*cat1*	*dfrA1*	*aadA1, aadA2, aadA5, aacC4, strA, strB*	*tetB, tetD*	*sul2*	*emr* operon *mdt* operon, *tolC*	*cusA*
140	ST46	IncFIB, IncN, IS26, IS903, IS903B, Tn21, *intI1*	bla_{TEM}, $bla_{ctx-m-15}$,	*bacA*	*baeRS*	*macA*	*qnrS1, arnA*	*pmrCAB*				*drfA5, dfrA14*	*aac(3)-I, strA, strB*	*tetA, tetG*	*sul2*	*emr* operon, *mdt* operon *tolC, marR*	*cusA*
142	ST73	IS26, Tn21, *intI1*	bla_{TEM}, $bla_{ctx-m-15}$,	*bacA*	*baeRS*		*qnrB1*		*vanG*			*dfrA6*	*aadA2, aac(3)-I, strA, strB*	*tetC, tetD*	*sul2*	*emr* operon, *mdt* operon, *tolC*	*cusA*

6.3.7.12 Stress, acid response genes and metal resistance genes

The stress response genes *cpxAR* were identified in 26 isolates and acid resistance associated genes were found in eighteen strains. As well as additional stress response genes, 71 % of strains carried *merA* conferring resistance to mercury and one strain carried, *terA* and *cusA* conferring resistance to tellurium and copper respectively.

6.3.8 *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-15}$ and associated MGEs

Consistent with previous studies the *bla*$_{CTX-M-15}$ was associated with the IS*Ecp1* genetic element (Partridge et al., 2011a, Zong et al., 2015, Tian et al., 2011). In strains 50, 87 and 142 an interrupted Tn2/3 carrying *bla*$_{CTX-M-15}$, was associated with a Tn6029-like genetic element carrying *sul2, strA* and *strB*. This one MGE carries resistance to ampicillin, 3GCs, sulphonamides and streptomycin (Figure 6.8).

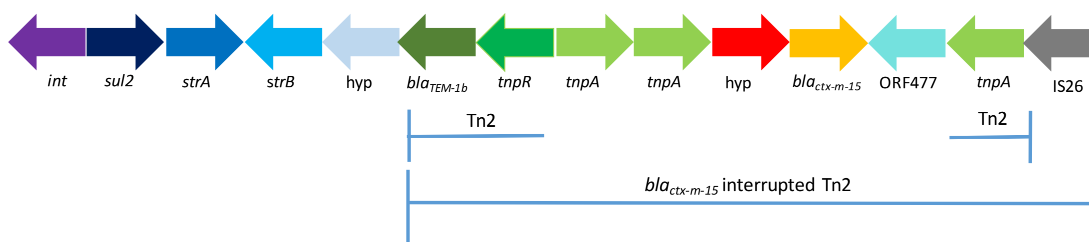


Figure 6.7 Genetic arrangement of an interrupted Tn2/3 carrying the ESBL gene *bla*$_{CTX-M-15}$ identified in 3 strains

The *bla*$_{CTX-M-14}$ gene was found associated with the IS*Ecp1,* IS*903C* and *IS26* consistent with previous records of genetic context of this gene (Figure 6.8). No other resistance genes were found on this MGE.

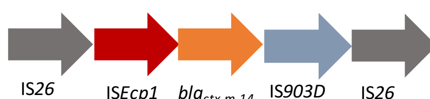


Figure 6.8 IS-associated *bla*$_{CTX-M-14}$ gene identified from strain 61, consistent with previous reports of this ESBL gene

6.3.9 Class 1 and class 2 integron structure

The class 1 and class 2 integrons identified in the study using BLAST searches were investigated regarding structure (Figure 6.9 and Figure 6.10). Only one structure of the class 2 integron was found which carried the *dfrA1, sat2* and *assfA1* genes consistent with previous reports of this integron (Figure 6.9).

None of the class 1 integrons carried the *qacE*Δ*1* gene characteristic of clinical integrons. All integrons identified carried trimethoprim resistance cassettes (Figure 6. 10)

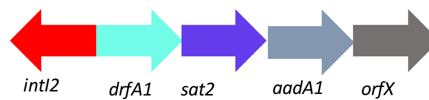


Figure 6.9 Class 2 integron structure identified in isolates carrying *bla*$_{CTX\text{-}M\text{-}15}$

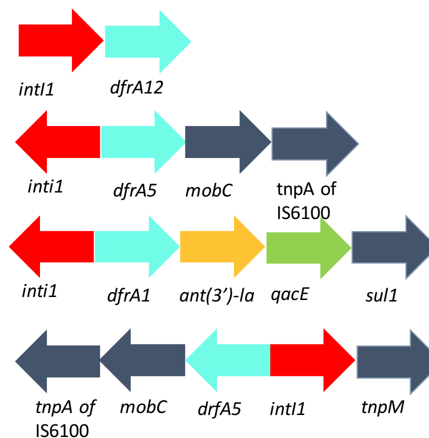


Figure 6.10 Class 1 integron structures identified in strains carrying ESBL gene

6.3.10 Virulence genes from strains carrying *bla*$_{CTX\text{-}M}$

E. coli isolated in the current study carried a number of virulence genes (Figure 6.11). Virulence genes included genes for toxin production including *hlyE and sheA* producing a haemolysin gene and in one isolate carrying *cnf1*, a cytotoxic necrotizing factor. No Shiga toxins were found in any of the strains in the current study.

100 % carried at least one siderophore, copper acquisition genes (*copA* and *cue0*), a genetic island associated with new-born meningitis (*gimB*) and a porcine-associated virulence gene (*paa*) (Figure 6.11).

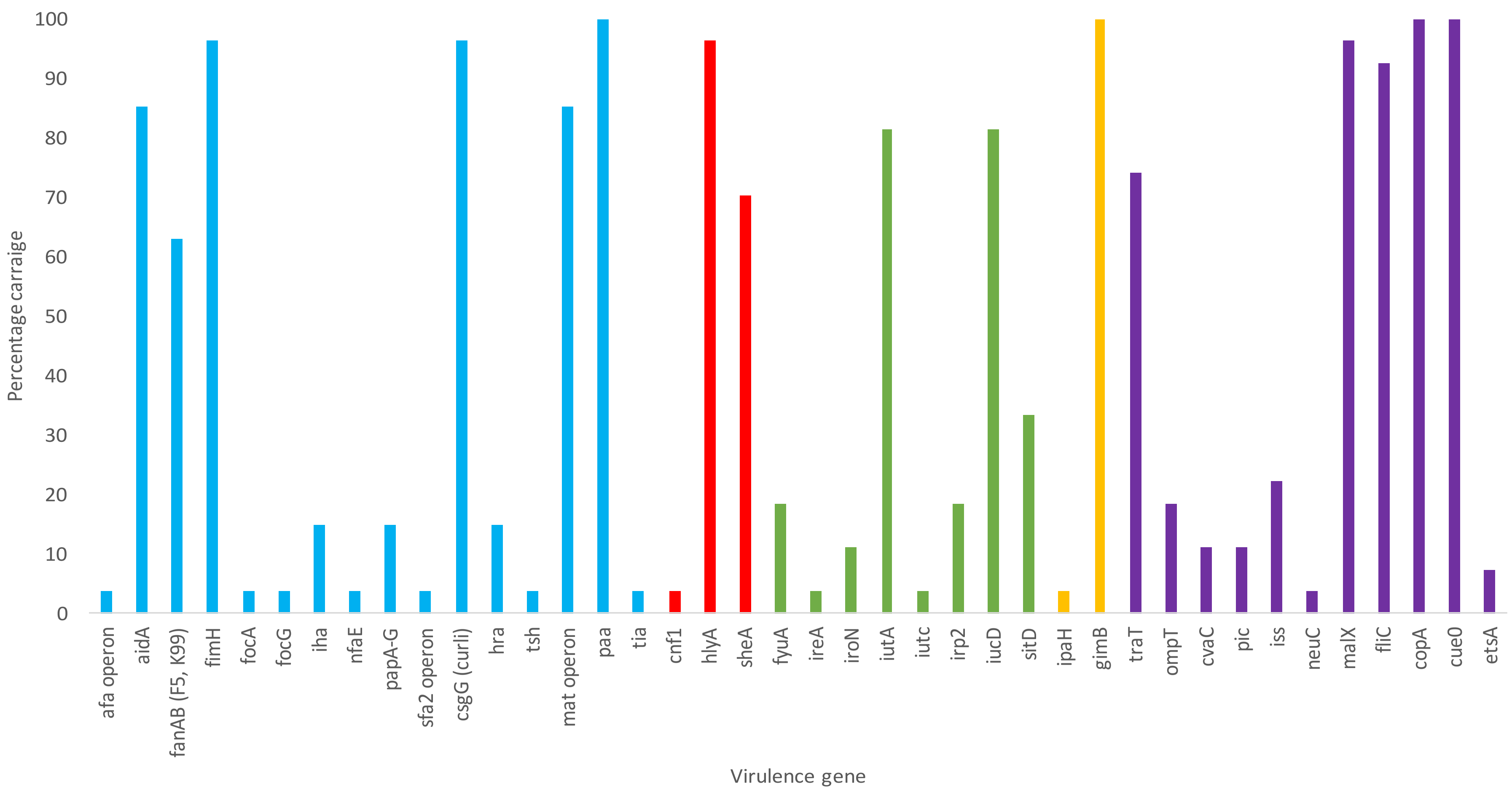


Figure 6.11 Virulence genes identified in *E. coli* strains carrying *bla*$_{CTX\text{-}M\text{-}15}$ gene. Blue = adhesins, red = toxins, green = siderophores, orange = invasins and purple = other

6.3.11 Comparison of ST131 *E. coli* isolates

E. coli identified as ST131 was further investigated to determine comparative resistance profiles between the 5 strains identified from the complete 77. Strains 40 and 102 did not carry $bla_{CTX\text{-}M}$ genes (Figure 6.12). As expected all strains carried the mutated *gyrA* conferring resistance to quinolones and the multidrug efflux pump *tolC*. The mutated *gyrA* was one of the first identified mechanisms of quinolone resistance so it not surprising that all strains investigated carried this gene conferring low level resistance. The TolC pump is a chromosomally encoded resistance efflux mechanisms and therefore would be expected to be found in all strains identified in the present study.

Genes that have recently emerged (within the last ~20 years) such as the $bla_{CTX\text{-}M}$ genes would be expected not to be carried by all strains. The carriage of these genes in 3 of the 5 identified ST131s is concerning and suggests a substantial dissemination of this ESBL gene in the environment. This gene, although believed to have originated in an environmental bacteria is predominantly associated with clinical infections (Canton, 2012). It is also interesting that strains carrying these ESBL genes also carry additional β-lactamases ($bla_{TEM\text{-}1}$ and $bla_{OXA\text{-}1}$) providing extensive resistance to the β-lactam antibiotics as well as streptomycin resistance conferring genes, sulphonamide resistance genes, tetracycline resistance genes and metal resistance genes, the majority of which are likely carried on the same MGE due to structure of integrons and transposons which often carry these genes.

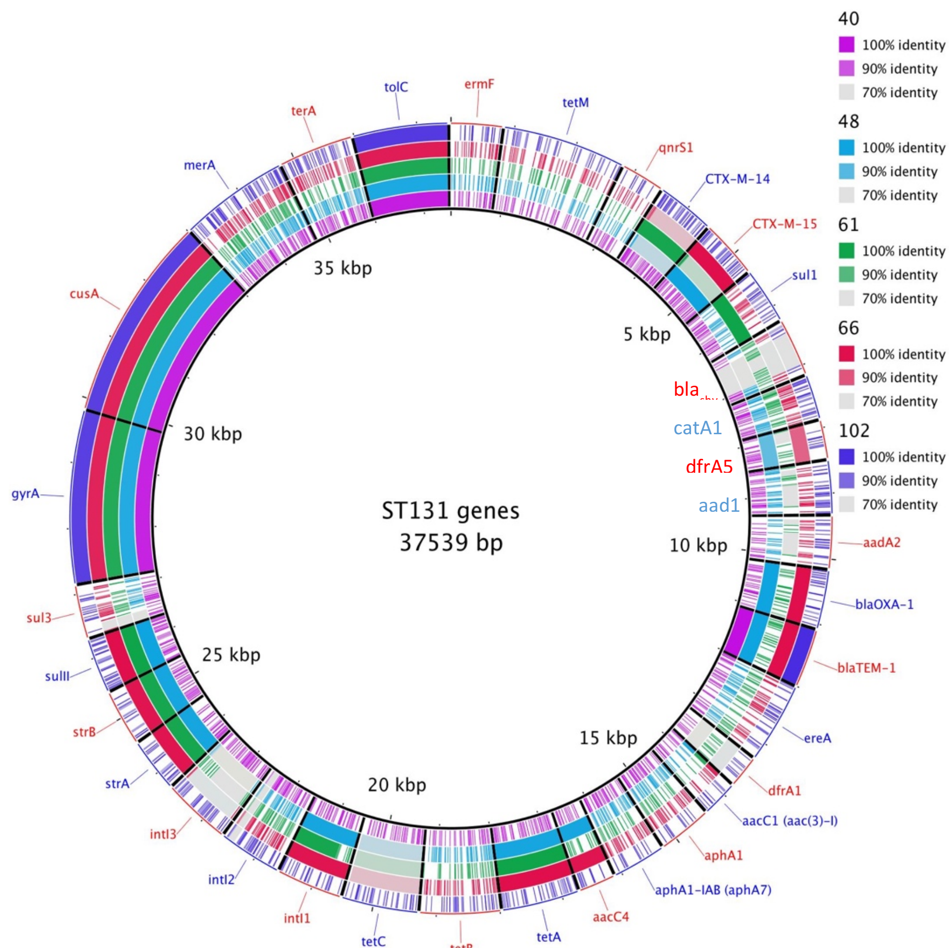


Figure 6.12 Comparison of AMR genes, metal resistance genes and integrase genes from ST131 *E. coli*. Generated using BRIG (Alikhan, 2011).

6.3.12 Comparative genomics of ST131

Relatedness between *bla*$_{CTX\text{-}M}$ carrying strains of ST131 and ST940 were separately investigated using MAUVE. Reference strains were compared with river isolates to determine potential regions of interest for further analysis. Strains identified as ST131 by MLST and confirmed by phylogenetic analysis, were aligned against closest related reference strain: 48 was alignment to JJ1897. MAUVE analysis showed that 48 had multiple scaffolds unable to align with the reference strain suggesting large number of accessory genes, presumably plasmid-borne. The alignment returned 46 LCBs with a minimum weight of 39. Alignments between ST131 between 61 and 66 were also run showing high conservation. The number of LCBs for the alignment between strain 61 and ST131 JJ897 was nineteen with a minimum weight of 479

(Figure 6.13) and the alignment between strain 66 returned 30 LCBs with a minimum weight 136. Alignments between 66 and JJ897 and 48 and JJ897 can be found in the Appendix 6 and Appendix 7.

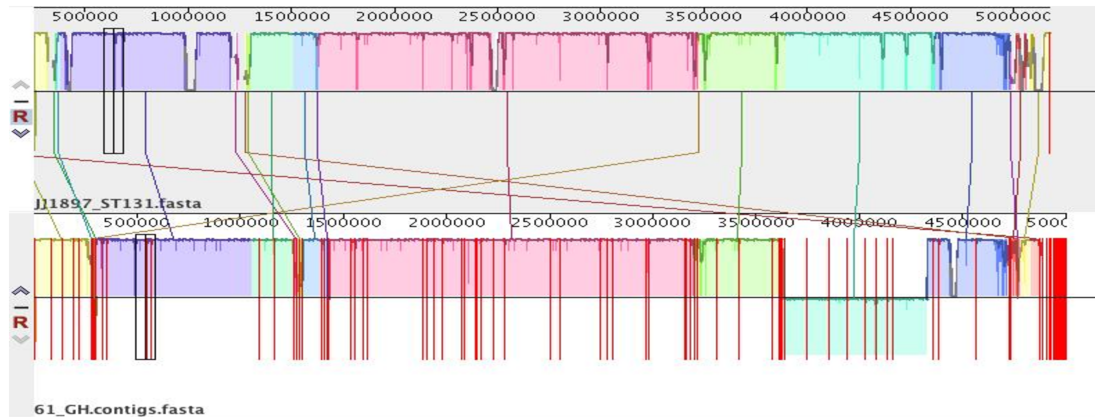


Figure 6.13 MAUVE alignment between strain 61 identified as ST131 and known ST131, JJ897. High level of conservation shown between the reference strain and strain 61. Multiple scaffolds unaligned on the right suggesting additional genetic elements not in the reference strain aligned suggesting either plasmid or phage acquisition. Coloured blocks represent genome sequences that align free of genomic rearrangement: locally collinear blocks (LCBs). The height of the profile within each LCB represented the degree of conservation within the aligned region. For most LCBs the conservation is high between strain 61 and ST131.

