# Selection for antibiotic resistance in the aquatic environment: novel assays to detect effect concentrations of micropollutants

Submitted by Aimee Kaye Murray, to the University of Exeter as a thesis for the degree of *Doctor of Philosophy* in Medical Sciences, August 2017.



I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

(Signature) ……

**Abstract**

The environment is increasingly recognised as a key player in the emergence and mobilisation of antibiotic resistance, which negatively impacts human health, healthcare systems, and farming practices worldwide. Recent work has demonstrated concentrations of antibiotics in the natural environment may select for resistance *in situ*, but a scarcity of meaningful data has prevented rigorous environmental risk assessment of antibiotics. Without such data, mitigation strategies, such as improved antibiotic stewardship or environmental discharge limits, cannot be effectively designed or implemented.

This thesis designed and developed two methods for determining effect concentrations of antibiotics in complex microbial communities, thereby generating a significant amount of data to address this knowledge gap. Minimal selective concentrations (MSCs) were determined in long term selection experiments for four classes of antibiotic at concentrations as low as 0.4 µg/L, which is below many measured environmental concentrations. Lowest observed effect concentrations were determined using a short term, growth based assay which were highly predictive of MSCs. A novel finding was significant selection for cefotaxime resistance occurred at a wide range of antibiotic concentrations, from 125 µg/L - 64 mg/L, which has important clinical implications. Determination of MSC in single species assays was also shown to be a poor predictor of MSC in a complex microbial community.

Co-selection for antimicrobial resistance was demonstrated in selection experiments and through improved understanding of class 1 integron evolution, assessing selective effects on resistance gene acquisition using a novel PCR method and next-generation sequencing. In the final study, a novel resistance determinant (UDP-galactose 4-epimerase) conferring cross-resistance to biocides and antibiotics was discovered, providing a target for further study.

These findings indicate selection and co-selection for antimicrobial resistance is likely to occur in the environment, and provides the means to rapidly generate further data to aid in the development of appropriate mitigation strategies.

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## Author's declaration

All the work within is the author's own work, under the supervision of the named supervisors. Where advice has been sought or other people's data has been used, this has been noted in the text.

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**Definitions**

| Term | Definition |
|---|---|
| *A priori* | Knowledge of a fact based on theoretical deduction rather than experimental evidence. |
| Antibiotic | A chemical compound used to treat bacterial infections in humans or animals by killing the bacterium or preventing its growth. |
| Antibiotic resistance | Ability of a bacterium to survive antibiotic therapy due to presence of mutations/mobile genetic elements containing resistance genes which prevent cell death or growth inhibition. |
| Antimicrobial | A chemical compound used to kill micro-organisms (including bacteria, fungi, protozoa and viruses) or inhibit their growth. Includes antibiotics and biocides. |
| Antimicrobial resistance | Ability of a micro-organism to survive exposure to an antimicrobial due to presence of mutations / mobile genetic elements containing resistance genes which prevent cell death or growth inhibition. |
| Array | Referring to gene cassettes – several adjacent gene cassettes in an integron form an array. |
| Bacteria | A large group of prokaryotic organisms. |
| Bactericidal | An antibiotic which kills bacteria. |
| Bacteriostatic | An antibiotic which prevents bacterial cell growth. |
| Beta-lactam (β-lactam) | A class of antibiotics which prevents cellular division and growth through binding penicillin binding proteins, thereby preventing cell wall synthesis. |
| Beta-lactamase (β-lactamase) | An enzyme capable of degrading members of the β-lactam antibiotic class. |
| Biocide | An antimicrobial not used in chemotherapy but as a disinfectant or detergent. |
| Biofilm | Extracellular matrix comprising of excreted proteins and DNA excreted by sessile bacteria to allow attachment to surfaces or particles, prevent desiccation, reduce susceptibility to antimicrobials etc. |

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| Cephalosporin | An antibiotic belonging to the beta-lactam class of antibiotics. There are five generations of cephalosporins (e.g. cefotaxime is a third generation cephalosporin). |
| Commensal | A non-pathogenic bacterial species which co-exists with other commensals, pathogens and opportunistic pathogens in a bacterial community. |
| Competent | Bacterial cells which are able to take up and integrate DNA. |
| Conjugation | A mechanism of horizontal gene transfer whereby a donor bacterial cell forms a pilus to connect to the recipient cell, through which DNA is exchanged (commonly plasmids). |
| Co-resistance | When a bacterium is resistant to two or more antimicrobial compounds due to co-localisation of two or more resistance genes (most commonly on mobile genetic elements such as plasmids or integrons). |
| Co-selection | The process whereby resistance to one or more antimicrobial compounds is selected, due to either co-resistance or cross-resistance. |
| Cross-resistance | When a bacterium is resistant to two or more antimicrobial compounds due to presence of a single resistance mechanism which confers resistance to more than one compound (e.g. a multidrug efflux pump). |
| *De novo* | Completely new or novel. |
| Dose-response relationship | Where a variable (e.g. resistance) increases with treatment dose (e.g. antibiotic concentration). |
| Environment | The natural environment (including soils, lakes, rivers, sediments, and the sea). |
| Environmental resistome | The reservoir of resistance genes in all bacteria in the natural environment. |
| Extended spectrum beta-lactamase (ESBL) | A type of beta-lactamase which has extended spectrum of activity i.e. can degrade 2$^{nd}$ and 3$^{rd}$ generation beta-lactams, as opposed to β-lactamases which degrade only 1$^{st}$ generation β-lactams. |

| | |
|---|---|
| Fitness advantage | The benefit conferred by a given trait on an organism's ability to survive, grow and / or reproduce. |
| Fitness cost | The disadvantage conferred by a given trait on an organism's ability to survive, grow and / or reproduce. |
| Gene cassette | A short, extracellular, circular piece of DNA containing one or more genes (often resistance genes) and the *attC* site to allow integration into integrons. |
| Gram positive bacteria | Classification term for bacteria which only have plasma membrane, periplasmic space and thick peptidoglycan layer in their cell wall. |
| Gram negative bacteria | Classification term for bacteria which have plasma membrane, a thin layer of peptidoglycan sandwiched between two layers of perisplasmic space, and an outer membrane (lipopolysaccharide layer) as constituents of their cell wall. |
| Horizontal Gene Transfer (HGT) | The movement or resistance genes and/or mobile genetic elements between bacteria of the same or even distantly related species by conjugation, transduction or transformation. |
| *In situ* | In place. |
| *In vitro* | In a laboratory setting; not within a living organism. |
| *In vivo* | In real life. |
| Indirect selection | When susceptible bacteria are able to grow following antibiotic administration due to presence of resistant bacteria which degrade the antibiotic, allowing susceptible bacteria to grow once the antibiotic concentration reaches a low enough concentration. |
| Integrase | An enzyme encoded for by the integrase ('*int*') gene on integrons which facilitates site specific recombination between the *attI* site in integrons and the *attC* site on gene cassettes. |
| Integron | A genetic structure comprising of an integrase gene, an *attI* site, and usually one or more gene cassettes. |

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|---|---|
| Isogenic | Refers to two or more bacteria which have completely identical genomes and accessory genomes. |
| Minimum inhibitory concentration (MIC) | The lowest concentration of an antimicrobial which kills / prevents growth of a bacterium. |
| Minimal selective concentration (MSC) | The lowest concentration of an antimicrobial at which positive selection for resistance occurs. |
| Microbiome | All of the bacteria living on or in another living thing (e.g. human / animal / plant microbiome). |
| Mobile genetic element (MGE) | A piece of DNA which can be transferred horizontally, e.g. plasmids, integrons. |
| Mobilisation | When a mobile genetic element moves from one bacterial cell to another, or gains the ability to be mobilised (e.g. by being relocated to a plasmid by a transposase). |
| Mutation | Used to describe changes in both DNA and amino acid sequence, which may be either advantageous or disadvantageous in terms of fitness. |
| Opportunistic pathogen | A bacterium which does not normally cause disease in a healthy host, but can infect immunocompromised hosts. |
| Pathogen | A bacterium which infects and causes disease in the host. |
| Plasmid | An extracellular, circular piece of DNA; which is also a mobile genetic element. |
| Polymerase chain reaction | A molecular technique used to amplify target DNA of interest exponentially. |
| Point mutation | A mutation occurring in one single DNA base. |
| Proto-resistome | All the genes in the environment which have the ability to form part of the resistome, either through a point mutation or mobilisation from the chromosome to a mobile genetic element. |
| Real-time PCR (qPCR) | Real-time or quantitative PCR is used to exponentially amplify target DNA in real time, quantitatively. This allows quantification of the numbers of target genes in the original DNA sample. |

| | |
|---|---|
| Resistome | The reservoir of resistance genes in a given environment. |
| Transduction | A mechanism of horizontal gene transfer, whereby bacteriophage capture bacterial DNA during the infection cycle and then release this DNA into a new bacterial cell upon infection. |
| Transformation | A mechanism of horizontal gene transfer. Competent bacterial cells can absorb DNA from the environment released by other bacteria e.g. during biofilm formation or cell lysis following death. |
| Transposase | An enzyme which can excise itself and surrounding genes and reintegrate itself into a new location. |

## Abbreviations

| Abbreviation | Definition |
|---|---|
| 3GC(s) | Third generation cephalosporin(s) (e.g. cefotaxime) |
| AVMA | American Veterinary Medical Association |
| βLI(s) | β-lactamase Inhibitor(s) (e.g. avibactam) |
| bp | Base Pairs (of DNA) |
| cfu | Colony Forming Units |
| CDC | Centre for Disease Control and Prevention |
| ECDC | European Centre for Disease Control and Prevention |
| ESBL | Extended-spectrum β-lactamase |
| EU | European Union |
| EUCAST | European Committee on Antimicrobial Susceptibility Testing |
| DNA | Deoxyribonucleic Acid |
| HGT | Horizontal Gene Transfer |
| kb | Kilo Base Pairs (of DNA) |
| LOEC | Lowest Observed Effect Concentration |
| MEC | Measure Environmental Concentration |
| MGE | Mobile Genetic Element |
| MIC | Minimum Inhibitory Concentration |
| MSC | Minimal Selective Concentration |
| OECD | Organisation for Economic Co-operation and Development |
| PEC | Predicted Environmental Concentration |
| PCR | Polymerase Chain Reaction |
| PICT | Pollution-induced Community Tolerance Assay |
| PNEC | Predicted No Effect Concentration |
| $PNEC^R$ | Predicted No Effect Concentration for Resistance selection |
| QAC(s) | Quaternary Ammonium Compound(s) |
| qPCR | Real-time Polymerase Chain Reaction |
| ROS | Reactive Oxygen Species |
| RQ | Risk Quotient |
| UBA | UmweltBundesamt (German Environment Agency) |
| WHO | World Health Organisation |
| WWTP(s) | Waste Water Treatment Plant(s) |

# Chapter one: Literature review

## 1.1. Background

Antibiotic resistance is ancient, as it has been detected in pristine environments which predate the Anthropocene (D'Costa et al., 2011, Allen et al., 2009, Hall and Barlow, 2004). However, since the first clinical use of antibiotics in the 1940's (Zaffiri et al., 2012), the rate that resistance is emerging has steadily increased (Morrissey et al., 2013). The 'golden age' of antibiotic discovery has ended, with few antibiotics developed for clinical use over the last few decades (CDC, 2013). With some isolated cases of pan-resistance already being documented (Chen et al., 2017), we are on the path towards a 'post-antibiotic' era where routine infections cannot be effectively treated and as a result, other modern medical practices (routine surgery, organ transplants, cancer treatment) will be compromised (Livermore, 2009, Laxminarayan et al., 2013).

Estimated figures for global mortality rates and financial losses paint a bleak picture, with 10 million estimated annual deaths and a $100.2 trillion dollar reduction in global GDP by 2050 if resistance continues to emerge at its current rate (O'Neill, 2014). Research to determine the causes of increased resistance and treatment failure is of critical importance, so novel mitigation strategies can be designed and implemented to enhance current antimicrobial stewardship.

Until recently, research has focused on selection for resistance in the clinic; despite the fact that environmental, antibiotic resistant bacteria may act as a reservoir of resistance mechanisms acquirable by pathogens (Wellington et al., 2013). Antibiotics are also present in the environment, albeit at much lower concentrations (Kummerer, 2009a) than at point of use and it is currently unknown whether these environmental concentrations of antibiotics can select for *de novo* resistance (or maintain and increase transfer of pre-existing resistance determinants, or alter their expression), *in situ*.

Below is a brief overview of antibiotic resistance. The different pathways of how antibiotics and antibiotic resistance genes / bacteria enter the environment are described, as well as how these resistance genes can be mobilised within bacterial populations. The concept that environmental concentrations of antibiotics can select for antibiotic resistance is introduced. Finally, methods to

research this area are evaluated and ideas for how the area can progress are discussed.

## 1.2. Environmental resistome

The environmental resistome is all environmental genes which can contribute to or confer antibiotic resistance. It has long been recognised as a potential reservoir for pathogenic bacteria to acquire novel resistance (Wright, 2010), for example by mobilisation of a previously non-mobile resistance gene situated on the chromosome. Soil archaea may also contribute to the resistome, but there is little work in this area (Chee-Sanford et al., 2009).

Resistance is ancient and widespread (even in pristine areas). For example, a metagenomic study conducted on soil taken from a remote, pristine, Alaskan site discovered ancient β-lactamase genes and a previously unobserved bi-functional β-lactamase (Allen et al., 2009). Phylogenetic analysis of β-lactamases revealed they evolved over 2 billion years ago (Hall and Barlow, 2004). D'Costa et al. (2011) also found resistance genes in ancient permafrost via metagenomic analysis, and suggest selection for these pre-existing resistance determinants may be a cause for the rapid emergence of resistance we observe today, rather than selection of completely novel resistance genes. Maintenance of these genes in absence of an antibiotic selective pressure is possible (see 1.2. Selection for resistance).

In fact, both ancient and modern, naturally-occurring antibiotics may have a variety of ecological functions in bacterial communities, aside from growth inhibition of nearby competitors. Antibiotics may act as signalling molecules at sub-inhibitory concentrations (Aminov (2009), and can also be used as a carbon source (Wright, 2007, D'Costa et al., 2006). Aminov (2009) suggests the spread of resistance in the modern era is a result of positive selection for genes performing these roles, which by chance, also confer resistance as a secondary function. Therefore anthropogenic pollution of the environment may simply increase selection for pre-existing resistance determinants, but further work is required before this can be confirmed.

## 1.3. Selection for resistance

The Minimum Inhibitory Concentration (MIC) is the measured antibiotic concentration at which bacterial growth is inhibited. The ecological cut-off value (ECOFF) is the upper limit MIC of the wild-type bacteria in the population, and is used to describe emergence of 'ecological' resistance (Turnidge et al., 2006, Olivares et al., 2013). This differs to clinical definitions of resistance based on the likelihood of therapy failure; and as such, ecologically resistant bacteria may be considered susceptible in terms of clinical break points (Olivares et al., 2013). From here on, 'resistance' is used in terms of both the ecological and clinical perspective, though clinical resistance will be specified by comparison to published clinical breakpoint antibiotic concentrations (EUCAST, 2014).

Resistance can be either intrinsic or acquired. Intrinsic resistance is innate and conferred by several mechanisms including absence or modification of the drug target, production of enzymes which inactivate the compound, reduced drug uptake due to decreased permeability or increased number of efflux pumps; or even sequestration of the antibiotic inside the bacterial cell (Olivares et al., 2013, Rodriguez-Rojas et al., 2013, Taylor et al., 2011). Intrinsic resistance does not arise as a consequence of horizontal gene transfer ('HGT' (Olivares et al., 2013)). Conversely, acquired resistance arises through horizontal acquisition of whole genes; acquisition of mutation(s) in housekeeping genes; or mutations in inessential duplicated genes. This pool of genes with the potential to become antibiotic resistance genes has been termed the 'hidden resistome' (Baquero et al., 2008).

Acquired resistance is often associated with a fitness cost. For example, a mutation which alters the antibiotic target may impair the original function of that target; resistance genes which code for antibiotic-degrading enzymes will also increase the metabolic burden on the bacterium (Enne et al., 2005). This is why the adaptive mutation mechanism (whereby gene copy numbers are increased, so each can acquire different mutations to generate slightly different resistance mechanisms) concludes with elimination of extra gene copies (Blazquez et al., 2012), to reduce the burden of superfluous metabolic processes. Additionally, metabolic costs as a result of resistance can be offset through reorganisation of metabolic pathways (Handel et al., 2013) or through compensatory mutations (Enne et al., 2005, Baquero, 2001, Durao et al., 2015).

In rare cases, resistance genes can even confer a fitness advantage as with *qnr*A3 (a quinolone resistance gene), which when present on a small plasmid carried by *Escherichia coli* resulted in greater bacterial growth than the same strain bearing the same plasmid, but without *qnr*A3 (Michon et al., 2011). Some resistance determinants can also have neutral fitness, meaning resistance (once emerged) can be difficult to eradicate (Enne et al., 2005).