6.3.13 Genetic comparison of three *bla*$_{CTX-M}$ carrying ST131

Progressive MAUVE alignments were carried out between the three ST131 strains (48, 61 and 66) to investigate sequence conservation between isolates of the same sequence type. High levels of conservation were shown between all three isolates, particularly 61 and 66 (Figure 6.14). 61 had a large inversion compared to strains 61 and 66 and had comparatively fewer MGEs.

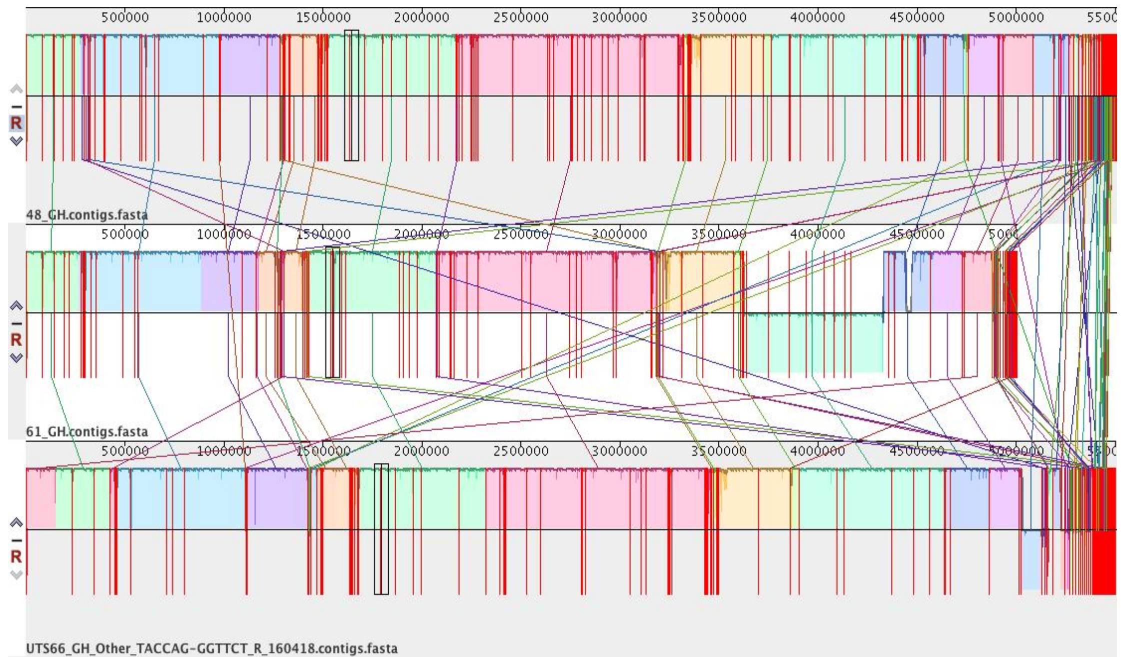


Figure 6.14 Alignment between ST131 strains 48, 61 and 66 using progressive MAUVE.

6.3.14 Investigating ST940 closest related *E. coli*

No reference strain could be found for *E. coli* ST940 so strains identified as ST940 by MLST were aligned to the closest relative (Figure 6.15) E24377A, an ETEC isolate. The alignment between strain 79 returned 50 LCB with a minimum weight of 210 showing a high level of similarity between these two strains of *E. coli* with a large number of additional MGE elements in the ST940 strain (Figure 6.15). All other ST940 strains were aligned to E24377A showing a high conservation of sequence excluding MGEs. All other alignments between ST940 strains and E24377A can be found in Appendix 8-19.

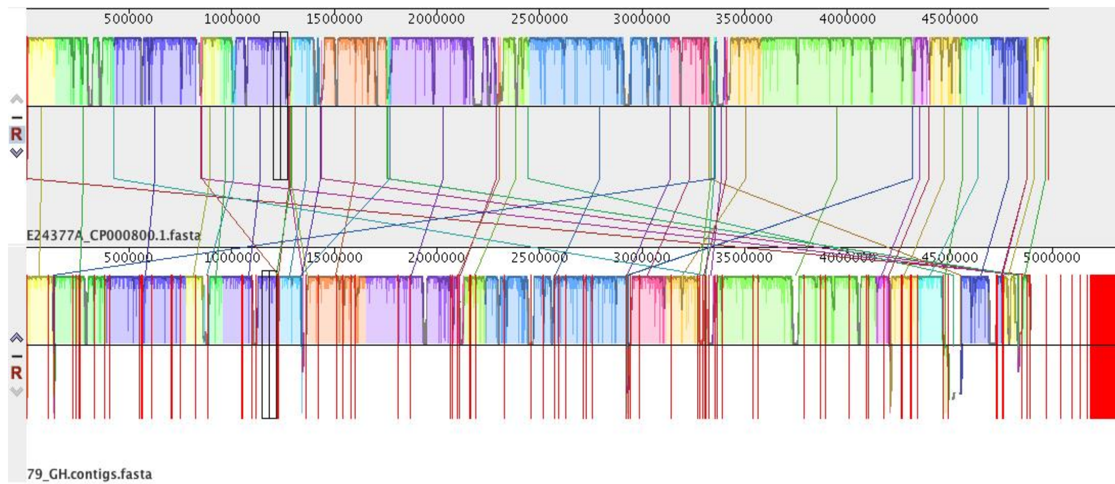


Figure 6.15 Alignment between strain 79 (ST940) and reference strain E24377A

Progressive MAUVE alignments were also performed to compare ST940 strains showing a high level of conservation between strains (Appendix 20). 72 was excluded from the analysis due the high number of scaffolds generated during genome sequencing. All strains were aligned to the reference sequence E24377A prior to aligning.

6.3.15 Toxin-antitoxin systems in isolates carrying *bla*$_{CTX\text{-}M\text{-}15}$

Isolates were searched for possible TA systems using the TAfinder (Shao et al., 2011). TA systems have been implicated in many cellular processes and may be involved in the persistence of bacterial cells. Persister cells are defined here as those cells able to enter a dormant like state in order to evade killing through stress, such as that induced by antibiotic presence. Of the 28 strains investigated in the current study 100 % carried the *relEBE* TA pair which has previously been associated with persister cell formation (Gelens et al., 2013). In addition, 96 % of strains also carried the *hipBA* TA system and 78 % carried the *hicBA* and 14 % *higBA* TA systems both of which have also been associated with persister function (Li et al., 2016). Isolates also carried TA systems which have been identified in plasmid maintenance including the PemKSa TA which was found in 32 % of isolates. MazEF which was present in 100 % of isolates and has previously been associated with SOS response (Sauert et al., 2016).

TA systems are associated with numerous roles, not exclusively persister cell formation which has only recently been elucidated (Page and Peti, 2016). They were first identified as meditating plasmid maintenance through post-segregationally killing (Gerdes, 1986, Ogura, 1983). The *pemK/I* genes were one of the first TA systems identified to play a role in plasmid maintenance, in the current study from the bla_{CTX-M} carrying strains 32 % carried this TA system (Tsuchimoto, 1988).

6.4 Discussion

A range of diverse *E. coli* were characterised with an impressive array of ARG, MGE and virulence genes showing enormous ability to adapt and evolve in to aggressive pathogens. The majority (72 %) of isolates were recovered from antimicrobial containing plates showing extensive recovery from environmental stresses. The 28 % that were isolated on HiCA only one isolate showed no phenotypic antimicrobial resistance to any of the antimicrobials tested. Of the 28 %, 25 % presented phenotypic to seven of the eight antimicrobials tested and 21 % presented resistance to six of the eight antimicrobials. The isolation process of *E. coli* required plating to purity over five times, the extensive resistance exhibited by isolates from non-antimicrobial selective plates suggests resistance mechanisms are not easily lost even in favourable conditions. The viability of these potentially pathogenic *E. coli* isolates is particularly concerning and highlights the need for more efficient wastewater treatment. The association between ST types and human infection suggests a strong WWTP impact and an important reservoir of pathogenic, resistant *E. coli*.

The *hylA* toxin was the most frequently detected toxin. This toxin encodes an α-hemolysin which is specific to ExPEC pathotypes and an important toxin in human and other mammalian infections (Lai, 2000). The increased serum survival (*iss*) virulence gene was found in 25 % of bla_{CTX-M} carrying strains. This gene is involved in resistance to extreme acid conditions of the intestines, enabling survival against the complement system and resulting in kidney epithelium damage (Johnson et al., 2008). Although these genes are also associated with animal infections they have the capability to cause human infection highlighting the potential risk associated with these environmental isolates. All strains also carried genes related to copper translocation (Vignaroli et al., 2012). The *cueO* gene is a chromosomal encoded gene whereas *copA* is plasmid-borne. Both function as copper oxidases and are involved in copper translocation and may be involved in protection against host copper toxicity as a method of innate immunity (Ladomersky and Petris, 2015). Genes responding to copper toxicity are likely a defensive mechanism and have been shown to be induced during phagocytosis by macrophages (Ladomersky and Petris, 2015).

This study did not detect any Shiga toxin producing *E. coli* (STEC) consistent with the study by Franz *et al* who similarly investigated the presence of ESBL producing *E. coli* in surface water impacted by wastewater. This may be because STEC are a rare phenomenon but is possibly a result of the isolation technique used (Hebbelstrup Jensen et al., 2014). The method used to culture *E. coli* is based on ability to produce β-D-glucuronidase and break down X-glucuronide. The combined cleavage of X-glucuronide and Salmon-GAL results in blue/purple colonies. The majority of STEC O157 are β-glucuronidase-negative *E. coli* and would therefore not form blue/purple colonies (Hayes, 1995, Doyle, 1984). Although O157:H7 has not yet been found in WWTPs, Shiga-like toxin I has been found in influent and effluents and the *stx1* and *stx2* phage have been detected in wastewater and river water suggesting STECs are also likely to be present (Bonetta et al., 2016, Dumke et al., 2006, Martinez-Castillo et al., 2012).

Five strains of *E. coli* O25:H4 ST131 were identified in the current study with three of these carrying $bla_{CTX\text{-}M}$ genes, two $bla_{CTX\text{-}M\text{-}15}$ and one $bla_{CTX\text{-}M\text{-}14}$. O25:H4 ST131 is considered a highly virulent human pandemic strain responsible for the widespread dissemination of $bla_{CTX\text{-}M\text{-}15}$ and is commonly associated with ciprofloxacin resistance (Woodford et al., 2009, Can et al., 2015). It is the predominant ST among ExPEC (Nicolas-Chanoine et al., 2014). Human commensal carriage of ST131 remains unknown but has been found in healthy individuals from China that between 7-51 % of faecal samples tested carried drug-resistant ST131, however these reports are inconsistent with large variation in percentage carriage (Zhong et al., 2015, Tian et al., 2011, Li, 2011). ST131 is generally regarded as a truly pathogenic *E. coli* therefore the number of healthy individuals carrying multidrug-resistant ST131 suggests that the $bla_{CTX\text{-}M}$ genes are widespread in both clinical infections and in healthy individuals and exist in pathogenic backgrounds which are able to cause infections (Nicolas-Chanoine et al., 2014). In Europe, one study reported commensal carriage at 40.5 % in a care home in Belfast which although is consistent with the studies in China presents an unrealistic estimate for the entire population where resistance is not as disseminated in Europe compared to Asian countries, however it highlights the high carriage of this strain and its extensive dissemination (Rooney et al., 2009). The

recent emergence of O25:H4 ST131 carrying *bla*$_{CTX-M-14}$ has been frequently detected in Europe and is also linked to clinical outbreaks in Japan (Suzuki et al., 2009). In the UK the *bla*$_{CTX-M-14}$ gene is not as prevalent as the *bla*$_{CTX-M-15}$ gene and has mainly been associated with animal infection (Ewers et al., 2010). O25b:H4 with *bla*$_{CTX-M-14}$ has previously been detected in the River Thame in 2011 when river water samples were taken. The first report of a ST131 carrying *bla*$_{CTX-M-15}$ in UK rivers was in 2014 from the same sites as the current study suggesting an increasing, or at least consistent, reservoir of this pathogenic ST type (Ewers et al., 2010, Dhanji, 2011, Amos et al., 2014).

Inconsistent with the study by Amos *et al* the ST type ST131 was not the most dominant ST type in the current study. Instead the ST940 was the dominant ST type. There have been no studies investigating the importance of ST940, with the only study mentioning prevalence which was coincidently the same study conducted by Amos *et al* (which investigated sites near the Finham WWTP) suggesting a persistent/recurrent ST in the River Sowe sediment most likely as a consequence of the Finham WWTP effluent. The Finham WWTP is a tertiary treatment WWTP which is the most effective type of wastewater treatment. The high counts of *E. coli* in Chapter 5 and the variety of *E. coli* ST types in the current study suggest a more vigorous wastewater process is required to reduce potentially pathogenic strains in UK rivers. In this study, fourteen isolates of ST940 were found with thirteen of these carrying *bla*$_{CTX-M-15,}$ suggesting that this ST is understudied and may play an important role in the dissemination of the *bla*$_{CTX-M-15}$ gene in the environment.

Other notable pathogenic ST types identified in this study include ST69, ST73, ST38 and ST453 all of which can cause human UTI infections (Coque et al., 2008, Hertz et al., 2016, Lau et al., 2008, Alghoribi et al., 2015, Minarini et al., 2007, Wu et al., 2013, Adbdallah, 2011, Sankar et al., 2009). The diversity of ST types was reduced when investigating the *bla*$_{CTX-M}$ genes with a reduction from 32 different ST types in the 77 isolates to eight. Within the 28 *bla*$_{CTX-M}$ containing strains, there were notable human strains including the previously mentioned ST73, ST38 and ST131. ST46, a clinically related ST type was also identified in the current study. This ST type has previously

been identified displaying the AmpC phenotype (Jorgensen et al., 2010). There has been emerging evidence reporting the widespread dissemination of ST38 carrying *bla*$_{CTX-M}$ associated with UTI infections further highlighting the importance of isolating this ST type from the river sediment (Chattaway MA, 2014).

Consistent with previous studies investigating *E. coli* isolated from non-host environments, the dominant phylogroups associated with AMR *E. coli* in the current study were B1 and A (Meric et al., 2013, Walk et al., 2007), inconsistent however was the association between *bla*$_{CTX-M-15}$ carrying strains which have previously been strongly associated with the A phylogroup (Franz et al., 2015). The study by Franz *et al* presented a similar sample number of 69 with 5 % of *bla*$_{CTX-M-15}$ carrying strains from the B1 phylogroup and 50 % of isolates from the A phylogroup. In the current study 48.15 % were from the B1 phylogroup and 33 % were from A. This difference in phylogroup prevalence within isolates is due to the high number of ST940 isolated.

Forsberg *et al* found MGE were rare in soil communities suggesting transfer is not likely to occur (Forsberg et al., 2014). Although it cannot be determined in this study whether the overall prevalence of MGE was low, there was a high prevalence of MGE recorded for each of the 77 *E. coli* isolates. There were thirteen isolates that did not carry any plasmids, however the majority carried at least two with the highest number found in one strain being four. Strain 36 carried IncHI1A, IncX4, IncR and IncA/C and strain 42 carried IncF, IncI1, IncHIB and IncX1 plasmids. Typically, the carriage of multiple plasmids within one strain is limited to two or three plasmids, but the strains identified in the current study demonstrated a large diversity of plasmids within single strains (Carattoli, 2009). All strains were reported to carry multiple IS and Tn suggesting a highly mobilisable resistome. Although, the rate of transfer from indigenous sediment communities is unknown, it is evident that the multidrug resistant *E. coli* reported here are capable of transferring ARG in the environment. The previous study by Amos *et al* demonstrated that isolates of *E. coli* from river sediment, carrying *bla*$_{CTX-M,}$ were able to transfer this gene to a mutated lab strain of *E. coli* on the IncF type plasmids (Amos et al., 2014). Further studies to investigate the rate of these transfers in the environment is important in furthering

the understanding the environmental resistome plays in disseminating clinically related ARG.