The lowest concentration at which selection can occur is known as the Minimal Selective Concentration ('MSC' (Andersson and Hughes, 2012)). The fact resistance can be maintained in the presence of very low antibiotic concentrations suggests the resistance determinant could confer a low fitness cost, and worryingly, is therefore more likely to become fixed in the population, particularly in the absence of antibiotic selection (Andersson and Hughes, 2012). It is also important to note that sub-MIC selection has clinical implications, as antibiotics have varying diffusion rates, resulting in different antibiotic concentrations in different body compartments. This may also allow selection for resistant bacteria *in vivo* at sub-therapeutic concentrations (Pena-Miller et al., 2013).

Antibiotics are hormetic compounds (meaning low doses can sometimes result in increases in bacterial growth, as shown previously with low level tetracycline treatment resulting in increased colony forming units (Migliore et al., 2013)). Therefore, they can have dose-dependent effects on bacteria (though these are not well described) and many of these can lead to resistance acquisition. For example, sub-inhibitory concentrations of β-lactams induce release of DNA by *Staphylococcus aureus,* which promotes biofilm formation and cell aggregation, thereby potentially increasing its own resistance levels and those of other community members indirectly (Kaplan, 2011). Sub-MIC concentrations of antibiotics can also upregulate expression of toxins in *E. coli* and *S. aureus*, increase rates of HGT and increase mutagenesis by generation of reactive oxygen species ('ROS' (Gutierrez et al., 2013)). ROS can stall the replication fork or inhibit DNA gyrase leading to many transcriptional changes (Lopez et al., 2007), the foremost of these being transcription of a specialised DNA polymerase which can bypass DNA lesions with reduced fidelity (Blazquez et al., 2012). This synchronized reaction that can lead to mutation (and potentially acquired resistance) is known as the 'SOS' response, which is induced by a multitude of different stresses, including some antibiotics (Baquero et al., 2013).

Antibiotic induced hormesis has also been shown to increase tolerance to heat shock and ROS (Mathieu et al., 2016) and so can increase general fitness.

Antibiotics can also increase levels of HGT. Tetracycline has been shown to increase transcription of the *rteC* gene which controls excision of genes and mobile genetic elements (Moon et al., 2005). Kim et al. (2014a) monitored transfer of the Inc resistance plasmid from *E. coli* to *P. aeruginosa* or sewage sludge recipients, and found an increasing dose-response relationship for transfer rates. However, there have also been cases where antibiotics have been found to decrease the rate of HGT (Riedl et al., 2000), or have no effect on transfer rates (Cottell et al., 2012).

The mechanisms are varied and complex, but there is enough supporting evidence to suggest that sub-MIC concentrations of antibiotics may not only select for pre-existing resistant clones, but also promote the generation of novel resistance mechanisms and the movement of these within bacterial populations.

## 1.4. Costs of resistance

Resistance to an antibiotic often arises soon after it is introduced as a therapeutic agent (Figure 1 (CDC, 2013)) which has significant clinical, social and economic repercussions. Infections caused by antibiotic resistant bacteria can increase mortality rates and cost of hospital care due to prolonged hospital stay (Neidell et al., 2012), requirement for multiple courses of treatment, and in extreme cases the need for patients to be isolated. It has been conservatively estimated that greater than 2 million cases of antibiotic resistant bacterial infections occur in the USA each year, with around 23,000 of these directly causing death. Around 26,000 of these infections are caused by extended-spectrum β-lactamase producing *Enterobacteriaceae*, which cost an extra estimated $40,000 per case (CDC, 2013).

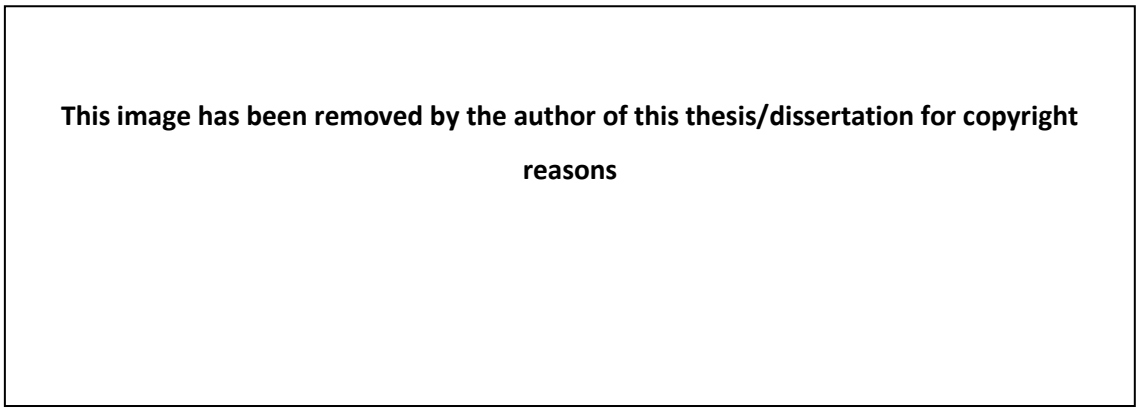


Figure 1. Based on the figure on page 28 from the report by the CDC (2013). Resistance to a drug soon arises after development and use in the clinic.

In Europe, a report by the European Centre for Disease Control and Prevention (ECDC) and the European Medicines Agency (EMA) found 25,000 deaths are caused by multi-drug resistant bacteria annually, with 2.5 million extra hospital days required for treatment of drug-resistant bacterial infections, amounting to estimated costs of resistance of €1.5 billion per year (ECDC/EMEA, 2009). Subsequently, the European Union (EU) funded the BURDEN project ('Burden of Resistance and Disease in European Nations') which found that mortality rates in patients infected with resistant bacteria were significantly higher than in patients infected with susceptible bacteria (Lambert et al., 2011). Specifically, for third generation cephalosporin-resistant *E. coli* blood stream infections, 30 day mortality rate was 2.5x higher and hospital stay was on average 5 days longer in patients infected with resistant *E.coli* compared to those infected with susceptible *E. coli* (de Kraker et al., 2011).

In 2014, a Review on Antimicrobial Resistance was commissioned by British Prime Minister David Cameron to propose concrete solutions to the global threat of antimicrobial resistance, which if left unaddressed, could be 'catastrophic' according to England's Chief Medical Officer Dame Sally Davies (McKenna, 2013). Chaired by economist Jim O'Neill, the committee produced several publications until its final report in May 2016 which estimated that should resistance continue to emerge at its current rate, by 2050 there will be a human casualty resulting from an antibiotic resistant infection every three seconds (O'Neill, 2015). Other doomsday scenarios predict that infections caused by resistant bacteria will be the leading cause of death worldwide with over 50 million

cases annually, and that this will cost the global economy in excess of $100 trillion (USD (O'Neill, 2014)).

Despite the obvious need for new antibiotics, very few are being developed; in fact, only 4 new classes have been introduced in the last 40 years (Cooper and Shlaes, 2011). One contributing factor is diminishing returns for pharmaceutical companies. With around a $70 million outlay to reach just the Phase III clinical trial on a drug which is prescribed usually only in short courses and to which resistance may quickly arise (Cooper and Shlaes, 2011), it's unsurprising companies prioritise investment elsewhere. Even most antibiotics currently in development are not novel, but rather the chemical groups surrounding the active core of the molecule are slightly altered (Baquero et al., 2009). This is more economical than developing a completely new compound and is argued to provide short term solutions; but resistance can quickly arise again (Altman, 2013), and it is therefore an inadequate long-term solution. New antibiotics are occasionally discovered, such as Teixobactin in 2015 (Ling et al., 2015), but the time and costs involved in reaching market makes investment high risk (O'Neill, 2014). In the final Review on Antimicrobial Resistance report, one of the ten final recommendations was to introduce funding incentives for development of new antibiotics (O'Neill, 2015) for these reasons.

### 1.5. Antibiotic uses and discharge to the environment

Antibiotics are not only used extensively as therapeutics and prophylactics in the clinic; they are also used in the community, farming, aquaculture and even horticulture (Andersson and Hughes, 2012). Prophylaxis in animal rearing can be at sub-therapeutic concentrations (Allen and Stanton, 2014) and antibiotics can also be used as growth promoters, though this is now banned in some countries (Martinez, 2009), including all countries in the EU (Laxminarayan et al., 2013).