Upon viewing the literature, the interrupted Tn2 arrangement carrying *bla*$_{CTX-M-15}$ had not previously been identified and therefore highlights the importance of WWTPs as a possible hotspot for gene movement and rearrangement. WWTPs have been suggested to promote HGT due to the selective pressures from collective wastes from hospitals, households and animal production farms often carrying antibiotic residues and biocides as well as a myriad of microorganisms providing optimal conditions for high levels of HGT (Renew and Huang, 2004, Yang et al., 2005, Brown et al., 2006, Bollmann, 2014, Munck et al., 2015, Schluter et al., 2007). The hybrid arrangement between the interrupted Tn2/3 and Tn6029-like element (lacking the *aphA1* gene) was found in 3 of the *bla*$_{CTX-M-15}$ carrying strains and may have resulted as a direct consequence of high selective pressures within the treatment plant. It carries resistance to sulphonamides, streptomycin, ampicillin and cefotaxime allowing selection of all these resistances on one MGE and links the Tn2/3 hybrid MGE carrying IS*Ecp1-bla*$_{CTX-M-15}$-*orf477Δ* with *sul1,* strA and strB.

It could not be conclusively determined if the Tn6029 was present in the *E. coli* isolates due to the sequence breaks resulting from the repetitive IS26 element. However, 26 of the 28 isolates carrying the *bla*$_{CTX-M-15}$ gene carried elements suggesting Tn6029 presence. These included the carriage of the *strA* and *strB*, *sul2* and *bla*$_{TEM-1}$ but excluded the *aphA1* gene which was only found in 2 strains. Although it could not be concluded if these isolates carry this transposon, the importance of the Tn6029 in disseminating ARG is increasingly evident from previous studies which have discovered a wide range of resistance genes carried on these transposons (Reid et al., 2015). They have been identified on a variety of different plasmid incompatibility groups including IncI, IncH, IncZ and IncF and often found in close proximity to class 1 integrons, however unlike class 1 integrons, the role of the Tn6029 is understudied in the environment (Roy Chowdhury et al., 2015, Cain and Hall, 2012).

Consistent with previous studies no *intI3* genes were detected in the *E. coli* isolates (Laroche et al., 2009). Unexpectedly, the current study recorded equal prevalence of *intI1* and *intI2* within the $bla_{CTX-M-15}$ carrying strains. This result contrasts to the study conducted by Laroche *et al* who similarly invested *E. coli* isolates from water impacted by WWTP effluent and recorded fewer *intI2* genes than *intI1* (present in 1.4 % and 8.9 % of isolates respectively) (Laroche et al., 2009). The study by Amos *et al* proposed a model to predict the prevalence of 3GC resistant bacteria in the river sediment based upon *intI1* prevalence (Amos et al., 2015). The current study does not support this approach considering only half of the $bla_{CTX-M-15}$ carrying strains carried *intI1* and no integrons carried ESBL genes. Combined with Chapters 4 and 5, these results suggest *intI1* should not be used as proxy for environmental ARG.

The class 2 integron was found in 14 strains (in addition to the $bla_{CTX-M-15}$ gene) with the same genetic structure identified in each strain. The class 2 integron carried resistance to trimethoprim, streptothricin, spectinomycin and streptomycin and had previously been identified in other studies (Ramirez et al., 2010) . The class 2 integron is not usually functional due to the intrinsic stop codon which prevents expression of genes on this MGE which has been suggested to explain why generally these MGE are not frequently detected, however the number of isolates carrying this integron in the current study suggests widespread dissemination. The lack of data available on class 2 integrons is not necessarily due to limited dissemination and may be due to the few studies which investigated the prevalence of this MGE. In fact, studies that have investigated prevalence have similarly found a relatively high number of isolates carrying this element suggesting this element is understudied and may contribute to ARG dissemination more frequently than currently reported (McIver et al., 2002, Crespo et al., 2005, Gonzalez, 1998).

As expected the most common plasmid type was IncF plasmid. Of the 27 Inc groups that exist within *Enterobacteriaceae,* the IncF plasmids are the predominant plasmid incompatibility type in *Enterobactericeae* and are primarily responsible for the global dissemination of the ESBL bla_{CTX-M} genes (Carattoli, 2009, Hawkey, 2015). Often, the IncF plasmids will additionally carry bla_{TEM-1}, bla_{OXA-1} and *aac(6)-Ib-cr*

and have consequently been detected in many clinical isolates but have also been recovered from animals (Carattoli, 2009). The highly virulent ST131 O25:H4 has been isolated from companion animals carrying IncF plasmids with *bla*$_{CTX-M-15}$ and the recent emergence of the mobilisable colistin resistance gene *mcr-1* was first identified from pig isolates and has since been detected on IncF plasmids encoding this last-resort antibiotic resistance gene (Ewers et al., 2010, Liu, 2015, McGann et al., 2016). IncF plasmids were identified in 84 % of the 77 *E. coli* isolates in the current study. In the 3GC resistant strains IncF plasmid carriage was 100 %. The *bla*$_{CTX-M}$ are commonly found on IncF plasmids (Carattoli, 2009, Yang et al., 2015, Phan et al., 2015). IncF plasmids are low-copy highly promiscuous plasmid types which often carry an array of antibiotic resistance genes conferring resistance to most classes of antibiotics. IncFII plasmids carrying *bla*$_{CTX-M-15}$ are often associated with ST131 but are not exclusively present in this *E. coli* sequence type; they have also been found present within ST405, ST354, ST28 and ST695. *E. coli s*trains possessing the IncFII plasmid and *bla*$_{CTX-M-15}$ gene were of sequence types ST46 (strain 29), ST38 (strain 47), ST131 (strain 48 and 61 carrying *bla*$_{CTX-M-14}$), ST940 (strains 72, 75, 79 and 96) and ST1421(strain 98). Although other plasmids were carried in these strains, the association between *bla*$_{CTX-M-15}$ and IncFII plasmids is likely to be responsible for the presence of this ESBL gene (Partridge et al., 2011b). Determining which plasmid this gene is present on requires further work to elucidate involving transfers experiments. The current work is limited by the sequencing read lengths which make plasmid assembly difficult. Further sequencing using long-read sequencing techniques should be performed to provide complete plasmid maps.

The 3 ST131 strains carrying *bla*$_{CTX-M}$ showed a large level of conservation. Strain 61 had a large rearrangement compared to 48 and 66 but overall genes were conserved. The plasmids carried by these 3 strains did vary however with all strains carrying IncF plasmids but 48 and 66 carrying in addition IncB/O/K and 48 carrying IncI (which has also been linked to carriage of *bla*$_{CTX-M-15}$ (Zong et al., 2015)).

IncX plasmids were present in 56 % of *bla*$_{CTX-M-15}$ carrying strains, IncX plasmids are narrow spectrum plasmids found in *Enterobacteriaceae* able to undergo conjugative transfer. The abundance of this particular plasmid group is likely underestimated (Johnson et al., 2012) however there are an increasing number of studies demonstrating the variability of resistance genes carried by this group with ESBL and fluoroquinolone genes identified on these plasmids (Partridge et al., 2011a, Bae et al., 2015, Sumrall et al., 2014).

Only 3 isolates carried the IncA/C plasmids, none of which were in strains carrying ESBL genes. IncA/C have previously been associated with 3GC resistance so it was surprisingly that none of isolates carrying the ESBL carried these plasmids however it suggests that these plasmids are not yet widely disseminated at these sites (Harmer and Hall, 2015).
Polymyxin resistance has recently received a lot of attention due to the recent publication in November 2015 of mobilisable *mcr-1* gene (Liu, 2015). In April 2016, the first human case was recorded in USA on a novel IncF plasmid also carrying *bla*$_{CTX-M}$ (McGann et al., 2016) and in June 2016 *mcr*-1 was reported for the first time in the UK from pigs (VMD, 2016, Anjum et al., 2016). The clinical use of colistin was stopped in the 1970s due to nephrotoxic side effects (Spapen et al., 2011). Increasing multidrug resistance in Gram-negatives however has led to an increase of colistin in the clinic after first-line antibiotics fail (Spapen et al., 2011). The widespread use of these antibiotics in farming has led to a large level of intrinsic resistance (Catry et al., 2015). Although the mobilisable *mcr-1* was not found in this current study, many other polymyxin genes were discovered with 82 % of strains resistance to 3GC also carried at least one gene conferring polymyxin resistance. The *pmrA* and *pmrB* genes were the most prevalent and are involved in the modification of lipid A with aminoarabinose and phosphoethanolamine which in turn confers resistance to polymyxin B. The precise mechanisms behind polymyxin B action are not fully understood but is thought to involve the interaction between the anionic surfaces of Gram-negative bacteria allowing entry in to cells. Resistance arises through modification of the lipopolysaccharide through neutralisation of the negative charge preventing initial binding of polymyxin B (Lee et al., 2004). the mechanisms involved

in resistance consequently provides resistance to antimicrobial peptides and proteins from human neutrophils (Shafer, 1984). This high level of resistance suggests that polymyxins are not likely to be an effective last-resort antibiotic.

Gram-negative bacteria are intrinsically resistant to vancomycin, however the *vanG* gene was found in 10 of the 28 ESBL producing strains possibly as a result of co-carriage on plasmids (O'Brien et al., 2015, Garcia-Solache and Rice, 2016) which may have been selected for in the WWTP process (Amos et al., 2014).

Aminoglycoside antibiotics have broad spectrum and potent bactericidal activity. Historically they have been underused in the clinic due to their toxicity levels but due to the imminent post-antibiotic era the interest in use of these antibiotics is remerging (Ramirez and Tolmasky, 2010). In the current study multiple aminoglycoside genes were found in the 28 ESBL producing strains which is consistent with previous environmental studies investigating aminoglycoside resistance prevalence (Heuer, 2002). The accumulation of multiple mechanisms of resistance including modifying enzymes such as the N-acetyltransferases (*aac*) and phopshotransferases (*aph*) is commonly found within clinical isolates and suggests a cumulative effect of resistance within cells (Ramirez and Tolmasky, 2010) .

The β-lactamase genes are arguably the most well-studied ARGs due to the worldwide use of the β-lactam antibiotics (Canton, 2012). The class A β-lactamases conferring resistance to 3GCs such as cefotaxime, ceftazidime and ceftriaxone are considered an urgent public health threat by the Centres for Disease Control and Prevention (CDC) . Particular ESBLs found in this study include $bla_{TEM,}$ $bla_{SHV,}$ and $bla_{CTX\text{-}M.}$ In addition to the $bla_{CTX\text{-}M}$ gene, 100 % of strains carried additional beta-lactamases, the most prevalent of these being bla_{CRP} and bla_{TEM} which were present in the 26 of the 28 $bla_{CTX\text{-}M\text{-}15}$ carrying strains. This high level of beta-lactamase in river sediment is not surprising and previous reports have consistently found these genes in the environment (Zhang et al., 2011, Korzeniewska and Harnisz, 2013, Dhanji, 2011, Tennstedt et al., 2003, Franz et al., 2015, Jang et al., 2013).

Multidrug-resistance conferring efflux pumps pose a significant threat to the increased resistance levels and multidrug resistance phenotype. The resistance-nodulation-division (RND) superfamily of pumps play a major role in this phenotype and is important in virulence and biofilm formation in Gram-negative pathogens. One of the most important multidrug RND efflux pumps within *E. coli* is the AcrAB-TolC transporter system and represents an important target for new developmental compounds. This system was detected in the majority of the isolates so far (100 % possessing *tolC*). Targeting this system would ultimately lead to a reduction in antibiotic resistance as well as decreased virulence. However so far, there have been no successful reports of an AcrAB-TolC inhibitor (Aron and Opperman, 2016).

The association between toxin-antitoxin systems and persister cell formation has previously been assessed (Page and Peti, 2016). The HipBA and RelBE systems have been linked to persister formation (Wen et al., 2014, Wang and Wood, 2011). In the current study genomes were investigated to determine if persister formation could provide a possible explanation for the high environmental levels of *E coli* that was detected in Chapter 5 during the sampling regime and consequently the high level of potentially pathogenic *E. coli* isolated in the current study. 96 % of $bla_{CTX\text{-}M}$ carrying strains carried persistence related genes which may in part explain why it was possible to isolate viable multidrug-resistant *E. coli* from the river sediment. It is currently unknown if *E. coli* is active in the environment but the presence of these genes presumably allows them to remain in a dormant-like state.

Although it cannot be determined for certain where these potentially pathogenic strains originated from, it is evident that the river sediment provides a significant reservoir of potentially virulent, multidrug-resistant, persistent *E. coli.* Many of the strains carrying the $bla_{CTX\text{-}M\text{-}15}$ gene conferring resistance to 3GCs also carry large numbers of virulence genes, additional ARG and persistence related genes which allow increased survival and tolerance to antibiotics. The expected correlation of (probable) pathogenic strains with AMR was evident in this study and demonstrated the dissemination of these strains in to the environment. It cannot be determined in this study alone to what extent and how virulent these strains are, but by association

of what is known about the ST types, it can be assumed that these strains do possess the potential to cause infection. The isolation of multidrug resistant ST131, ST69, ST38 and ST73 is concerning due to their association with UTI infections (Alghoribi et al., 2015, Wiles et al., 2008, Hertz et al., 2016).

Environmental surveillance is therefore important considering the wide array of pathogenicity determinants found in these isolates from the river Sowe. Methods involved in understanding transmission routes will ultimately aid in the reduction of the overall ARG and pathogen levels seen here in the environment

Berendonk *et al.* (Berendonk et al., 2015) suggested key genes that may be involved in the dissemination of resistance. Consequently, these genes were chosen for monitoring proposed in the Thame and Sowe (Chapter 4 and 5 respectively). The current study did not detect any *tetM* or *ermF* in the isolates sequenced here and only found *qnrS* in one isolate. Although, this study investigated *E. coli* it suggests, consistent with Chapter 4 and 5, that these genes may not be suitable marker genes for environmental monitoring. Instead, *tetA* or *tetB* may provide a more informative measure of tetracycline resistance, *macA* or *macB* may be a more suitable measure of macrolide resistance and *aac(6')-lb-cr* as a combined measure of fluoroquinolone and aminoglycoside resistance.

Chapter 7

Final discussion and conclusions

Antibiotic resistance presents one of the greatest challenges of the 21st century. It is estimated that by 2050, there will be 10 million death per year as a direct result of AMR infections (O'Neill, 2016). There is an urgent requirement to understand the mechanisms involved in the rapid dissemination and evolution of ARG, and to evaluate the extent to which ARG and ARB contribute to environmental pollution. From approximately the year 2000, studies began to report the extent of ARG spread in the environment (Amos et al., 2014, Li et al., 2015a, Li et al., 2015b). In addition the recent report by Lord O'Neil commissioned by the government on AMR identified the environment as one of key factors driving ARG dissemination (O'Neill, 2016).

Effluent from WWTPs is considered an important route of potential AMR, ARG and ARB in the environment. The mixing of large numbers of bacteria with potential selective agents has resulted in WWTPs being referred to as HGT "hotspots". Previous work in our lab has demonstrated that WWTPs are important reservoirs of clinically relevant ARG contributing to the dissemination of the $bla_{CTX\text{-}M\text{-}15}$ gene (Amos et al., 2014). The current study aimed to further this research to investigate the effects of WWTPs on river water and sediment and determine the effects of CSO release events on sediment with respect to prevalence and abundance of ARG and

ARB as well as investigate ARB diversity through culture and culture independent methods.