Most antibiotics are not fully metabolised in the body by humans or animals, resulting in their excretion as active parent or transformation products; in fact, 20 – 90 % of antibiotics excreted are estimated to still be active (Andersson and Hughes, 2012). When used for human therapy, these enter sewage or waste water treatment plants (treatment does not fully inactivate all antibiotics (Daghrir and Drogui, 2013)) and can then enter the environment in an active form. Additionally, 'stabilised solids' (i.e. treated waste solids) are used as

fertiliser or to improve soil quality (Ghosh et al., 2009) which may further facilitate dissemination of antibiotics or resistance genes in the environment (Calero-Caceres et al., 2014). In aquaculture, antibiotics can reach high concentrations in local waters and sediments (Kar and Roy, 2012), as around 80 % of ingested antibiotics can be excreted or secreted by fish. Excreta or antibiotics can also then be dispersed through aquatic environments (Cabello et al., 2013). For animals, run off from farms and the deliberate spreading of animal faeces as manure contribute to accumulation of active antibiotics and resistance genes in farmed and natural environments (Andersson and Hughes, 2012). Antibiotics and resistance genes may also pass back to humans via the food chain (CDC, 2013), though withdrawal periods are used to try reduce antibiotic concentrations in milk or meat before human consumption (Allen and Stanton, 2014). Finally, further dissemination may be facilitated by animals or birds, or abiotic factors such as wind or water currents (Allen et al., 2010).

Once in the environment, antibiotics can be degraded by photolysis, hydrolysis or thermolysis (breakdown by light, in water, or by heat, respectively), and rates for these vary greatly between antibiotics (Kummerer, 2009a). An antibiotic can even degrade at different rates in freshwater and salt water (Trovo et al., 2009). Bioavailability also differs greatly depending on the environmental context. Soil and sediment can act as an antibiotic sink, by removing antibiotics from water, and sorption rates (which can reduce antimicrobial potency) vary greatly for different compounds (Kummerer, 2009a). Conversely, antibiotic persistence increases with soil sorption rate, as when bound to soil particles biodegradation is reduced (Baquero et al., 2008). Biodegradation of antibiotics (by bacteria) is more common under anaerobic conditions than anaerobic, with rates differing once again between antibiotics (Kummerer, 2009a).

Active antibiotic concentrations tend to be very low in the environment. Liquid chromatography coupled to mass spectrometry (‘LC-MS’) as well as surface-plasmon resonance can be used as physio-chemical methods for detecting low concentrations of antibiotics. Immunological assays can also be used, and while they require less specialist equipment than the physio-chemical tests, they tend to be more expensive (Liu et al., 2011). It is therefore unsurprising that there is a relatively scarce amount of data on the concentrations of antibiotics found in the environment, particularly in developing countries. In a systematic review of measured pharmaceutical concentrations in freshwater rivers for

example, 80 % of the papers were from North America or Europe (Hughes et al., 2013).

In general, concentrations of antibiotics are highest in pharmaceutical industry (mg/L) and hospital effluents (high µg/L), lower concentrations (low µg/L) are found in waste water treatment plant (WWTP) effluent, and even lower concentrations (ng/L) are found in surface water, seawater, and ground water (Homem and Santos, 2011). Specifically, antibiotic concentrations in WWTP effluent is usually between 10 – 1000 ng/L for a range of antibiotics including β-lactams, sulphonamides and trimethoprim, amongst others (Le-Minh et al., 2010). Deblonde et al. (2011) showed that there is little if any difference between WWTP influent and effluent antibiotic concentrations by conducting a review of these measurements in the literature; Verlicchi et al. (2012) found similarly low antibiotic removal by treatment plant processes. Another study found higher levels of both antibiotic resistance genes and antibiotics in effluent compared to the receiving water (Xu et al., 2014). WWTPs can also impact the concentrations of antibiotics within biofilms found downstream, with concentrations ranging from 10.4 ng/g (trimethoprim) to 276 ng/g (levofloxacin) (Aubertheau et al., 2016). Higher antibiotic concentrations in WWTPs could reduce biodegradation efficacy if the bacteria responsible for this process are susceptible to the antibiotic concentrations present (Kar and Roy, 2012); for example, methane production has been effectively reduced when bacterial communities are spiked with antibiotics (Sun et al., 2012).

In the natural environment, Chen and Zhou (2014) measured the concentrations of 20 antibiotics with ultra-high performance liquid chromatography-tandem mass spectrometry, at 13 different sites in the Huangpu River, in China. Freshwater concentrations varied greatly between the sites, and antibiotics associated with veterinary use were higher when measured at sites with nearby farms. Average concentrations of sulfamethoxazole were 259.6 ng/L, and fluoroquinolone concentrations in general were much lower, between 34.2 ng/L and 327 ng/L. They also measured antibiotic concentration in river sediments. The highest concentration was of erythromycin at 24.6 µg/kg, and the lowest was of chloramphenicol at 0.7 µg/kg (Chen and Zhou, 2014).

Conversely, antibiotic concentrations can be extremely high in environments which receive effluent from pharmaceutical production facilities. In India, a WWTP serving 90 of these facilities had unprecedented levels of

antibiotic residues up to 31 mg/L (Larsson et al., 2007). Though this concentration was toxic, it has been shown that other environments contaminated with production facility effluent can have much higher resistance gene levels, for example an exposed Indian lake had a resistance gene abundance 7000 times greater than a comparative Swedish lake (Bengtsson-Palme et al., 2014).

This vast variability prevents any generalisation across compounds or environments, and therefore greater effort is required to quantify actual antibiotic concentrations in different environmental compartments, and also to begin to consider potentially bioactive transformation products. In summer 2016 an online database curated by the German environment agency UmweltBundesamt (UBA) went live, which comprehensively lists measured environmental concentrations (MECs) of a range of pharmaceuticals (including antibiotics), as part of their project "Pharmaceuticals in the environment - occurrence, effects and options for action" (UmweltBundesamt, 2016). However, due to the vast variability between measurements even in the same environmental compartment for the same compound in the same country, continued monitoring of environmental pharmaceuticals is necessary.

## 1.6. What is driving environmental prevalence of AMR – discharge of antibiotics or resistant bacteria?

Environmental antibiotic residues may be a driving force of selection for resistance, but conversely, persistence of antibiotic resistance genes (in either viable or non-viable bacteria) may be the real threat. Selection for resistant genes may occur in the body (human or animal), or even in treatment plants (Zhang et al., 2009b), and some of these may enter the environment. There is evidence of a clear relationship between the level of pollution and presence of resistance genes, for example prevalence of resistance genes and *intI1* (a gene which facilitates mobilisation) from different sites in a river in China decreased concurrently with anthropogenic impact (Chen et al., 2014), and *intI1* levels are increased in biofilms downstream of WWTPs (Aubertheau et al., 2016). The *intI1* gene has also been proposed as a proxy for antibiotic resistance and thereby anthropogenic pollution (Gillings et al., 2015, Berendonk et al., 2015, Berglund, 2015, Amos et al., 2015). Once through the WWTP system, resistance genes have been shown to persist in the environment in the complete absence of

anthropogenically introduced antibiotics (Allen et al., 2009, Tamminen et al., 2011, Muziasari et al., 2014), and half-lives of resistance genes in the environment in the absence of antibiotics have been reported as up to three months (Burch et al., 2014). Additionally, multi-drug resistance plasmid bearers can persist without nutrients at very low antibiotic concentrations (Bien et al., 2015), and at a range of pH and temperature (Calero-Caceres and Muniesa, 2016). Resistance gene host identity can also impact persistence, with phage-associated genes being more persistent than those in bacteria (Calero-Caceres and Muniesa, 2016).

The greatest concern is that resistance genes can persist and be mobilised into environmental bacteria (Yang et al., 2013a) and then back into pathogens, many of which are naturally competent and can readily uptake extracellular DNA (Mao et al., 2014). There is compelling evidence that clinically relevant resistance genes originate in environmental bacteria (Walsh, 2013a, Humeniuk et al., 2002, Poirel et al., 2004, Poirel et al., 2005, Potron et al., 2011), and have up to 100 % sequence similarity to genes in environmental bacteria (Forsberg et al., 2012) which worryingly indicates recent HGT transfer between environmental bacteria and human pathogens.

Three steps have been proposed for fixation of environmental resistance genes in human pathogens (Martinez, 2009): firstly, resistance evolves in environmental bacteria over evolutionary time, secondly, this resistance is transferred to human associated bacteria in an environment where they are in direct contact with environmental bacteria, and finally this resistance is transferred to human pathogens, possibly within the gut or WWTPs which are both considered to be HGT hotspots (Broaders et al., 2013, Rizzo et al., 2013). Beaches have been suggested as an environment which can allow contact between human commensals (faecal indicator organisms) and resistant environmental bacteria (Alm et al., 2014), as have recreational waters which can contain ESBL-producing, multi-drug resistant bacteria (Blaak et al., 2014, Leonard et al., 2015). Additionally, certain human populations are more at risk of exposure and transmission e.g. those working in animal farming (Huijbers et al., 2014), or those with frequent contact with contaminated waters such as surfers (Leonard et al., 2015).

Antibiotic resistance plasmids have been detected in treated waste water (Rahube and Yost, 2010) and resistance genes have been found in greater

relative quantities in the effluent than the influent of a WWTP (Marti et al., 2013). These increased numbers of resistance genes in the effluent can be similar to that in the downstream receiving water (Berglund et al., 2015). This relationship is found to hold for the majority of different treatment plants tested (Du et al., 2014), and even tertiary treated effluent can still contain significant amounts of resistance genes (LaPara et al., 2011).

However, greater than 90 % of integrons present in sewage influent have been found to be removed by treatment processes, showing some DNA may be successfully eliminated (Zhang et al., 2009a). Even when integrons are not removed successfully, gene cassette diversity (frequently antibiotic resistance genes) may be reduced by treatment processes (Stalder et al., 2013). Other studies have found no significant difference in sulphonamide, tetracycline or beta-lactam resistance genes following treatment in WWTPs, with different loads and different treatment stages having no impact on prevalence (Laht et al., 2014).

Some studies have directly investigated how effective different treatments are at removing resistance genes. Thermophillic treatment can decrease resistance gene abundance, whereas mesophillic treatment results in reduced removal of the same genes (Ghosh et al., 2009). UV treatments may increase the prevalence of antibiotic resistance genes, despite being bactericidal, which may be because UV also induces the SOS response, which can increase the number of point mutations (Blazquez et al., 2012) and even rates of HGT (Beaber et al., 2004) and gene cassette recombination within integrons (Guerin et al., 2009, Guerin et al., 2010, Guerin et al., 2008). Additionally, upon re-exposure to light, the enzyme photolyase may repair damaging DNA lesions (Jungfer et al., 2007, Oguma et al., 2001) allowing bacterial survival. Chlorination has also been demonstrated to increase antibiotic resistance, in particular to ampicillin, cephalothin and tetracycline (Murray et al., 1984, Huang et al., 2013). Bacteria can still be viable after treatment (Amos et al., 2014a), including significant clinical pathogens such as *E. coli* ST131 harbouring the resistance gene $bla_{\text{CTX-M-15}}$ (Dolejska et al., 2011). In fact, numbers of viable, resistant bacteria may increase at each stage of treatment, resulting in a significant increase in resistance prevalence in the effluent compared to the influent (Zhang et al., 2009b).

Treatment processes should be assessed for efficacy of resistance gene removal; yet recently, pilot studies for new treatment processes still use removal of viable enterococci (an indicator of water quality) as a measure of treatment

effectiveness (Michael et al., 2012) and fail to take into account removal of genes which may be in other host backgrounds or even exist as free DNA. An inexpensive post treatment strategy has been suggested using qPCR to screen waste water for levels of antibiotic resistance genes before releasing the effluent into the environment (Nam et al., 2013).

Most research conducted to date has been correlative; i.e. it has not been empirically determined in controlled experiments whether antibiotics in the natural environment select for resistance; or if a given resistance gene becomes more widely disseminated due to low fitness cost (or benefit, as a result of other potential ecological functions). It has also not been empirically determined whether antibiotics influence 'stochastic' events such as co-localisation with other genes under positive selection ('co-selection'), or mobilisation into a fitter plasmid, etc. In any case, the pertinent issue from a human health perspective is when a resistance mechanism is transferred to a human pathogen.

## 1.7. Mobilisation of resistance genes

HGT is the movement of DNA between bacteria (which need not be closely related (Rodriguez-Rojas et al., 2013)). There are three main mechanisms by which HGT occurs (Figure 2). The first, transformation, is when naturally competent bacterial cells take up DNA directly from the environment. The second involves transport of bacterial DNA by bacteriophage, viruses which infect bacteria. Transport of DNA in this way is limited by the type of phage, but the length of DNA mobilised can be greater than 100 kb (Muniesa et al., 2013), a sufficient length to contain many resistance genes, and phage isolated from hospital and urban effluents as well as river water have all been found to harbour high numbers of *bla*$_{\text{CTX-M}}$ and *qnr* genes (Balcazar, 2014). The final mechanism involves formation of the conjugation or 'sex' pilus, through which some mobile genetic elements (MGEs) can be transported (Thomas and Nielsen, 2005, Furuya and Lowy, 2006).


Figure 2. Taken from Furuya & Lowy (2006). The three main mechanisms of horizontal gene transfer ('a, b' and 'c').

Recent horizontal movement of genes can be detected when genes in different species have very high sequence similarity (Chee-Sanford et al., 2009), and association with MGEs can be used as an indicator of further mobilisation potential. Biofilms, WWTPs or within filter-feeding organisms (Yang et al., 2013a, Lupo et al., 2012) are considered 'hot-spots' for transfer (as bacteria are in close proximity, with ample nutrient sources). Rates of HGT can also be affected by physiological factors such as temperature (van Elsas and Bailey, 2002).

MGEs can include plasmids, integrons, transposons, integrative conjugative elements (ICEs), and insertion sequence common regions (IS*CR*s), amongst others. IS*CR*s are associated with genes conferring resistance to many different antibiotics. If inserted adjacent to resistance genes, upon excision they

can carry these genes to a new location (Toleman et al., 2006). Transposons are sections of DNA which encode a transposase, which allows excision and recombination of itself and any associated genes (often antibiotic resistance genes (Toleman et al., 2006)). ICEs are chromosomally-located but can still be mobilised via conjugation (Wozniak and Waldor, 2010). These MGE's are not covered in any greater detail here; however it is important to note transposons cannot undergo conjugation alone (Wozniak and Waldor, 2010) but are frequently incorporated into plasmids, and integrons can be found within both transposons and plasmids (Rahube and Yost, 2010) and so on.

Plasmids are extra-chromosomal pieces of DNA, and those conferring antibiotic resistance are often large and self-replicating. They can be difficult to eradicate, having clear implications for managing spread of antibiotic resistance (Carattoli, 2013); in fact, some plasmids exhibit parasitic behaviour, in that they are maintained without conferring any benefit to the host (Brown et al., 2013). They can be divided into different compatibility groups; incompatible plasmids (Inc plasmids) cannot replicate when they are in the same bacterial cell as they both have the same replicon and therefore the same replication mechanism (Carattoli, 2013). Amplification of plasmid DNA with replicon-specific primers can be used to determine different incompatibility groups, though successful primer binding is limited (Tennstedt et al., 2003). Additionally, plasmids can have broad or narrow host ranges, meaning they can persist in many or only a few bacterial species, respectively. Plasmids can also be self-transmissable or mobilisable, meaning they can transfer themselves or require a 'helper' plasmid, respectively (Smillie et al., 2010). Some plasmids ensure their survival during replication by encoding 'addiction systems' which kill daughter cells that no longer contain the plasmid following cell division (Carattoli, 2013).

All these features can have varying effects on plasmid fitness, which in turn affect likelihood of selection for antibiotic resistance. Potentially, a very fit plasmid (for example, it is small, autonomously replicating, has a broad host range and high compatibility with other plasmids, and encodes an addiction system) which may also be carrying one or more antibiotic resistance genes could be maintained due to the characteristics of the plasmid itself and not due to the resistance being conferred by the antibiotic resistance genes (Carattoli, 2013). In addition, in terms of antibiotic resistance, plasmid-mediated resistance has been shown on average to confer a smaller fitness cost than chromosomal, mutation-

based resistance (Vogwill and MacLean, 2015). Antibiotics can in turn also affect the success of a plasmid by altering transfer rates, which have been shown to be higher at 'medium' concentrations of antibiotics (32 – 64 mg/L) compared to very high or low antibiotic concentrations (Schuurmans et al., 2014).

Integrons comprise of the *intI1* gene (which encodes the integrase enzyme) and an *attI* recombination site, as well as one or more gene cassettes. Gene cassettes exist extracellularly in the environment, often code for antibiotic resistance genes and include the palindromic *attC* recombination site (Hall, 2012). Integrase facilitates site-specific recombination of any number of gene cassettes between the *attI* and *attC* sites (Hall, 2012), with more than one gene cassette forming an 'array' (Partridge et al., 2009). Integrons often carry multiple antibiotic resistance gene cassettes, for example, 28 of 30 integron-carrying strains isolated from Lake Taihu in China were resistant to more than one antibiotic tested (Yin et al., 2013).