The CGNF investigated to determine the ARG levels within this sub population hypothesising that important (clinically and agriculturally) ARG would reside within this clinically important group of bacteria. Although metagenomics is a powerful tool able to identify known and novel ARG it provides no context and cannot deduce if ARG are actively expressed or are even in a viable host. Using selective plates, the current study proved a significant number of culturable Gram-negative bacteria, with the capacity to express phenotypic resistance could be retrieved from the river. This work identified that the ARB resistant to ciprofloxacin formed a distinct community compared with the other antimicrobial conditions, which may be as a result of mutations increasing survival of these ARB (Fuzi, 2016). It was also found that the targets chosen to explain phenotypic resistance (i.e *qnrS* for ciprofloxacin resistance, *tetM* for tetracycline resistance, *ermF* for erythromycin resistance, $bla_{CTX\text{-}M\text{-}1}$ for 3GC resistance and *qacE* for biocide resistance) were not primarily responsible for the AMR observed and therefore the observed phenotypic resistance was attributed to other unknown mechanisms. The use of culture based techniques relying on phenotypic resistance is limited by this as it cannot be determined which genes are responsible for resistance, however, the diversity of bacteria highlights the widespread AMR in the river environment and suggests further work should investigate which key genes are responsible for the resistant phenotype.

Investigating the culturable fraction presents numerous problems with inconsistent counts and overestimation of coliforms (McLain et al., 2011). In the current study HiCA media was used to isolate the culturable fraction. This medium is designed to isolate coliforms and *E. coli*, however 16S analysis showed that *Pseudomonas spp.* were the most dominant in cultured water and sediment samples. It has previously been reported that chromogenic media can lead to errors in culturability with non-specificity resulting in false positives up to ~48 % (McLain et al., 2011). It was therefore expected that other bacteria would grow on the HiCA plates resulting in a high level of OTU abundance from non-coliform bacteria. The sediment culturable

fraction consisted of 61 % *Pseudomonas* species compared to 33 % in water samples highlighting the extent of non-specificity of HiCA plates which may also be due to the culture process which involved an incubation period of 2 days without purification before DNA extraction. It was not the primary aim of the current study to investigate *Pseudomonas spp.* within the river however it is an important clinical pathogen and the large abundance of species within the river is concerning (Wright et al., 2014). Due to the phenotypic mucoid growth on antibiotic plates it cannot be determined whether *Pseudomonas spp.* carried ARG or if phenotypic resistance was instead a protective property (Pritt et al., 2007, Owlia, 2014). The ability to grow on antibiotic containing plates is of interest and relevance considering the level in the current study and subsequent experiments should investigate the importance of *Pseudomonas spp.* and characterise phenotypic resistance.

A drawback of using 16S to identify bacteria is that within the *Enterobacteriaceae* 16S sequences are very similar and so determining the species present in samples was not possible for most *Enterobacteriaceae* (Pham et al., 2007). Other targets should be optimised for targeted sequencing to potentially include *dnaJ* to identify down to species level of the *Enterobacteriaceae* to determine key species showing antibiotic resistance (Pham et al., 2007).

As part of the current study it was hypothesised that increased prescription and rainfall in the Winter months would drive selection of ARG in the environment, therefore one of the aims was to evaluate temporal effects on river sediment at sites impacted by CSO release events and WWTP effluent. The changes in ARG and ARB abundance with season were consistent with previous studies reporting a higher abundance in the Autumn/Winter months and a higher prevalence of *E. coli* and $bla_{CTX\text{-}M\text{-}1}$ at the site impacted by CSO release events (Garcia-Armisen et al., 2014, Caucci, 2016b). The higher number of *E. coli* detected in Autumn/Winter may be due to of large volumes of untreated human waste entering the river via the CSO. Although it cannot be conclusively determined if spikes in *E. coli* abundance are a direct result of CSO release events due to the unmonitored amount of release events

occurring from this WWTP (Robert Huxham, Severn Trent Water, personal correspondence, 13th July 2016), previous studies have reported a significantly higher number of release events in Winter months (Jalliffier-Verne, 2016). If true seasonal effects are to be determined with respect to changes in ARG and ARB within river systems, the number of CSO release events must be more rigorously monitored by the water authorities to investigate the volume of water released and the frequency of events during the year. The current guidelines on CSO release from the EA state that spills can only take place when the flow to the treatment exceeds the "pass forward flow" which is defined as the volume of water that must proceed to full treatment at the WWTP. The formula defining the pass forward flow takes in to account the population served, water consumption per head per day, infiltration and trade effluent (EA, 2014). The guidelines also state that the operator may model the effects of discharge on the environment to justify CSO release events by modelling the frequency and volume to be released and to confirm that it will not result in 'significant deterioration' (EA, 2014). These guidelines are modified for release in to bathing waters which stipulate that no more than three spills can occur within one bathing season (specified as between May-September) where a single spill is defined as any discharge within a 12-hour period and spills lasting longer than 12 (up to 36 hours) count as two spill events and thereafter additional release up to additional 24 hours counts as one spill (EA, 2014). There are no guidelines stating how much untreated wastewater can be released, only that the number of spills should be recorded. Clearly, the number of spills is not always monitored however so unknown quantities are released in the river at an unknown frequency (Robert Huxham, Severn Trent Water, personal correspondence, 13th July 2016). The water authorities are responsible for the prevention of CSO spill events and this issue has been addressed by Thames water in London with the development of the Thames Tideway which is currently underway but there are no known plans to improve the sewage treatment by Severn Trent (ThamesWater, 2015b, Amos et al., 2014).

Class 1 integrons have previously been suggested as suitable markers for anthropogenic pollution (Gillings et al., 2015) and have more recently been used in models in an attempt to predict 3GC resistance based on *intI1* as a proxy (Amos et

al., 2015). The widespread distribution of *intI1* was investigated in a number of studies and was detected at high levels in all river samples in the current study (Table 5.3). Integrons may be marker of anthropogenic inputs but using them to precisely predict ARG will overestimate contamination. Whilst the levels of *intI1* detected matched a wide range of studies of polluted environments, it is clear from the current work that in UK rivers carriage of ~10 % is common and therefore substantially higher than carriage of ARG which generally less than 5 %. No reports of the prevalence of *intI1* in pristine environments could be found, therefore evaluating the extent of anthropogenic contamination based solely on this gene cannot be determined (Table 5.3). The suggestion that this gene may be suitable as a proxy is based only the observation that the class 1 integrons are associated with clinical infections, however the carriage of *intI1* is not necessarily indicative of carriage of clinical integrons with a diverse range of integrons carrying this gene recently reported carrying additional ARG but not the characteristic *sul1* and *qacEΔ1* (Amos *et al,* in press) (Gillings et al., 2015). Furthermore, *intI1* prevalence did not show any variation with seasonal effects contrasting to other ARG targets and the class 2 integrons integrase gene was found within equal numbers of ESBL producing strains in Chapter 6 with 50 % of strains carrying *intI1* and 50 % carrying the *intI2* gene. Although class 1 integrons are primarily associated with clinical infections the fact that they were detected at the same frequency as class 2 integrons (which have previously been found at comparatively low abundances in clinical and environmental strains) suggests the class 2 integron may be an under represented marker of resistance and may be equally important in disseminating resistance in the environment and clinic (Deng et al., 2015, Ramirez et al., 2010, Mirnejad et al., 2013, Stange et al., 2016). It also suggests that rather than using the *intI1* gene as a marker of pollution and predictor of resistance, key AMR should instead be monitored to represent the important clinical classes of antibiotics still used in the treatment of human infection or if clinical class 1 integrons are of interest then primers designed to capture the 3' conserved region consisting *qacΔ1* (which is exclusively characterised to occur on class 1 integrons) and *sul1* genes may be more effective in evaluating the true dissemination of these MGE.

Berendonk *et al.* set out key ARG targets that should be monitored in the environment consistently to gain a cohesive picture of AMR between research studies. These targets were not supported in the current study. Combined, results showed very low levels of the *ermF* and *tetM* genes at sample sites in both in the Thames and Sowe sampling regimes and WGS did not identify these genes in *E. coli* strains in Chapter 6 where *tetA* and *ereA* were detected suggesting a lack of importance in these clinically important strains. Of course, it cannot be concluded if erythromycin and tetracycline resistance genes were not present in the environments tested, but given the extensive use of these antibiotics in veterinary medicine and aquaculture combined with the frequent detection of tetracycline antibiotics in the environment, resistance was expected (Chantziaras et al., 2013, Harnisz et al., 2015, Qiao M, 2012, Sarmah et al., 2006, Singer et al., 2014). Many *tet* genes were sequenced from the *E. coli* carrying $bla_{CTX\text{-}M\text{-}14/15}$ isolates suggesting clinical relevance of tetracycline resistance is not related to *tetM* and that more suitable targets to evaluate clinically important ARG dissemination in the environment should include *tetA* (to monitor tetracycline resistance) and *macB* (for erythromycin resistance). Previous studies have demonstrated these genes are present in the environment with metagenomic studies showing higher levels of *tetA* in soils and sediments than *tetM* (Li et al., 2015b). Although *tetA* and *macB* may be more important in the dissemination of resistance within clinically important bacteria, the choice to monitor only a select few genes clearly won't work with the variation of resistance genes between sites based on geographical differences. Instead, if monitoring of environmental contamination is properly evaluated a more extensive list of genes must be considered to capture all diversity of ARG within the environment.

WGS of *E. coli* was performed to elucidate the full resistance and virulence profiles of exotic *E. coli* found in the river sediment and allowed an insight in to the full genomic potential of strains to characterise isolates based on resistance and virulence profiles (Dahms et al., 2015). It was previously reported that 13 % of *E. coli* in the river were pathogenic STs but the current study found a much higher percentage of pathogenic STs with 72 % of the total 77 *E. coli* strains of pathogenic

STs (both human and animal) and 63 % human-associated pathogenic STs (Gomi et al., 2015) . The current study is however biased by the selection process used which isolated *E.* coli based on antimicrobial properties. The association between AMR and pathogenic STs is obvious therefore it was expected that most *E. coli* isolated on antimicrobial agents would be pathogenic STs. There were a number of strains however that were not isolated on antimicrobial agents and of these 21 isolates ~20 % were pathogenic ST types including ST131 and ST69 strains associated with UTI infections which was higher than the 13 % reported in Japan by Gomi *et al.* who used a similar method of isolation (using XM-G coliform selecting agar) but focussed on water as a source of pathogenic *E. coli* (Gomi et al., 2014, Gomi et al., 2015).

E. coli ST940 was the most frequently isolated ST and all but one carried the ESBL $bla_{CTX\text{-}M\text{-}15}$ gene yet there are no published reports of this ST type so determining its importance in the dissemination of 3GC resistance is currently unknown. The majority (92 %) carried the K99 fimbriae virulence determinant which is associated with animal ETEC infection suggesting animal origin. ST940 may therefore have a significant role in disseminating $bla_{CTX\text{-}M\text{-}15}$ within animals and may present a risk in the food chain (Kaper et al., 2004). Other important ST types isolated from the river sediment included the human pandemic ST131 O25:H5 strain and other clinically important UPEC strain causing UTI infections (ST73 and ST46). UTI infections are the most commonly presented bacterial infection in women in the UK in primary care and approximately 40-50 % of women will experience at least one episode during their lifetime (Flower et al., 2016). As a direct result of this high prevalence of infection, between 1-3 % of all consultations in general practise are related to UTI infections and frequently result in prescription of antibiotics (Flower et al., 2016). Recurrent UTI infections are particularly problematic and often result in antibiotic prophylaxis which is not always effective with 50 – 60 % of women suffering from recurrent infection between 3 – 6 months after treatment is completed (Flower et al., 2016). The frequent prescription of antibiotics and prophylaxis for UTI infections has consequently led to the widespread dissemination of pathogens such as ST131 and no doubt contributes to the environmental pathotypes recovered from the sediment in the current study (Dhanji, 2011, Can et al., 2015). The first line of

treatment is normally trimethoprim and sulphamethoxazole, if this fails then nitrofurantoin and cephalexin are prescribed and as a last resort, quinolones and fluoroquinolones are used (NHS, 2015). In the clinic, trimethoprim resistance is predicted at ~20 %, in the ESBL producing *E. coli* isolates presented in the present study 100 % carried ARG conferring resistance to trimethoprim, sulphonamides and quinolones and 21 % carried fluoroquinolone resistance genes which would make treatment difficult if infection with any of these *E. coli* strains occurred (Flower et al., 2016).

Of the 77 *E. coli* isolates presented in the current study 35 % of these carried the ESBL $bla_{CTX\text{-}M\text{-}15}$ gene in addition to other clinically important ARG conferring resistance to quinolones, streptomycin, sulphonamides, aminoglycosides, polymyxins, tetracyclines and additional beta-lactams. This represents one of the first reports to investigate the full ARG profile of ESBL producing *E. coli* from a UK river and defines extensive resistance in opportunistic pathogenic strains. Although no pan-resistant strains were detected in the current study, resistance to most clinically relevant antimicrobials was reported with multiple genes conferring resistance to beta-lactams in each strain. Consistent with previous studies investigating *E. coli* from the environment no carbapenem resistance was detected in any of the isolates (Ferreira da Silva et al., 2007). The use of carbapenems in the clinic is restricted due to the accelerated resistance dissemination of primarily $bla_{NMD\text{-}1}$ and $bla_{OXA\text{-}48}$ genes however, this study concludes that carbapenems are still effective against the majority of *E. coli* infections that could result from environmental exposure.

The $bla_{CTX\text{-}M\text{-}14}$ gene was present in one *E. coli* isolate and is the second most prevalent $bla_{CTX\text{-}M}$ gene worldwide. It is predominantly associated with human infection and is the primary ESBL in Spain and China but has been detected more frequently in animals than humans in the UK with approximately only 10 % of UK *E. coli* isolates from clinical infections carry $bla_{CTX\text{-}M\text{-}14}$ (Tyrrell et al., 2016, Hawkey and Jones, 2009, Carattoli et al., 2005). Consistent with most reports in the UK, the current study found the most prevalent ESBL was the $bla_{CTX\text{-}M\text{-}15}$ gene clearly supporting the UK trends of 3GC resistance gene dissemination. The ESBL genes have

been increasing in prevalence over the last 20 years with increasing studies demonstrating up to 65 % carriage in faecal samples in South East Asian communities (Woerther et al., 2013). The dissemination of genes conferring 3GC resistance are most widespread in developing countries but have been detected worldwide (Woerther et al., 2013). Molecular tracking of certain genotypes may help understanding of local and global dissemination trends and is required if the rate of spread is to be decreased.

The study of WGS in Chapter 6 found the majority of *E. coli* isolates recovered from sediment carried multiple plasmids. The IncF and IncK have been associated with the dissemination of the $bla_{CTX\text{-}M}$ genes, although due to the sequencing method used, it is not possible to elucidate which plasmids carried these ESBL genes (Perry and Wright, 2013, Dhanji et al., 2012, Yang et al., 2015, Dhanji, 2011). All strains characterised as ESBL-producing strains carried IncF plasmids and therefore are likely to be responsible for gene presence. The global dissemination of $bla_{CTX\text{-}M\text{-}15}$ has previously been attributed to the association with IS*Ecp1* which has resulted in mobilisation on to IncF plasmids. It was therefore expected that the $bla_{CTX\text{-}M\text{-}15}$ genes would be associated with this IS element and would be mobilised to the IncF plasmids. The IncF plasmids are highly promiscuous plasmids which have acquired a wide array of ARG resulting in the global dissemination of many clinically relevant ARG (Carattoli, 2009).

One of the major limitations of Illumina sequencing is the short read lengths which arise due to repetitive stretches of DNA, (often in multiple copies) which make assemblies difficult and sometimes unreliable (Forde et al., 2014, Salzberg et al., 2012, Nagarajan, 2010, Kingsford et al., 2010). This is particularly problematic when trying to investigate plasmid gene carriage because numerous repetitive elements including IS and transposons result in sequencing breaks producing many contigs. The strains identified in the current study in Chapter 6 carried multiple plasmids (some as many as 4) and therefore identifying gene carriage on specific plasmids was not possible. To determine the plasmid associated with specific ARG carriage, transfer experiments should be carried out, although this is still limited by the

potential transfer of multiple plasmids. Sequencing with PacBio may provide an alternative and due to the long reads may provide full plasmids maps which will allow ARG location to be determined. In the current study, associations with transposons and IS elements were elucidated but full MGE could not be determined. The presence of integrons (class 1 and 2), IS26 and Tn21 were prevalent within this sample set, combined with the high frequency of plasmids suggests a highly mobilisable resistome with these viable, potentially pathogenic *E. coli*. Previous work in our lab has demonstrated that isolates from the same sites show transferable antibiotic resistance, with $bla_{\text{CTX-M-}15}$ shown to transfer from environmental isolates to a mutated lab strains of *E. coli* with frequencies ranging from 10^{-4} to 10^{-6} (Amos et al., 2014). Strains in the study by Amos *et al.* were not characterised extensively for carriage of plasmids and it cannot be determined if the plasmids transferred from the environmental strains were of the IncF group, however all strains that were able to transfer plasmids did carry plasmids of this incompatibility group.