Carriage of arrays which confer resistance to a range of compounds is believed to be a mechanism by which co-selection may occur. Co-selection can be described in two ways: cross-resistance is selection for a gene because it confers resistance to more than one compound (e.g. multidrug efflux pump) and only one of those compounds needs to be present for the gene to be maintained (Andersson and Hughes, 2011). The second mechanism of co-selection is co-resistance, whereby a resistance gene can 'hitchhike' along with another resistance gene, or genetic locus (or loci, e.g. on a plasmid) undergoing positive selection (Gullberg et al., 2011, Nesme and Simonet, 2014).

Gene cassettes can confer resistance to biocides and detergents; for example quaternary ammonium compound, or 'QAC', resistance is conferred by the *qacE* gene. *QacE* or its derivatives (e.g. *qacE*Δ1) are commonly found on integrons isolated from the environment (Gaze et al., 2011). So for co-selection to occur, a QAC resistance gene located in the same array as an antibiotic resistance gene will result in maintenance of this gene cassette when the bacterium is exposed to QACs, even if it is not being exposed to antibiotics. A paper by Gaze et al. (2011) found more than half of all integrons detected in QAC-contaminated soil harboured semi-functional *qacEΔ1*, which is always associated with *sul1*, a sulphonamide resistance gene. In a previous study, bacterial isolates from reed-bed soil and river sediment contaminated with QACs had a significantly greater proportion containing class 1 integrons compared to controls, and *qacE,*

the fully functional multi-drug efflux pump, was detected in 18 of 19 of class 1 integron-containing isolates (Gaze et al., 2005). The *qacE* gene itself is significant, as both a multidrug efflux pump to confer cross-resistance (Gaze et al., 2005) and because it contains its own promoter (Partridge et al., 2009) it can confer a strong co-selective force for antibiotic resistance in the presence of biocides, even if it is distal from the integron promoter.

## 1.8. Issues with current research methods aiming to study resistance in the environment

Evidence suggests resistance genes can be maintained in the environment when concentrations of antibiotics are very low, despite their 'expected' fitness cost. However, it is unknown whether these concentrations can select for resistance *in situ* (Tello et al., 2012). The majority of studies outlined (as mentioned previously) have provided only correlative details; for example, the relative increase of abundance of resistance genes in effluent from treatment plants compared to the influent: Lachmayr et al. (2009) found sewage treatment may enrich for β-lactamase producing bacteria, but the mechanisms of how this occurred (e.g. direct selection, or uptake of free resistance genes, or co-selection) are unknown. Another study examined fate of *bla*$_{TEM}$ genes, which are an abundant family of β-lactamase encoding genes. The ratio of *bla*$_{TEM}$ genes to 16S rRNA genes was monitored by real-time PCR (qPCR) and found to increase after sewage treatment. It is unknown whether this is simply due to enrichment of pre-existing resistant bacteria, or resistance acquisition in previously susceptible bacteria, or both; and whether this was due to the selective compounds, the fitness costs of the genes; or their genetic location was not investigated.

Transfer experiments have largely neglected the role plasmid fitness may play in maintenance or transfer frequency of resistance genes located on plasmids (Carattoli, 2013). However, plasmid persistence, virulence, growth and fertility (conjugation rate) was investigated for the IncK pCT plasmid, both carrying and not carrying *bla*-$_{CTX-M14}$ (an ESBL), and no difference in fitness was observed (Cottell et al., 2012), indicating in this case, the plasmid itself may be responsible for the success of the resistance gene. However, the question remains: are transfer rates comparable between the laboratory and the

environment, and in particular, in environmental matrices contaminated with antibiotics, with a more complex (and therefore realistic) bacterial community?

In terms of the current methods employed for testing the ecotoxicity of antibiotics which inform environmental risk assessment, most studies use the standard toxicity tests: measuring $CO_2$ production as a proxy for algal growth or bacterial growth, and reduction in bioluminescence of *Vibrio fischeri* (Donner et al., 2013), as well as tests on fish and invertebrates (Verlicchi et al., 2012). Tests on more complex organisms seem unlikely to provide information on how antibiotics may affect bacteria, and specifically, in terms of selection for resistance. Even *V. fischeri,* as a marine microorganism, lacks the ability to be tested in freshwater, is generally sensitive only to higher concentrations of compounds (Fatta-Kassinos et al., 2011), and is unaffected by some antibiotics (Brandt et al., 2015). Additionally, using $CO_2$ production in bacteria as a proxy for toxicity (or selection of resistance) is misleading as bacteria can persist in a bacteriostatic state (Demoling et al., 2009). Despite these disadvantages, this is the only bacterium described when assessing ecotoxicity of the antibiotics in water compartments in a review by Santos et al. (2010).

There are a few ecotoxicological studies where resistance is considered. For example, ecotoxicity of antibiotics commonly used in fish farming was assessed by testing on algae, *V. fischeri*, and activated sludge. Turbidity was used as a proxy for toxic effect on the sludge bacteria but 100 % inhibition of growth was never observed (Munch Christensen et al., 2006).

Some studies have used pollution-induced community tolerance (PICT) as a proxy for toxicity. If any bacteria in the community are tolerant, they survive and therefore the combined tolerance of the community increases. Translated into microbial ecological terms, resistant bacteria survive and begin to take over the population, or potentially transfer their resistance to previously susceptible bacteria. This approach can be used to test tolerance of bacterial communities in both aquatic and terrestrial environments (Demoling et al., 2009). In a recent review it was recommended that communities used for environmental risk assessment should not have been exposed to the test compound previously (Brandt et al., 2015), but such a community would be difficult to find and may need to be synthetic; and would therefore primarily allow tracking of only *de novo* mutations, and not selection of pre-existing resistance determinants. Conversely, if single species were used, the strains would indeed need to be carefully selected

based on their susceptibility profile (Brandt et al., 2015), but again this would not take into account mobile and / or pre-existing resistance determinants.

A paper by Isidori et al. (2005) used the SOS chromotest on *E. coli* (which quantitatively measures SOS pathway induction) and the Ames Test (which assesses genotoxic potential) on *Salmonella* Typhimurium to test toxicity of a range of antibiotics. Only one antibiotic gave a positive result for the SOS chromotest and three of the six antibiotics tested possessed genotoxic potential. This is a rational approach as it is a standardised method which could be used to assess potential of an antibiotic to select for resistance. However, no ecotoxicity papers assess genotoxicity of environmental concentrations of antibiotics or their degradation products on a range of environmental (and ideally opportunistically pathogenic) bacteria.

Many ecotoxicological studies also investigate the toxicity of antibiotic degradation products generated through different degradation pathways. Some products lose their antimicrobial activity, for example photolytic or photocatalytic degradation compounds of ciprofloxacin lose their potency with increasing treatment when tested against *E. coli* K12 (Paul et al., 2010). However, some degradation products retain their activity and can even be more potent than the parent compound, for example photodegraded products of tetracycline exerted greater toxic effects on *V. fischeri* (Jiao et al., 2008), and trimethoprim solar $TiO_2$ photocatalysis intermediates are still moderately toxic to *V. fischeri* (Sirtori et al., 2010).

Few have considered antimicrobial effects of antibiotic degradation products on bacteria. Photodegradation products of different fluoroquinolones have been shown to retain antimicrobial activity against clinically relevant strains (such as *Staphylococcus aureus* subsp. *aureus*, *P. aeruginosa,* and *Klebsiella pneumonia* subsp. *ozaenae*) (Kusari et al., 2009) and environmental strains (*E. coli*, *Klebsiella oxytoca, Enterobacter cloacae*) (Sunderland et al., 2001). Oxolinic acid (used in aquaculture and veterinary medicine) and its transproducts produced by photocatalysis both in, and in absence of $TiO_2$ were applied to *E. coli*, and toxicity on *V. fischeri* was also measured. It was found that the degradation products retained antimicrobial activity, but had reduced toxicity on *V. fischeri* (Giraldo et al., 2010), further illustrating the inadequacy of this method when aiming to consider a compound's potential to select for resistance. Similarly, ofloxacin and trimethoprim and their degradation products were not

toxic to *Daphnia magnia* at µg/L concentrations (Michael et al., 2012), which shows this standard ecotoxicity assay may be ineffective for detecting selection of antibiotic resistance, which has been shown to occur at such levels (Gullberg et al., 2011, Negri et al., 2000). Inhibition of bacterial luminescence other than *V. fischeri* has also been monitored. Toxicity of antibiotics in pig manure was estimated by measuring inhibition of luminescence in *Photobacterium phophoreum*. Reduction of luminescence was observed at 'trace concentrations' (Sun et al., 2012), suggesting it may be more suitable for risk assessments attempting to determine MSCs, though again not necessarily for bacteriostatic antibiotics if luminescence is independent of growth.