Individual strain analysis has provided useful information for potential of mobilisation of ARG and the carriage of plasmids already implicated in ARG gene transfer. In addition, many of the surviving strains recovered from sediment showed virulence factors that could be hypothesised, when considering ST type as well, to contribute to pathogenesis of these strains. Although these ST types are primarily commensals (with very few true pathogenic *E. coli* strains known), the high numbers of opportunistic pathogens recovered suggests a high commensal carriage in the community served by the Finham WWTP. There are only a limited number of studies that have investigated the commensal carriage of opportunistic pathogenic *E. coli* but figures suggest ~ 60 % of healthy individuals carry potentially pathogenic STs highlighting the potential for infection from commensal carriage (Rooney et al., 2009, Zhong et al., 2015, Leflon-Guibout et al., 2008). Although infection from *E. coli* most commonly presents as UTI infections, most cases of septicaemia are from host microbiota, which in this case, septicaemia resulting from the strains in this current study would result in severe infections that would not respond to many antimicrobial agents (Lobo et al., 2016). The transfer of resistance has previously been investigated in the human gut showing that it is likely to occur, further highlighting the importance

of commensal carriage of these MDR bacteria which are able to transfer ARG allowing movement of genes between potential pathogens and commensals within the human gut (Karami et al., 2007, Trobos et al., 2009). One study demonstrated that the gut microbiota can harbour up to 53 different ARG highlighting the extensive carriage of resistance and vast potential for HGT in the human gut (Ghosh et al., 2013). The potential for HGT is something that needs to be addressed both in the human gut and in the environment with studies of the plasmidome. Investigating the plasmidome to elucidate only the mobilisable fraction of the resistome is an important area of research that is only just starting to emerge. The first studies were focussed on the culturable fraction and movement of gene within this subpopulation however studies are progressing to investigate the full plasmid metagenome with the most recent study by Li *et al.* investigating the plasmidome from WWTPs (Li et al., 2015a). This study discovered a substantial diversity of resistance genes that were missed in the total metagenome highlighting the importance of studies in this new field of research. Currently however, the technique requires considerable optimisation as recent papers have reported that large plasmids and linear plasmids (which may play important roles in Gram-negative pathogens) were omitted from plasmid specific extraction (Dib et al., 2015, Szczepanowski et al., 2009, Li et al., 2015a, Zhang et al., 2011). Large plasmids (over 150 kb) cannot be purified and plasmids around 45-50 kb are not readily eluted (Li et al., 2015a). Therefore the IncA/C plasmids, known to carry extensive AMR, including bla_{TEM-3}, bla_{TEM-21} and bla_{TEM-24}, *qnrA*, are typically between 128-130 kb in size will therefore not be isolated on their own, and only with chromosomal DNA (Harmer and Hall, 2015).

A number of studies by Wright and others have aimed to provide evidence that resistance genes are highly prevalent in the environment and are generally borne on the chromosome suggesting that they are therefore unlikely to be mobilisable and do not provide any threat to human health (Bhullar, 2012, D'Costa et al., 2011). The study by Forsberg *et al.* reported that HGT does not occur at high levels in the environment and instead phylogeny is more important in the spread of environmental resistance (D'Costa et al., 2011, Forsberg et al., 2014). Of course, the majority of the soil and sediment microbiome will not undergo extensive HGT,

however it is the presence of exotic clinically relevant species, such as *E. coli* that are hypothesised to carry out gene transfer in the environment and although they do not predominate these environments they no doubt contribute to environmental levels of ARG. Many of the *E. coli* isolates identified in the current study were reported to carry multiple plasmids which will accelerate gene exchange between these mobilisable elements via transposons which allow intra- and inter- species movement of genes therefore allowing gene movement between plasmids in the host (Bennett, 2008). Some strains carried four different plasmids and therefore possess a large potential for gene exchange. It remains to be investigated whether these plasmids can and will transfer to indigenous bacteria but it is evident that the plasmids in exotic species have accumulated large numbers of resistance and may undergo conjugation when bacterial hosts enter more favourable environments, such as the human gut (Kurokawa et al., 2007, Huddleston, 2014). This method of gene movement is particularly concerning when considering the IncF carrying bla_{CTX-M} genes (McGann et al., 2016, Smillie et al., 2011).

The process of co-selection was not evaluated in the current study, however the carriage of multiple genes conferring resistance to metals and biocides was observed using WGS. The carriage of both ARG and BRGs/MRG suggests a large potential for co-selective effects. Biocides are present in a wide range of environmental locations and are particularly prevalent in WWTPs which has resulted in a recent study reporting BRG and MRG levels of 30-300 times more than ARG in WWTP influent (Bengtsson-Palme, 2016). Although BRG are reported to be substantially higher than ARG in the WWTP process, the carriage of BRG and ARG is commonly associated on the same MGE which would result in selection of BRG/MRG from biocide/metal selective pressures and consequently select for ARG (Deng et al., 2015, Pal et al., 2015). No studies could be found investigating the occurrence of co-selective events in the environment, however the study by Whitehead *et al.* demonstrated that biocide exposure could select for multidrug resistance using *in vitro* experiments in *Salmonella enterica* Serovar Typhimurium through the selection of a multidrug efflux pump therefore highlighting the selective effects of biocides that will occur in the environment (Whitehead et al., 2011). The co-selective effects of separate ARG and

BRG has yet to be conclusively shown in the environment because multiple selective pressures will occur and the cumulative effects of these pressure are evidently important in sustaining resistance metals, biocides and antibiotics , but co-occurrence has been documented and it is widely accepted to occur (Pal et al., 2015).

The current work compared the sediment and water to determine which carried a greater load of resistant Gram-negatives. It found that although the prevalence was comparable when investigating the viable plate counts, the absolute abundance in the sediment (per g) was greater than water (per ml) with a higher number of ARG and ARB in samples tested, suggesting that sampling should focus on sediment but should aim to sample both to determine the risk to animals and humans. Previous risk assessments have taken in to account water or sediment but in most scenarios, both sediment and water will contribute to the risk of colonisation with the disruption of sediment leading to increased numbers in the water. Assessing the risk of ARB exposure from the river is however difficult and will depend on the treatment plant, the population it serves, the type of treatment plant and the number of CSO releases. These factors will all vary according to location and season as evidenced by the current study. The risk of colonisation will vary with each person based on numerous factors including weight, age, gender and microbiota (Wijetunge et al., 2015, Pasche et al., 2005, Round and Mazmanian, 2009). Determining risk associated with river ARB should therefore be undertaken with caution not to overestimate risk which will vary considerably across river catchments. The ST type of *E.* coli is also an important consideration when determining risk where EHEC strains typically have an infectious dose of between 10-100 whereas EPEC strains require between 10^8 – 10^{10} to cause infection (Kaper et al., 2004, Mellies et al., 2007). Risk will relate to the type of recreational use of water and clearly swimming is less likely in the Autumn/Winter months therefore it is important that future studies take in to account all possible factors when evaluating the risk posed by ARB in the environment and to assess the likelihood of human contact at a contaminated site. The risk of ARB in the environment has previously been assumed, however, it is unlikely that direct transmission is a frequent occurrence (Amos et al., 2014). Instead, it is more likely

that infection/colonisation is via indirect transmission from the river to animals and via zoonotic transmission to humans (Verraes et al., 2013, McEwen and Fedorka-Cray, 2002, Dahms et al., 2015, Guardabassi et al., 2004). The likelihood of animals being colonised by pathogenic ARB from the river is inevitably higher than the risk of human colonisation due to drinking of water from rivers. It will be important and useful to understand the exact relationship between prevalence, abundance and transmission to establish risk presented by environmental ARB and although this is difficult to study it is very important to understand the transmission routes to fully understand the risk. As an initial study faecal samples from animals near WWTP effluent and CSO spill-polluted rivers should be tested for ARG to determine whether this presents a viable route of transmission from river to animal and then further to this if the same environmental ARB are present in animal faeces the transmission between animals and humans should be researched.

Abstraction from rivers for the irrigation of farm land is another potential route of transmission of ARB from effluent and CSO event polluted sites. Current laws state that licences must be obtained if more than $20m^3$ of water is to be abstracted per day (EA, 2013). Despite the large population and heavy industrial impacts, 90 % of landuse in Warwickshire is for agriculture and from the River Sowe licenses can be obtained to extract a total of 5.6 Ml/d which is available to take over 153 days of the year as long as 143 Ml/d of water is flowing in the river (EA, 2013). The irrigation of polluted water will disseminate ARB and can consequently end up in the food chain with numerous reports having recorded outbreaks of pathogenic *E. coli* O157 with consumption of food contaminated from water irrigation sources. For example outbreaks in 2006, from contaminated spinach occurred in California, in 2005 in Sweden from contaminated lettuces and in 2013 in the UK, were all traced back to polluted water used for irrigation purposes (Gelting et al., 2011, Söderström A, 2008, Jenkins et al., 2015). If water is abstracted from the sites samples in the current study, ARB will result in the food chain.

Most reports present prevalence (ARG relative to 16S count) as a measure of the resistome however the work presented here suggests that abundance (absolute ARG

count) is likely more important in determining the overall risk (Gaze et al., 2011, Byrne-Bailey et al., 2011). Investigations attempting to evaluate the river as a potential source of ARB use a relative measure for comparisons between sites however using 16S will not give a suitable measure due to the varying copy number between each bacterial strain which ranges from 1-15 (Klappenbach et al., 2001). In the current study 2.5 copies was chosen based on the previous publication by Gaze *et al.,* however from the rrnDB the mode is 2, median is 4 and the mean number of copies is 4.12 therefore justifications for any of these chosen numbers would be valid (Gaze et al., 2005). No other studies could be found specifying the number of 16S copies used to standardise results so it is unclear if all studies use 2.5 and therefore whether results are in fact comparable. In addition to this, environmental samples will vary in their mode, mean and median number of 16S with populations due to different dominant bacteria in different environments. A standard set of 16S copies should be determined for each site investigated if 16S is to be used. Alternatively, abundance should be used for comparisons to provide a better indication of environmental contamination. Not all indigenous bacteria will carry ARG and therefore environmental ARG levels will be more attributed to a subpopulation of exotic species. Measuring ARG prevalence with respect to the total bacterial communities does not provide any insight to what is occurring in the environment and is a misleading way of presenting ARG contamination as it suggests a random percent of the total community will carry ARG whereas it is in fact only a subpopulation that are likely to carry these genes.

In the current study high levels of viable ARB were reported in all samples. Determining whether high abundance is due to accretion or persistence requires further investigations. An important part of the study was to evaluate the extent of accretion of exotic species such *E. coli* in sediment and the potential of these strains to persist in the environment. The level of accretion cannot be determined due to the unknown amount of CSO events that took place during the sampling regime however It can be hypothesised that the possession of certain TA genes, including *hipA*, may allow the exotic bacteria that enter the environment to form persister cells in the environment with little replication, surpassing environmental stressors (Kussell

et al., 2005, Nierman et al., 2015). *E. coli* has previously been shown to persist in soils for up to 9 years and mechanisms of persister cell formation have previously been associated with TA systems in *E. coli* (Brennan et al., 2010, Dorr et al., 2010, Sauert et al., 2016, Schumacher et al., 2009, Li et al., 2016). TA systems which had previously been associated with persister cell formation were present in all 28 *E. coli* isolates carrying the ESBL $bla_{CTX\text{-}M}$ genes and may play a role in the survival of these exotic species in the river sediment. Previous studies have demonstrated the ability of persister cells to form in water and under environmental stressors including nutrient restriction, sub inhibitory levels of antibiotics and metal oxyanions (Nierman et al., 2015, Harrison et al., 2005). Studies demonstrate the possibility of *E. coli* forming persister cells in an environment contaminated with WWTP effluent which is likely to carry sub inhibitory levels of antibiotics, biocides and metals. No studies could be found definitively demonstrating the formation of persister cells in the environment but the stresses induced in the WWTP process suggest they are likely to occur. Investigations in to persister occurrence should determine if possession of TA systems contributes in the environment. This will determine whether persistence is more important in the recovery of *E. coli* from sediment or if accretion from the WWTP is more important.

The current study evaluated the ARG and ARB potential in rivers contaminated with WWTP effluent and raw sewage released from CSOs. A diverse range of *E. coli* ST types were isolated in sediment communities and these had attributes of virulence and resistance. It is evident that WWTPs contribute to the environmental contamination of river systems which consequently can be detected 8 km downstream of effluent release. For all gene targets (except *ermF*) WWTP effluent contributed to the prevalence of genes detected at the sampling site as well as the surrounding agricultural impact. This study concludes that the *intI1* gene is not a suitable predictor of 3GC-resistant bacteria and was found at substantially higher numbers contrasting to all other gene targets. The current study suggests the wastewater treatment process should be more efficient in removing ARB and selective (antibiotics) and non-selective agents (metals and biocides) from the effluent and highlights the damaging effects of CSO release events highlighting the

importance of monitoring (and regulating) spills to prevent further ARG dissemination.
Inferences from the current study are limited by the fact it consisted of 2 sites located near one WWTP along one river system. To determine conclusively the contribution of CSO release in ARG dissemination, different sites must be investigated to cover different treatment plants using different levels of wastewater treatment (i.e primary and secondary), different population sizes served and different locations (such as agricultural regions). These factors are all assumed to influence levels of ARG and ARB, with some studies demonstrating differences in gene prevalence with different treatment plants however there are few comparative studies that conclusively report differences in prevalence and abundance of ARG and ARB as studies are primarily focussed on assessing impact at one WWTP (presumably chosen based on accessibility) (Li et al., 2015a). Monitoring of other river catchments must also be included to determine whether at different sites there are factors, other than season, which may predominate changes in river sediment populations. Other studies have previously shown variable OTU number based on the type of treatment for example, so investigating changes in treatment type with season will be of interest to determine which factors are most important (Hu et al., 2012).

In conclusion, the work presented demonstrates widespread environmental ARG and ARB dissemination consistent with previous studies. What is evident from this study is that viable potentially pathogenic *E. coli* carrying extensive AMR are able to survive in the environment with the ability to cause infection if a suitable host is present. The extensive AMR demonstrated has been documented in many other studies and emphasises the importance of finding new antimicrobial agents with activity against Gram-negative bacteria. Predominantly the work on antibiotic discovery is focussed on the discovery of compounds based on peptide antibiotics, however due to the Gram-negative cell wall these antibiotics are unlikely to be effective (Donadio et al., 2007). The recent discovery of teixobactin was the first new antibiotic class discovery using the promising iChip technology which uses a membrane to allow diffusion of antimicrobial products but so far has only identified antimicrobials with activity against Gram-positive bacteria (Ling et al., 2015). Between 2000-2015 only 30 new

antibiotics and antibiotic-inhibitor combinations were introduced and predominantly these were active against Gram positives (Butler, 2015). If the 10 million deaths per year predicted by the recent report on AMR are to be prevented then novel antibiotics need to be discovered with activity against Gram-negative bacteria (O'Neill, 2016).

Appendix

Chapter 6 Diversity within genomes of selected *E. coli* isolates

Appendix 1 Phenotypic resistance profiles of the 77 *E. coli* isolates from the river Sowe sediment

Isolate	Sequence Type	Ampicillin (25ug)	Cefotaxime (5ug)	Imipenem (10ug)	Tetracycline (10ug)	Chlorampenicol (30ug)	Erythromycin (8ug)	Sulfurazole (300ug)	Nalidixic acid (30ug)
4	ST1286	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
25	Unknown	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
26	ST453	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
27	ST3727	Yes	No	No	No	No	Yes	No	No
28	Unknown	No	No	No	No	No	Yes	Yes	No
29	ST46	Yes	Yes	No	Yes	No	Yes	Yes	Yes
30	ST212	Yes	No	No	Yes	No	Yes	Yes	Yes
31	ST4105	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
32	ST162	Yes	No	No	No	No	Yes	Yes	Yes
33	ST3576	No	No	No	No	No	No	No	No
34	ST295	Yes	Yes	No	Yes	No	Yes	Yes	No
35	ST1431	Yes	No	No	Yes	No	Yes	Yes	Yes
36	ST607	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
37	ST69	Yes	No	No	Yes	No	Yes	Yes	Yes
38	ST399	No	No	No	No	No	Yes	No	No
39	ST399	No	No	No	No	No	Yes	No	No
40	ST131	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
41	ST1421	Yes	Yes	No	Yes	No	Yes	Yes	Yes
42	ST455	Yes	Yes	No	Yes	Yes	Yes	Yes	No
47	ST38	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
48	ST131	Yes	Yes	No	Yes	No	Yes	Yes	Yes
50	ST3202	Yes	Yes	No	Yes	No	Yes	Yes	Yes
51	ST940	Yes	Yes	No	Yes	No	Yes	Yes	Yes
59	ST607	Yes	Yes	No	Yes	No	Yes	Yes	Yes
60	ST4105	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
61	ST131	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
62	ST1421	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
63	ST1421	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
64	ST3202	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
65	ST1421	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
66	ST131	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
67	ST940	Yes	Yes	No	Yes	No	Yes	Yes	Yes
70	ST154	Yes	No	No	No	Yes	Yes	No	Yes
71	ST716	Yes	No	No	No	No	Yes	Yes	Yes
72	ST940	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
75	ST940	Yes	Yes	No	Yes	No	Yes	Yes	Yes
76	ST58	No	No	No	Yes	No	Yes	Yes	Yes
77	ST940	Yes	Yes	No	Yes	No	Yes	Yes	Yes
78	ST1421	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
79	ST940	Yes	Yes	No	Yes	No	Yes	Yes	Yes
80	ST1421	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
81	ST940	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
85	ST69	No	No	No	Yes	No	Yes	Yes	No
86	ST3568	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
87	SST3202	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes

89	ST940	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
92	ST940	Yes	No	No	Yes	No	Yes	Yes	Yes
95	ST940	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
96	ST940	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
98	ST1421	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
99	ST940	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
101	ST940	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
102	ST131	Yes	No	No	No	No	Yes	Yes	Yes
103	ST3202	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
104	ST155	Yes	Yes	No	Yes	No	Yes	Yes	No
106	Unknown	Yes	Yes	No	No	Yes	Yes	Yes	No
109	ST940	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
112	Unknown	Yes	Yes	No	Yes	Yes	Yes	No	Yes
115	ST80	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
117	ST3747	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
119	ST10	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
120	ST5128	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
121	ST46	Yes	Yes	No	No	ny	Yes	Yes	No
122	ST46	Yes	Yes	No	No	Yes	Yes	Yes	No
123	ST46	Yes	Yes		Yes	Yes	Yes	Yes	No
124	ST399	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
125	ST3574	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
127	ST69	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
128	ST582	Yes	Yes	No	Yes	Yes	Yes	Yes	No
129	ST2302	Yes	Yes	No	No	Yes	Yes	Yes	Yes
130	ST3574	Yes	Yes	No	No	Yes	Yes	Yes	Yes
131	ST744	Yes	No	No	Yes	Yes	Yes	Yes	Yes
134	Unknown	No	No	No	No	No	No	No	Yes
135	ST46	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
140	ST46	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
142	ST73	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
143	Unknown	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes

Appendix 2 Illumina sequencing statistics summary

Isolate	**Contigs**	**Genome Size**	**N50**	**Median cov**	**% GC**
4	522	5380207	91724	61	50.7
25	451	4860083	22473	21	50.2
26	177	4921132	186677	57	50.2
27	175	5062444	73668	37	50.2
28	423	4943308	52968	38	50.7
29	162	5195975	156992	54	50.6
30	595	5259020	144469	62	50.5
31	145	5031929	193417	52	50.6
32	104	4718644	88662	43	50.7
33	264	5165913	43919	29	50.7
34	137	4952054	154683	65	50.7
35	679	5819703	119015	41	50.2
36	260	5317249	56030	37	50.7
37	267	5278234	70773	60	50.5
38	696	5416568	56989	39	49.8
39	192	5074375	157554	40	50.7
40	276	5105125	79288	56	50.6

41	776	5889234	73518	45	50
42	1199	6098924	65093	45	51.3
47	167	5509356	88846	49	50.6
48	290	4958431	41920	53	50.9
50	231	5280782	106592	38	50.3
51	237	5576739	156267	49	50.7
59	162	4847688	127892	60	50.5
60	117	5005130	138943	40	50.7
61	353	5160759	79288	59	50.6
62	259	5105185	82184	43	50.6
63	286	4958474	41415	88	50.9
64	272	5103727	79288	47	50.6
65	194	5504245	159178	39	50.6
66	330	5264737	43808	15	50.3
67	103	4645868	209822	75	50.5
70	294	5216878	33724	27	50.8
71	858	5114859	10025	13	50.3
72	204	5295516	70252	48	50.3
75	297	5047152	32268	24	50.5
76	238	5286544	106592	44	50.4
77	264	5103188	69051	36	50.6
78	167	5311527	95622	76	50.3
79	263	5102908	71683	39	50.6
80	235	5281842	104347	36	50.3
81	68	4842182	566858	50	50.5
85	63	4400792	308073	79	50.7
86	298	4961041	41920	76	50.9
87	236	5281588	106592	50	50.3
89	228	5280641	103941	53	50.3
92	236	5282915	103995	56	50.3
95	187	5307968	88085	47	50.3
96	218	5131883	60569	41	50.6
98	226	5278604	117998	50	50.3
99	228	5280743	103952	52	50.3
101	126	5012714	239922	82	50.7
102	259	4759293	45637	35	50.7
103	139	4807979	133537	55	50.7
104	175	4911813	144492	39	50.5
106	225	5279146	103918	39	50.3
109	225	5279146	103918	39	50.3
112	477	5155942	18371	17	50.2
115	105	4867718	222456	91	50.3
117	156	4592101	120802	37	50.7
119	115	5590136	198820	42	51.3
120	206	4774778	81344	66	50.7
121	210	4774140	81344	88	50.7
122	216	4778594	81344	89	50.7
123	315	5357216	54003	64	50.4
124	146	4803883	73724	25	50.7
125	195	5104403	146638	42	50.7
127	98	4756764	219158	53	50.5
128	55	4806836	469368	59	50.8
129	159	4817980	218129	82	50.3
130	138	4761349	119201	50	50.6
131	204	4748758	89414	35	50.7
134	280	4825041	112945	28	61

135	253	4726903	55347	79	50.8
140	200	5274466	68343	30	50.3
142	242	4817822	47219	25	50.4
143	242	4817822	185779	25	50.4

Appendix 3 MGE and plasmid incompatibility groups identified in ESBL producing strains

Isolate	Sequence Type	IS26	intI 1	intI 2	incb/o/k	IncFIA	IncFIA (HI1)	IncFII	IncFIB	IncFIC(FII)	IncI1	IncHI1B	IncX1	IncX4	IncQ1	IncN	IncR
29	**ST46**	Yes	Yes			Yes	Yes	Yes		Yes							
41	**ST1421**	Yes	Yes			Yes		Yes	Yes		Yes				Yes		
42	**ST455**	Yes		Yes				Yes	Yes		Yes	Yes	Yes				
47	**ST38**	Yes	Yes			Yes		Yes	Yes	Yes				Yes			
48	**ST131**	Yes	Yes		Yes	Yes		Yes	Yes	Yes	Yes						
50	**ST3202**	Yes	Yes					Yes	Yes								Yes
51	**ST940**	Yes		Yes				Yes	Yes			Yes	Yes				
61	**ST131**	Yes	Yes			Yes	Yes	Yes	Yes	Yes							
62	**ST1421**	Yes	Yes			Yes		Yes	Yes		Yes				Yes		
63	**ST1421**	Yes	Yes			Yes		Yes	Yes		Yes				Yes		
66	**ST131**	Yes	Yes		Yes	Yes		Yes	Yes								
67	**ST940**	Yes		Yes				Yes	Yes			Yes	Yes				
72	**ST940**	Yes		Yes				Yes	Yes	Yes			Yes				
75	**ST940**	Yes		Yes				Yes	Yes	Yes		Yes	Yes				
77	**ST940**	Yes		Yes				Yes	Yes			Yes	Yes				
78	**ST1421**	Yes	Yes			Yes		Yes	Yes		Yes				Yes		
79	**ST940**	Yes		Yes				Yes	Yes	Yes		Yes	Yes				
87	**SST3202**	Yes	Yes					Yes	Yes								Yes
89	**ST940**	Yes		Yes				Yes	Yes			Yes	Yes				
92	**ST940**	Yes		Yes				Yes	Yes			Yes	Yes				
95	**ST940**	Yes		Yes				Yes	Yes			Yes	Yes				
96	**ST940**	Yes		Yes				Yes	Yes	Yes		Yes	Yes				
98	**ST1421**	Yes	Yes		Yes	Yes		Yes	Yes	Yes	Yes				Yes		
99	**ST940**	Yes		Yes				Yes	Yes			Yes	Yes				
101	**ST940**	Yes		Yes				Yes	Yes			Yes	Yes				
109	**ST940**	Yes		Yes				Yes	Yes			Yes	Yes				
140	**ST46**	Yes	Yes						Yes							Yes	
142	**ST73**	Yes	Yes														

Appendix 4 Antibiotic class and associated genes identified in WGS of *E. coli* isolates from the Sowe sediment.

Class	Gene
Beta-lactamase	bla_{oxa}
	bla_{tem}
	bla_{DH2}
	bla_{CRP}
	bla_{ACT-7}
	bla_{CMY-63}
	$bla_{cxt-m-15}$
	$bla_{cxt-m-14}$
	$bla_{cxt-m-99}$
bacitracin	*bacA*
novobiocin and deoxycholate	*baeR*
	baeS
stress response	*cpxA*
	cpxR
	evgA
acid resistance	*evgS*
	marA
	gadX
	macA
macrolide	*macB*
	mphA
	qepA
fluoroquinolone	*qnrB1*
	qnrS1
	gyrA
	K.pneu acrR
	parC
polymyxin	*arnA*
	pmrA
	pmrB
	pmrC
	pmrE
	pmrF
	phoP
	phoQ
vancomycin	*vanG*
	TriC
streptothricin	*sat-1*
	cat
chloramphenicol	*cat1*
	catA1
trimethoprim	*drfA1*

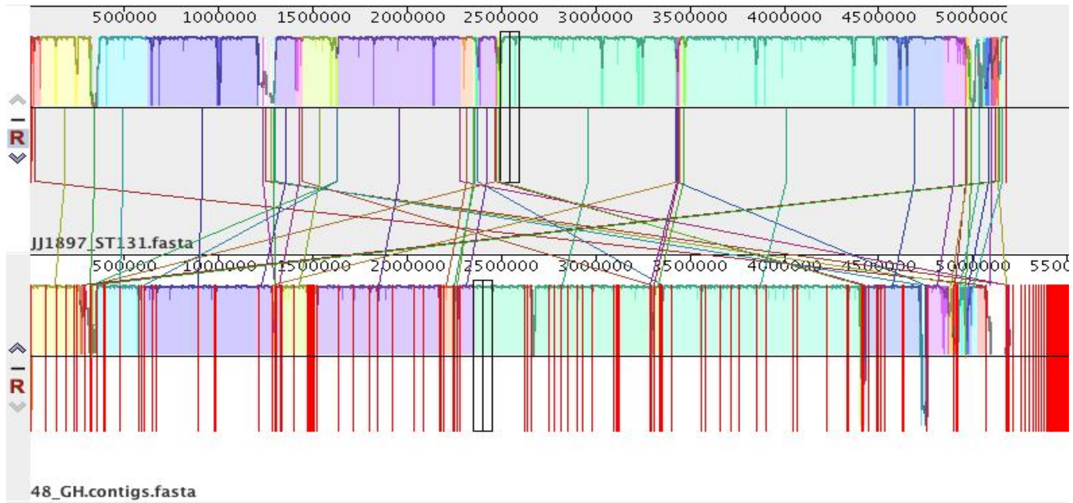
	dfrA5
	dfrA14
	dfrA17
aminoglycoside	*aada*
	aadA1
	aadA2
	aadA5
	aadA25
	aacC4
	aac(6')-lb-cr
	aac(3)-lla
	aac(3)-l
	aph(3")-lb
	aph(3')-la
	aphA1
	aphA1-lAB
	aph(6)-id
	kdpE
streptomycin	*strA*
	strB
streptothricin	*sat-1*
tetracycline	*tetA*
	tetB
	tetG
	tetc
	tetD
sulphonamide	*sul1*
	sul2

Appendix 5 Antimicrobial genes strain summaries for the 27 ESBL carrying *E. coli* isolates, continued over page.