Inhibition of growth is another assay used to assess pollutant effect; for example, new veterinary antibiotics must have a PEC below 100 µg/Kg, which must be tested according to the OECD/OCDE 2000 guidelines. Finally, standard test lengths for measuring inhibition of luminescence or $CO_2$ production have been suggested to be currently too short for assessing toxicity of antibiotics, as they do not reflect the generation times of different bacteria (Kummerer et al., 2004).

There appears to be a significant lack of knowledge regarding selection for antibiotic resistance in natural environments and little progress towards developing a standardised method to test for toxicity of antibiotics and their degradation products, and their potential to select for resistance in a range of environmental bacteria. This limits the development of rigorous environmental risk assessment and mitigation strategies.

## 1.9. Problems for risk assessment of antibiotics using current methods

In a review by Roos et al. (2012), ranking methods used to prioritise pharmaceuticals for environmental risk assessment were compared. Nine different methods defined a total of 32 different pharmaceutical compounds which should be prioritised for risk assessment. None of these were antibiotics. This suggests the current ranking methods may not be well suited to considering the selective effects of antibiotics on bacteria; or that the data in this area is scarce; or indeed that the area is largely neglected in ecotoxicity evaluations. The authors go on to state: "A first tier prioritisation scheme should be as widely applicable as possible and therefore ideally be based on data that is readily available for all

pharmaceuticals" (Roos et al., 2012), but data on effects of antibiotics on bacteria is considerably lacking. Though all new pharmaceuticals require environmental risk assessment during market authorisation, the problem is that current methods do not assess the potential to select for resistance (Brandt et al., 2015). Analysis of a compound's ecological effects and exposure potential is essential to risk assessment (Aurelien et al., 2013), but without sufficient methods to determine the 'toxicity' of antibiotics, or probability that antibiotics can select for resistance in bacteria, it will be impossible to design an appropriate risk assessment with management strategies to reduce antibiotic release.

Unfortunately, this is not the only example of how current risk assessment methods underperform. Assessments of exposure to pharmaceuticals is often considered only in terms of the weight of the compound in sales, but more data is required to design an accurate risk assessment, for example the compound's degradation rate, and whether it is removed by treatment plants (Roos et al., 2012). Additionally, little or no work has investigated entantiomeric antibiotics. Enantiomers are two mirror copies of a single compound, and they may have different degradation rates and differ in their toxicity (Lopez-Serna et al., 2013). For example, ofloxacin is a racemate comprising of the 'active' entantiomer, levofloxacin, and its inactive entantiomer (Kummerer et al., 2004). Could degraded, previously inactive entantiomers become toxic? In these cases, could quantification of racemates overestimate exposure risk, or quantification of active, parent racemates underestimate risk?

Munch Christensen et al. (2006) also highlight the importance of complex mixtures of pharmaceuticals versus simple mixtures, which may have different "additive, synergistic, and antagonistic effects". Different effects can occur if the group of compounds affect different biological sites with different mechanisms ('concentration addition'), or if they each have independent mechanisms (Munch Christensen et al., 2006).

It has been stated that risk assessment of antibiotics should take into consideration the potential for selecting for resistance (Tello et al., 2012). Tello et al. (2012) calculated the wild-type cut-off value ($CO_{WT}$) for bacterial growth inhibition using previously determined MICs, and compared this to the environmental concentrations of a range of antibiotics measured in different environmental compartments, including waste water and sewage treatment plants, manure and streams. Their results suggested the current cut-off value

outline by the Veterinary International Conference on Harmonisation (VICH) phase I guidance document (VICH, 2000) for the discontinuation of veterinary drug development (<1 part per billion in water, and <100 part per billion in soil) is not low enough to prevent selection for resistant bacteria (Tello et al., 2012). However, predicted environmental concentrations and predicted no effect concentrations ('PECs' or 'PNECs') are the standard approaches to assessing a drug's risk to the environment (Roos et al., 2012). They are used to determine the 'Risk Quotient' ('RQ' obtained by dividing PEC by PNEC), which if equal to or greater than 1, a significant environmental risk is assigned to the compound (Aurelien et al., 2013, Jesus Garcia-Galan et al., 2009).

As little work has investigated whether sub-inhibitory concentrations of antibiotics select for resistance (Gullberg et al., 2011, Negri et al., 2000), the 'effect' of these PNEC's is likely cell death or cell survival; not mutations or resistance gene acquisition or maintenance. In fact, pharmaceuticals for human use only require Phase II risk assessment (that is, investigation of their fate and environmental effects) if their PEC in surface water is greater than 0.01 µg/L (Bialk-Bielinska et al., 2011), and though all new pharmaceuticals require environmental risk assessment during market authorisation, current methods do not assess the potential to select for resistance (Brandt et al., 2015). For example, PEC, PNEC and RQ values were calculated for a range of pharmaceuticals including antibiotics, from hospital waste water. The antibiotic with the highest RQ of 0.8 (assigned as an 'insignificant' risk) was amoxicillin, with a PEC of 499 µg/L in the hospital effluent (Escher et al., 2011), which is much higher than sub-inhibitory concentrations of antibiotics shown to select for resistance (Gullberg et al., 2011). This RQ approach is inadequate for antimicrobial stewardship in a time when novel antibiotics are few and far between. Other measures of antimicrobial activity may be more suitable, for example $EC_{10}$ (concentration at which 10 % of bacterial growth is inhibited) or $EC_{50}$ values, which can be sensitive down to the µg/L level when assessing antibiotic toxicity of sulphonamides to soil microcosms and activated sludge (Jesus Garcia-Galan et al., 2009).

It is clear "current methods do not monitor effects of chemicals on microbes well" (Backhaus et al., 2012) and the best approach for developing a meaningful risk assessment of antibiotics requires a combination of current ecoxtoxicity approaches (in that a standardised test yielding reproducible results needs to be created to generate enough data to inform the risk assessment), and

other methods which are more tailored to investigating bacteria in detail. It has been suggested an assay should be developed to measure MSCs, which can be used in risk assessment to determine a threshold level for development of antibiotic resistance (Ashbolt et al., 2013). This must first be approached with an 'experimental evolution' method, which allows monitoring of evolution under highly controlled, easily manipulated laboratory conditions in order to separate cause and effect (Jansen et al., 2014), before being applied to more environmentally relevant conditions. Development of such an assay is the primary focus of this PhD.

## 1.10. New microbiological methods to inform risk assessment

In order to design an assay for the risk assessment of antibiotics for selection for resistance, low cost, high throughput, transferable methods are required that can generate reproducible results across different laboratories.

Methods can be culture dependent or culture independent. The former requires growth of bacteria in laboratory media which can present significant bias when investigating antibiotic resistant bacteria from natural populations (Garcia-Armisen et al., 2013). Additionally, culture based methods generally cannot be used as a substitute for environmental microcosms, where nutrient levels and physiological conditions are very different. However, laboratory-based culture dependent methods are necessary to unravel the complex processes occurring (Jansen et al., 2014), in natural communities.

Culture independent methods are free from culture bias, but often require *a priori* knowledge of targets. For example, conventional molecular methods such as polymerase chain reaction (PCR) and qPCR require knowledge of previously characterised and sequenced resistance genes. Yin et al. (2013) investigated the resistance profile of Lake Taihu in China and found a high prevalence of ampicillin (β-lactam) resistance through standard culturing methods. However, only 8 of the total 28 resistant isolates yielded positive results for PCR amplifying *bla*$_{TEM}$ and *bla*$_{SHV}$ (both coding for β-lactamases), meaning the majority of resistance genes or mutations were not identified in this study.

There are ways to circumvent this issue, for example if integrons are targeted. Primers designed for different integron classes based on differences in

the *intI1* gene (Hall, 2012) can allow tracking of resistance gene movement and allow sequencing of potentially novel genes, with no prior knowledge required of the resistance genes' sequence present in the gene cassette(s) (Rizzo et al., 2013). However, these methods are currently laborious and do not yield long enough products to study large arrays. Integrons have been suggested as a potential indicator of human impact on the mobilome (Ashbolt et al., 2013, Gillings et al., 2015) and as markers for antibiotic resistance (Amos et al., 2015). In general, MGE's isolated from HGT hot-spots can be argued as better targets then antibiotic resistance genes for this reason (Lupo et al., 2012). HGT hot-spots, for example in sewage sludge, can have continuously increasing rates of HGT when exposed to antibiotic concentrations from the ng/L level to lethal concentrations (Kim et al., 2014a).