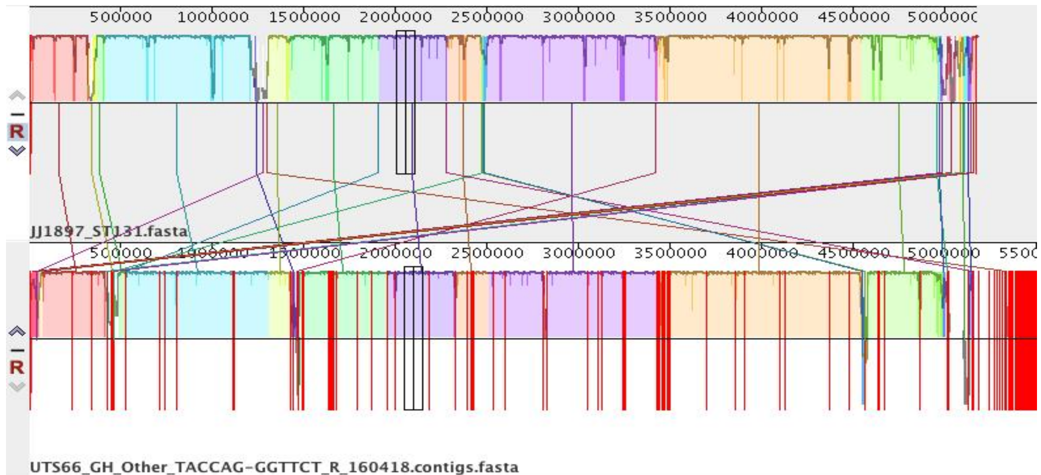
Isolate	Sequence Type	OXA	TEM	DHA-2	ctx-m-15	CRP	ctx-m-99	ctx-m-14	ACT-7	cmy-63	bacA	baeR	baeS	cpxA	cpxR	evgA	evgS	marA	K.pneu acrR	gadX	macA	macB	mphA	s.aur rpoB	m.tb rpoB	m. tb katG	qepA	qnrB1	qnrS1	gyrA	parC	arnA	pmrA	pmrB	pmrC	pmrE	pmrF	phoP	phoQ	vanG	TriC	kdpE	sat-1	cat	cat1	catAI	drfA1	dfrA5	dfrA14	dfrA17
29	ST46	Yes	Yes		Yes	Yes			Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes		Yes	Yes		Yes	Yes				Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes										Yes
41	ST1421		Yes		Yes	Yes				Yes			Yes					Yes		Yes	Yes		Yes							Yes		Yes			Yes							Yes				Yes		Yes		
42	ST455/940	Yes	Yes		Yes	Yes				Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes		Yes	Yes	Yes	Yes	Yes						Yes				Yes	Yes	Yes	Yes					Yes	Yes	Yes			Yes			
47	ST38				Yes	Yes			Yes		Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes			Yes		Yes		Yes			Yes	Yes		Yes							Yes	Yes				Yes	Yes		Yes		
48	ST131	Yes	Yes		Yes	Yes					Yes	Yes			Yes	Yes		Yes		Yes	Yes	Yes			Yes	Yes	Yes			Yes	Yes	Yes								Yes		Yes						Yes	Yes	
50	ST3202	Yes	Yes		Yes	Yes					Yes		Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes		Yes		Yes		Yes		Yes		Yes	Yes	Yes		Yes								Yes				Yes	Yes	
51	ST940	Yes	Yes		Yes	Yes				Yes	Yes	Yes	Yes	Yes	Yes		Yes		Yes	Yes	Yes	Yes	Yes			Yes				Yes	Yes	Yes		Yes	Yes	Yes	Yes					Yes	Yes	Yes			Yes			
61	ST131				Yes	Yes	Yes	Yes	Yes		Yes	Yes		Yes	Yes		Yes	Yes			Yes		Yes	Yes		Yes				Yes										Yes										
62	ST1421		Yes		Yes					Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes			Yes				Yes		Yes		Yes	Yes	Yes	Yes					Yes			Yes	Yes		Yes	Yes	
63	ST1421		Yes		Yes	Yes				Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes			Yes				Yes				Yes	Yes	Yes						Yes			Yes			Yes	Yes	
66	ST131	Yes	Yes		Yes	Yes				Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes				Yes				Yes	Yes	Yes		Yes	Yes	Yes	Yes					Yes		Yes				Yes		
67	ST940	Yes	Yes		Yes	Yes				Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes				Yes	Yes	Yes		Yes	Yes	Yes	Yes					Yes	Yes	Yes			Yes			
72	ST940		Yes		Yes	Yes			Yes		Yes	Yes	Yes	Yes		Yes	Yes	Yes		Yes	Yes	Yes	Yes		Yes	Yes				Yes	Yes	Yes								Yes			Yes				Yes	Yes		
75	ST940	Yes	Yes		Yes	Yes			Yes		Yes	Yes	Yes	Yes	Yes					Yes			Yes							Yes										Yes					Yes		Yes			
77	ST940	Yes	Yes		Yes	Yes				Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes			Yes				Yes	Yes	Yes		Yes	Yes	Yes	Yes					Yes	Yes	Yes			Yes			
78	ST1421		Yes		Yes	Yes				Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes			Yes				Yes		Yes		Yes	Yes	Yes	Yes								Yes	Yes		Yes	Yes	
79	ST940	Yes	Yes	Yes	Yes	Yes			Yes		Yes	Yes	Yes	Yes	Yes					Yes			Yes							Yes										Yes					Yes		Yes			
87	SST3202	Yes	Yes		Yes	Yes				Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes				Yes		Yes		Yes		Yes		Yes	Yes	Yes	Yes					Yes		Yes				Yes	Yes	
89	ST940	Yes	Yes		Yes	Yes				Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes						Yes	Yes	Yes		Yes	Yes	Yes	Yes					Yes	Yes	Yes			Yes			
92	ST940	Yes	Yes		Yes	Yes					Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes		Yes				Yes	Yes	Yes		Yes	Yes	Yes	Yes					Yes	Yes	Yes			Yes			
95	ST940	Yes	Yes		Yes	Yes				Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes				Yes	Yes	Yes		Yes	Yes	Yes	Yes					Yes	Yes	Yes			Yes			
96	ST940	Yes	Yes		Yes	Yes			Yes		Yes	Yes	Yes	Yes	Yes					Yes			Yes							Yes										Yes								Yes		
98	ST1421		Yes		Yes	Yes			Yes		Yes	Yes	Yes	Yes	Yes					Yes			Yes							Yes										Yes					Yes	Yes		Yes		
99	ST940	Yes	Yes		Yes	Yes				Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes				Yes	Yes	Yes		Yes	Yes	Yes						Yes	Yes	Yes			Yes			
101	ST940	Yes	Yes		Yes	Yes				Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes		Yes				Yes	Yes	Yes		Yes	Yes	Yes	Yes					Yes	Yes	Yes			Yes			
109	ST940	Yes	Yes		Yes					Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes				Yes	Yes			Yes	Yes	Yes						Yes	Yes	Yes			Yes			
140	ST46		Yes		Yes					Yes	Yes	Yes	Yes	Yes	Yes					Yes	Yes					Yes			Yes	Yes		Yes		Yes	Yes	Yes	Yes											Yes	Yes	
142	ST73		Yes		Yes	Yes			Yes		Yes	Yes	Yes	Yes	Yes					Yes								Yes		Yes										Yes								Yes		

Isolate	Sequence Type	aada	aadA1	aadA2	aadA5	aadA25	aacC4	aac(6')-Ib-cr	aac(3)-IIa	aac(3)-I	aph(3")-Ib	aph(3')-Ia	aphA1	aphA1-IAB	aph(6)-id	strA	strB	tetA	tetB	tetG	tetc	tetD	sul1	sul2
29	ST46				Yes		Yes	Yes	Yes	Yes	Yes				Yes	Yes	Yes	Yes		Yes			Yes	Yes
41	ST1421								Yes	Yes		Yes		Yes	Yes	Yes	Yes	Yes	Yes			Yes		Yes
42	ST455/940		Yes	Yes			Yes	Yes	Yes		Yes				Yes	Yes	Yes	Yes	Yes			Yes		Yes
47	ST38		Yes	Yes		Yes				Yes								Yes	Yes				Yes	
48	ST131						Yes	Yes	Yes	Yes	Yes				Yes	Yes	Yes	Yes		Yes				Yes
50	ST3202						Yes	Yes	Yes	Yes	Yes				Yes	Yes	Yes	Yes		Yes				Yes
51	ST940		Yes	Yes			Yes	Yes	Yes		Yes				Yes	Yes	Yes		Yes			Yes		Yes
61	ST131									Yes	Yes				Yes	Yes	Yes	Yes		Yes			Yes	Yes
62	ST1421						Yes		Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes			Yes		Yes
63	ST1421								Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes			Yes		Yes
66	ST131						Yes	Yes	Yes	Yes	Yes				Yes	Yes	Yes	Yes		Yes				Yes
67	ST940		Yes	Yes			Yes	Yes	Yes		Yes				Yes	Yes	Yes	Yes	Yes			Yes		Yes
72	ST940		Yes	Yes							Yes				Yes	Yes	Yes	Yes	Yes			Yes		Yes
75	ST940		Yes	Yes			Yes									Yes	Yes		Yes					
77	ST940	Yes	Yes	Yes			Yes	Yes	Yes		Yes				Yes	Yes	Yes	Yes	Yes			Yes		Yes
78	ST1421								Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes			Yes		Yes
79	ST940		Yes	Yes	Yes		Yes									Yes	Yes		Yes			Yes		Yes
87	SST3202						Yes	Yes	Yes	Yes	Yes				Yes	Yes	Yes	Yes		Yes				Yes
89	ST940	Yes	Yes	Yes			Yes	Yes	Yes		Yes				Yes	Yes	Yes	Yes	Yes			Yes		Yes
92	ST940	Yes	Yes	Yes			Yes	Yes	Yes		Yes				Yes	Yes	Yes	Yes	Yes			Yes		Yes
95	ST940		Yes	Yes			Yes	Yes	Yes		Yes				Yes	Yes	Yes	Yes	Yes			Yes		Yes
96	ST940		Yes	Yes			Yes									Yes	Yes		Yes					Yes
98	ST1421									Yes			Yes	Yes		Yes	Yes		Yes					Yes
99	ST940	Yes	Yes	Yes				Yes	Yes		Yes				Yes	Yes	Yes	Yes	Yes		Yes			Yes
101	ST940	Yes	Yes	Yes			Yes	Yes	Yes					Yes	Yes	Yes	Yes	Yes	Yes			Yes		Yes
109	ST940		Yes	Yes			Yes	Yes	Yes		Yes				Yes	Yes	Yes		Yes			Yes		Yes
140	ST46									Yes	Yes				Yes	Yes	Yes	Yes		Yes				Yes
142	ST73									Yes						Yes	Yes							Yes

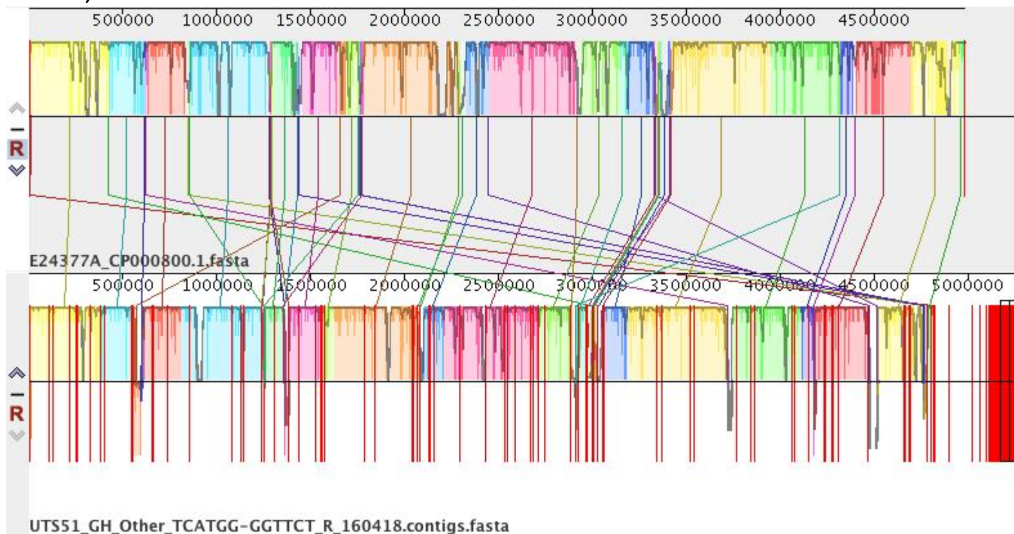
Isolate	Sequence Type	emrA	emrB	emrD	emrE	emrK	emrR	emrY	mdtC	mdtD	mdtE	mdtG	mdtH	mdtK	mdtL	mdtM	mdtN	mdtO	mdtP	mdtF	mdtA	mfd	tolC	marR	robA	soxR	smeA	cusA	merA	terA	cusA
29	ST46	Yes		Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes			Yes	Yes		
41	ST1421	Yes	Yes			Yes			Yes	Yes	Yes			Yes			Yes	Yes	Yes	Yes	Yes	Yes	Yes					Yes	Yes		
42	ST455/940	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes		Yes			Yes	Yes		
47	ST38	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes		Yes			
48	ST131	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes			
50	ST3202	Yes		Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes		Yes			Yes			
51	ST940	Yes				Yes	Yes	Yes	Yes	Yes			Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes		Yes	Yes	Yes			Yes	Yes		
61	ST131	Yes							Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes			Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes
62	ST1421	Yes	Yes	Yes	Yes	Yes			Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes				Yes	Yes		
63	ST1421	Yes	Yes	Yes	Yes	Yes			Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes			Yes	Yes		
66	ST131	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes			Yes			
67	ST940	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes			Yes	Yes		
72	ST940	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes		Yes			Yes			
75	ST940	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes					Yes	Yes		
77	ST940	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes		Yes	Yes		Yes	Yes	Yes		Yes			Yes	Yes		
78	ST1421	Yes	Yes	Yes	Yes	Yes			Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes				Yes	Yes		
79	ST940	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes					Yes	Yes		
87	SST3202	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes			Yes			
89	ST940	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes			Yes	Yes		
92	ST940	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes			Yes	Yes		
95	ST940	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes			Yes	Yes	Yes	Yes				Yes	Yes		
96	ST940	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes					Yes	Yes		
98	ST1421	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes					Yes	Yes		
99	ST940	Yes	Yes	Yes	Yes				Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes				Yes	Yes		
101	ST940	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes				Yes	Yes		
109	ST940	Yes	Yes	Yes	Yes	Yes			Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes				Yes	Yes		
140	ST46	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes				Yes			
142	ST73	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes					Yes			



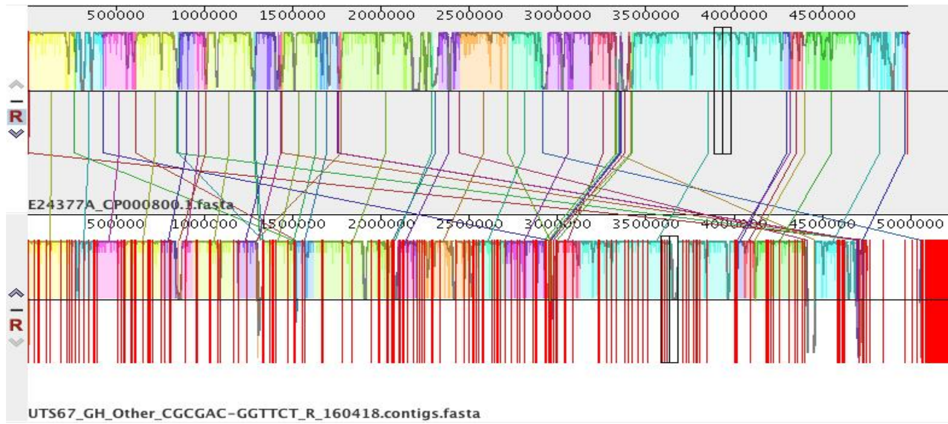
Appendix 6 MAUVE alignment between strain 48 identified as ST131 and known ST131, JJ897



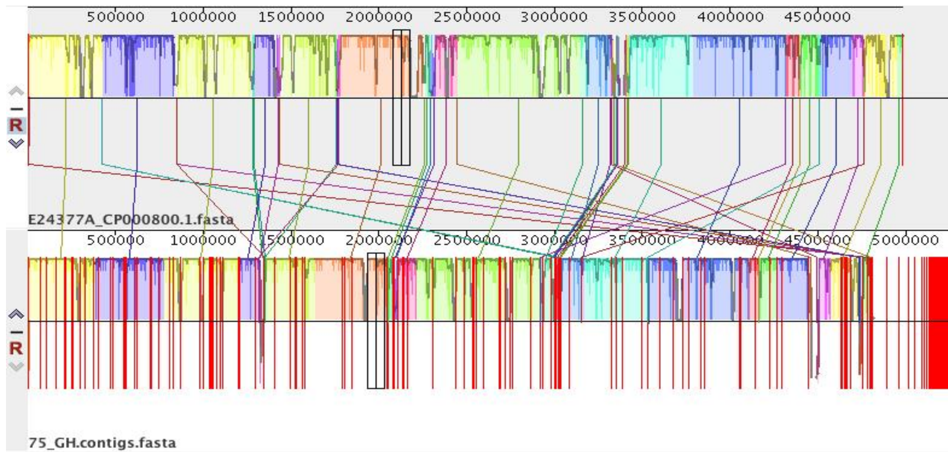
Appendix 7 MAUVE alignment between strain 66 identified as ST131 and known ST131, JJ897



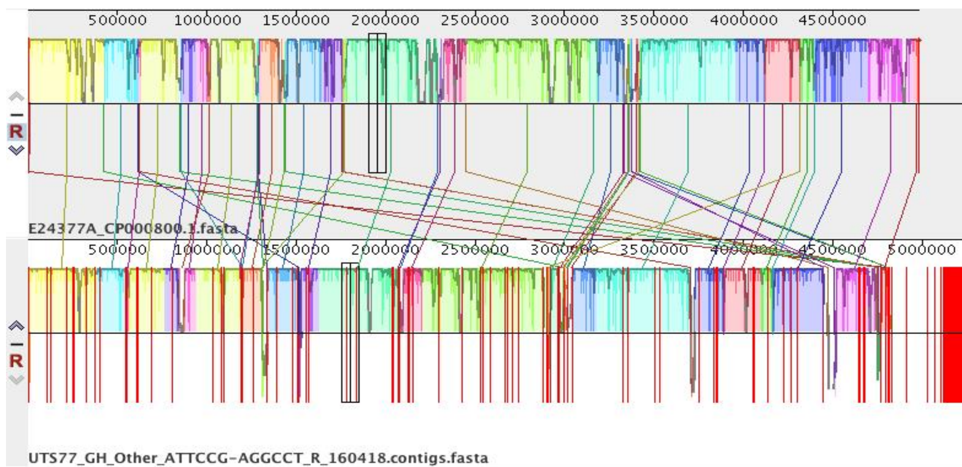
Appendix 8 Alignment between strain 51 (ST940) and reference strain E24377A



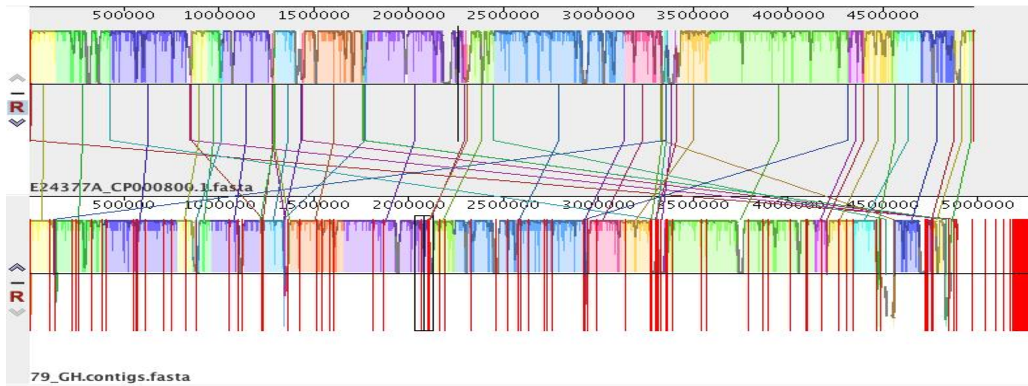
Appendix 9 Alignment between strain 67 (ST940) and reference strain E24377A



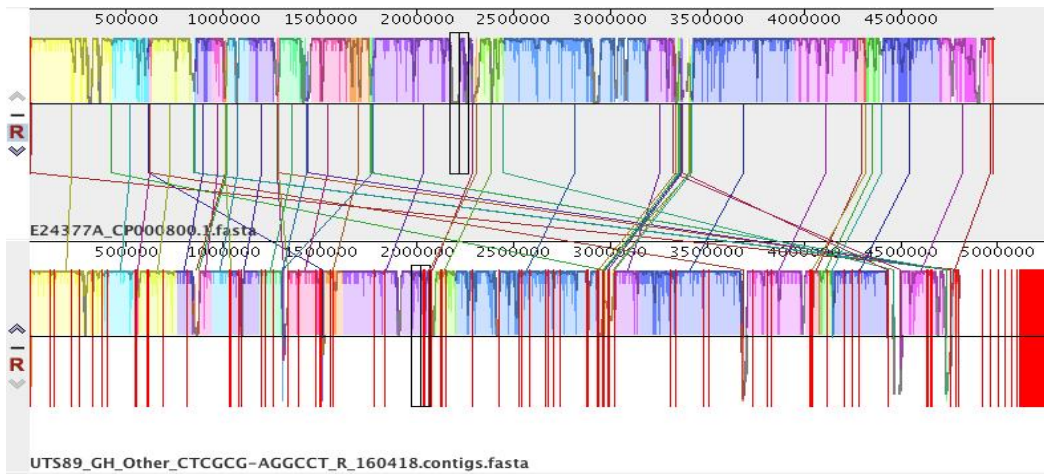
Appendix 10 Alignment between strain 75 (ST940) and reference strain E24377A



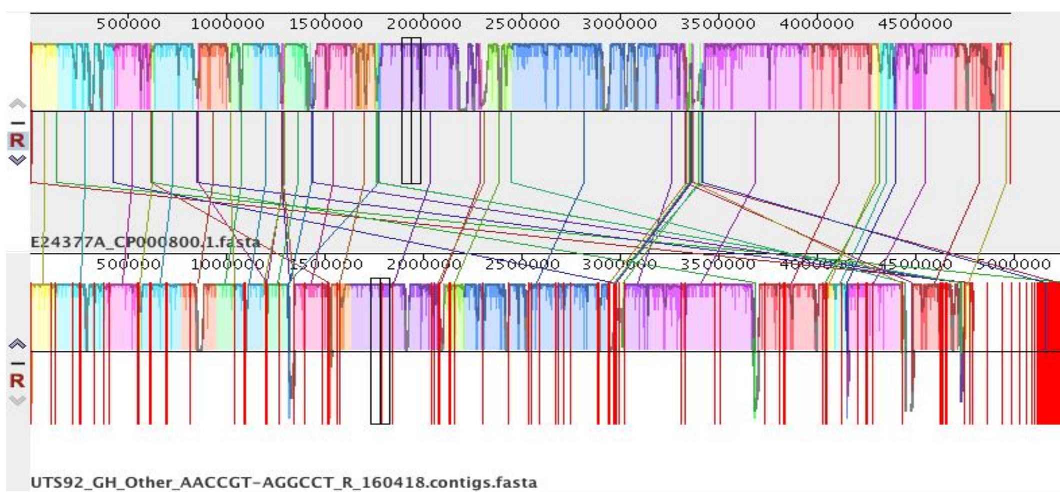
Appendix 11 Alignment between strain 77 (ST940) and reference strain E24377A



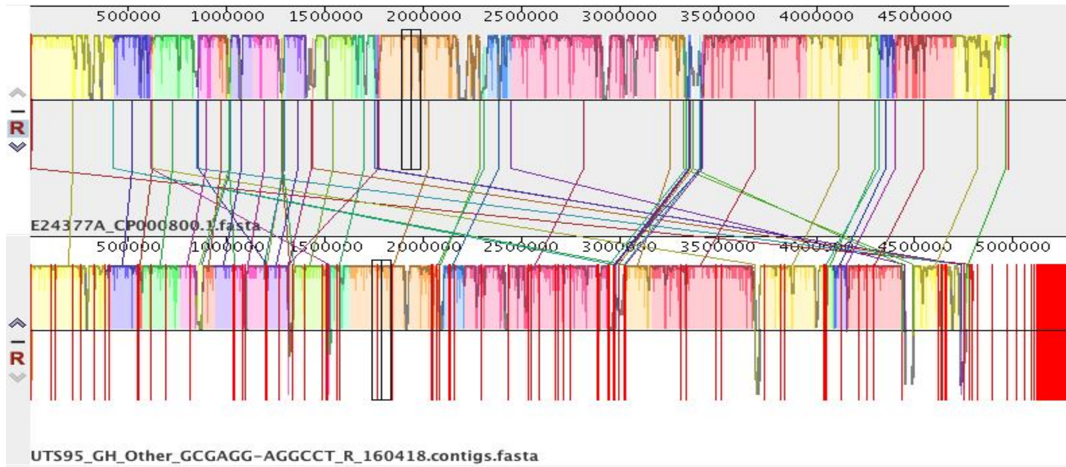
Appendix 12 Alignment between strain 79 (ST940) and reference strain E24377A



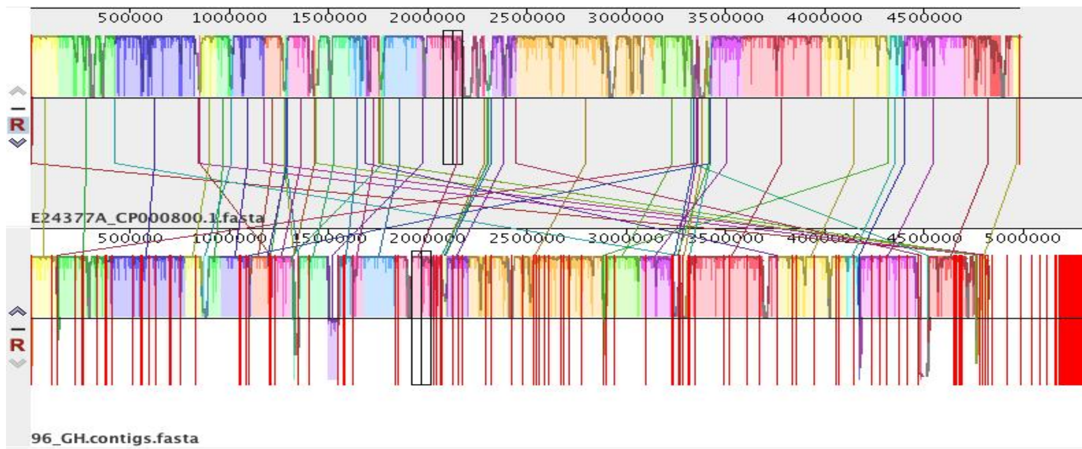
Appendix 13 Alignment between strain 89 (ST940) and reference strain E24377A



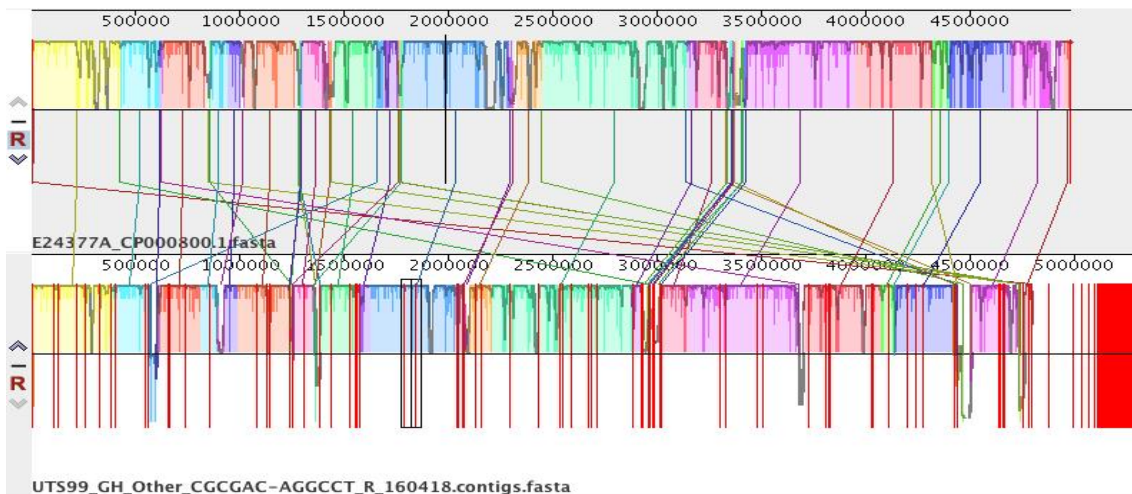
Appendix 14 Alignment between strain 92 (ST940) and reference strain E24377A



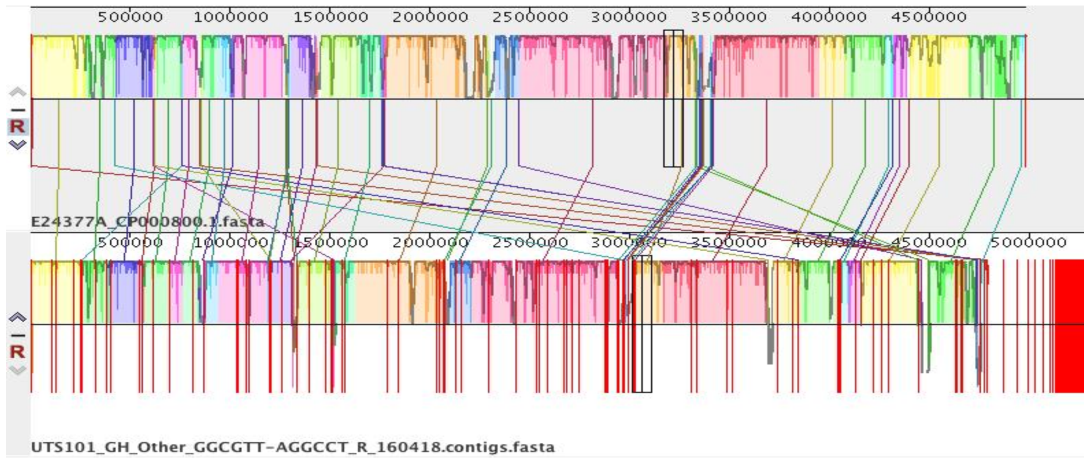
Appendix 15 Alignment between strain 95 (ST940) and reference strain E24377A



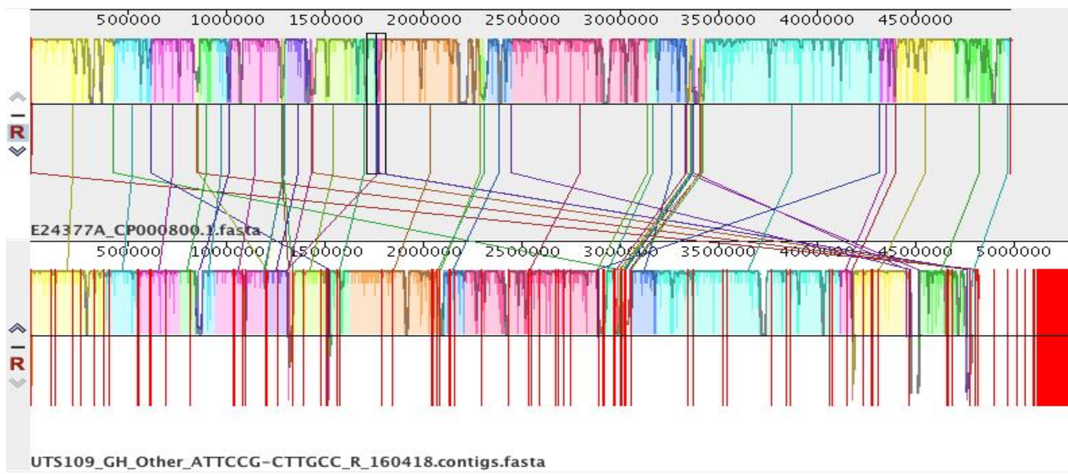
Appendix 16 Alignment between strain 96 (ST940) and reference strain E24377A



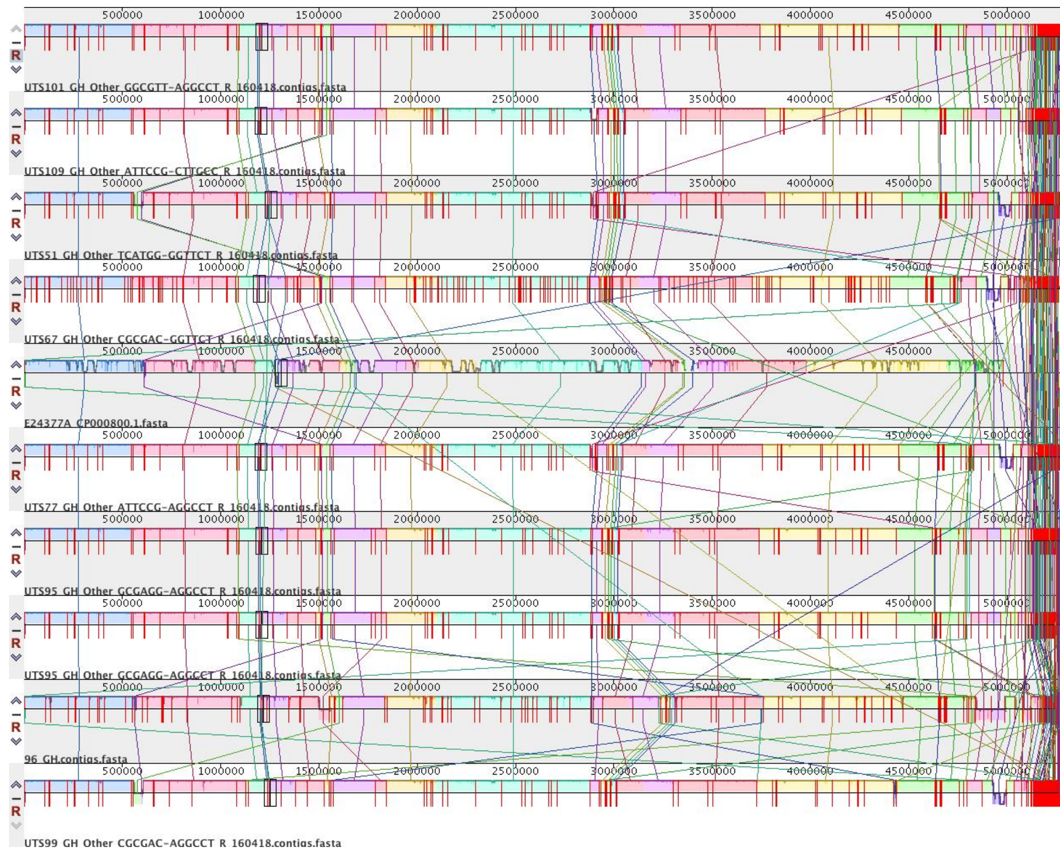
Appendix 17 Alignment between strain 99 (ST940) and reference strain E24377A



Appendix 18 Alignment between strain 101 (ST940) and reference strain E24377A



Appendix 19 Alignment between strain 109 (ST940) and reference strain E24377A



Appendix 20 Aligned ST940 strains using progressive MAUVE

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