QPCR can be used to track abundance or expression levels of 16S rRNA genes to indicate the number of bacteria present in the sample. This can then be used to calculate percentage of resistant bacteria, through comparing its relative abundance or expression with that of the 16S rRNA genes, as well as information on bacterial species diversity. 16S rRNA gene sequences differ between species, but broad-range primers can still target the gene for amplification (Grice and Segre, 2012). However, when comparing relative gene abundance, it is necessary to consider that some bacterial isolates carry more than one copy of the 16S rRNA gene (Lachmayr et al., 2009). Similarly, when performing qPCR or PCR, it should be considered that some bacteria can harbour more than one copy of the same resistance gene, which would increase the expression or number observed respectively (Volkmann et al., 2004). DNA quality is crucial for PCR reactions (Rizzo et al., 2013), and it is likely when amplifying directly from environmental samples (to take into account unculturable bacteria), the reaction will be inhibited by environmental contaminants which could affect PCR efficiency. However, this can be mitigated by generating environmental DNA dilution standards (Lachmayr et al., 2009, Marti et al., 2013), or through addition of extra reagents such as bovine serum albumin (BSA), to bind inhibitors.

There has recently been an increase in next generation sequencing methods (e.g. Illumina HiSeq, PacBio, Oxford Nanopore) facilitating more metagenome wide sequencing efforts. Metagenomic analyses has been used successfully to elucidate the resistance profile of bacterial communities (Yang et al., 2016), but the process is relatively new and still has some limitations. Large

amounts of data are produced which exceed standard computational storage and power to analyse; and gene identification depends upon the presence of similar genes already annotated in the database, necessitating careful and time-consuming analysis of the sequences and vigilant database curation. Unfortunately, the quality appraisal of these is not always sufficient.

Functional metagenomics is a bridge between 'classical' culturing microbiology methods and the newer sequencing approach, which allows phenotypic and molecular characterisation of all bacteria in an environmental sample, by cloning all DNA into an expression vector in a culturable organism (usually *E. coli*). This is another method which can overcome culture bias, but still allows functional screening which is infinitely more useful than sequencing based approaches alone, as gene function can be experimentally determined concurrently.

The best methods to investigate this area would combine all of the above, to represent both culturable and unculturable bacteria, to allow investigation for novel genes, whilst being able to alter the level of complexity of the assay (number of species, nutrient levels and physiological conditions). In terms of an ecotoxicological assay - simplicity, reproducibility, accuracy and applicability to a wide range of compounds and environments is desirable.

## 1.11. Thesis overview

While competition assays focusing on single species are an elegant method to determine MSCs, they do have limitations in their ability to predict what occurs in natural environments. The use of a natural, bacterial community inoculum would provide a more realistic representation of what may occur in the environment.

It would also be useful from a regulatory perspective to determine if there is a relationship between MSC and MIC, and whether this varies with host, antibiotic class, or individual antibiotic compounds. If a relationship can be elucidated, it will greatly facilitate toxicity testing of antibiotics and subsequently risk assessment. Therefore the main body of work in this thesis is based around an experimental evolution system, where a natural, complex community is exposed to varying concentrations of different antibiotics over several days, and compared to single host species competition assays using culture dependent and

independent methods. This forms chapter two. A simpler growth based assay based on this work that could be used for environmental risk assessment was also evaluated, which comprises chapter three.

As mentioned previously, class 1 integrons can be used as markers for anthropogenic pollution and for antimicrobial resistance in the environment (Ashbolt et al., 2013, Gaze et al., 2011, Gillings et al., 2015, Berendonk et al., 2015). Could rate of recombination of novel gene cassettes containing resistance genes into the integron - or shuffling of pre-existing resistance gene cassettes closer to the promoter, increasing the strength of their expression – be affected by different antibiotic concentrations? This was investigated with a new long range PCR method and PacBio sequencing in combination, which forms chapter four.

To further investigate co-selection by QACs, functional metagenomic libraries generated from biocide contaminated environments were screened for QAC resistance. These underwent transposon mutagenesis to simultaneously allow functional screening and sequencing to identify novel biocide resistance genes. The resistant clones were screened on a range of antibiotics to determine whether co-selection occurs in biocide-contaminated environments. This study forms chapter five.

### 1.11.1. Co-authored papers

In addition to the papers resulting from and outlined for each chapter, I was also co-author on four other publications during my PhD studies.

I am co-author on the cross-sectional study assessing the risk of coastal water exposure leading to colonisation of surfers and non-surfers by antibiotic resistant bacteria (‘The beach bum survey’). For this, I performed several hundred PCRs to determine the phylotype of *Escherichia coli* isolated from bathing water samples, to assess potential pathogenicity.

I will be second author on the paper which will publish the long term experimental data for macrolides used in chapter three. I contributed through designing the long term experimental assay and the plating experiment which confirmed the experimental system did not bias towards Gram negative organisms; and advised on all aspects of data collection and data analyses.

The final two papers, the first of which I was second author, and the second of which I was first author, was on a side project in collaboration with the Microbiology Department of the Royal Cornwall Hospital Treliske. The first paper confirmed a zoonotic opportunistic pathogen (*Staphylococcus pseudintermedius*) was routinely unidentified using the standard, clinical microbiological testing of infections (Lee et al., 2015). For this I performed PCR of two housekeeping genes, sequenced these and performed sequence and phylogenetic analyses, as well as several standard clinical microbiology phenotypic tests. The second paper was based on my observation that one of the strains from a non-local patient was highly diverse, and might be a new species. This was confirmed by whole genome sequencing, and I named the species *Staphylococcus starkensis*. This is due to the species being isolated from a patient from the North of England, and potentially in a previous publication from Norway (though it was not confirmed as a new species). '*Stark'ensis* refers to one of the key family of characters in the popular book series and TV show Game of Thrones, the 'Starks', which rule in the North.

## Chapter two: Selection and co-selection for antibiotic resistance in a complex community, at low antibiotic concentrations

### 2.1. Introduction

This study comprises two main parts – Minimal Selective Concentration (MSC) determination in a natural complex community, and studying indirect selection for susceptible bacteria.

### 2.1.2. MSC determination

The concept of sub-lethal or sub-inhibitory selection contradicts many long-standing concepts about resistance. The primary issue is the presumption that selective pressure (i.e. the antibiotic concentration) must be sufficient to offset the fitness cost of resistance. In the 'traditional selective window' (Gullberg et al., 2011), resistance is perceived as being so costly it is only beneficial in a scenario where the antibiotic concentration is sufficiently high, so that all susceptible organisms are killed or inhibited. However, this is based on a false assumption that resistance is always costly, and that these costs are always substantial. In fact, there are several studies which have shown that harbouring resistance confers no measurable fitness cost on the host organism (Enne et al., 2005); and in some cases resistance genes themselves (Michon et al., 2011) or the plasmids they are carried on can actually confer a fitness benefit (Carattoli, 2013). Additionally, compensatory mutations can arise which may offset the cost of resistance (Andersson and Hughes, 2012).

Once it is accepted that resistant bacteria are not always at a significant disadvantage compared to their susceptible competitors, it is conceivable that some resistant bacteria would have a slight fitness advantage compared to these competitors, at low selective pressures (i.e. low antibiotic concentrations). Therefore, resistance mechanisms that have low or neutral fitness cost could be selected for at low as well as high antibiotic concentrations. This may explain the presence of some clinically important resistance genes in the environment or the human microbiome in the absence of antibiotic selection (e.g. *bla*$_{\text{CTX-M}}$ bearing *E. coli* (Canton and Coque, 2006)).

Despite all of these possible mechanisms to mitigate or reduce the cost of resistance, it has long been assumed that selection for antibiotic resistance occurs only above the MIC of the susceptible bacteria of the population and below that of the resistant bacteria in the population ((Gullberg et al., 2011) Figure 3). In this 'Traditional Selective Window', high antibiotic concentrations are required in order for the resistance to be maintained despite its fitness cost. However, selection for resistance at sub-inhibitory antibiotic concentrations was demonstrated in an elegant study by Gullberg et al. (2011), which used isogenic susceptible and resistant, fluorescently tagged mutants that were counted by fluorescently-activated cell sorting (FACS). An increase in the numbers of resistant bacteria indicated positive selection, and the lowest concentration which selected for resistance was termed the MSC. The most concerning possibility arising from this finding is that selection could occur along a massive spatial and temporal range, from point of use (including antibiotic concentration gradients in different compartments in the human (or animal) body (Pena-Miller et al., 2013)), all the way through passage through waste water treatment plants (WWTPs) into soil and aquatic environments. Gullberg et al. (2014) later determined MSCs for resistance genes on a clinically isolated multi-drug resistant plasmid, illustrating sub-lethal selection can act on entire genes and mobile genetic elements (MGEs) which, in theory, confer a substantial fitness cost. This proof of concept has paved the way for several other studies, including the one below, which aims to further explore sub-inhibitory selection.

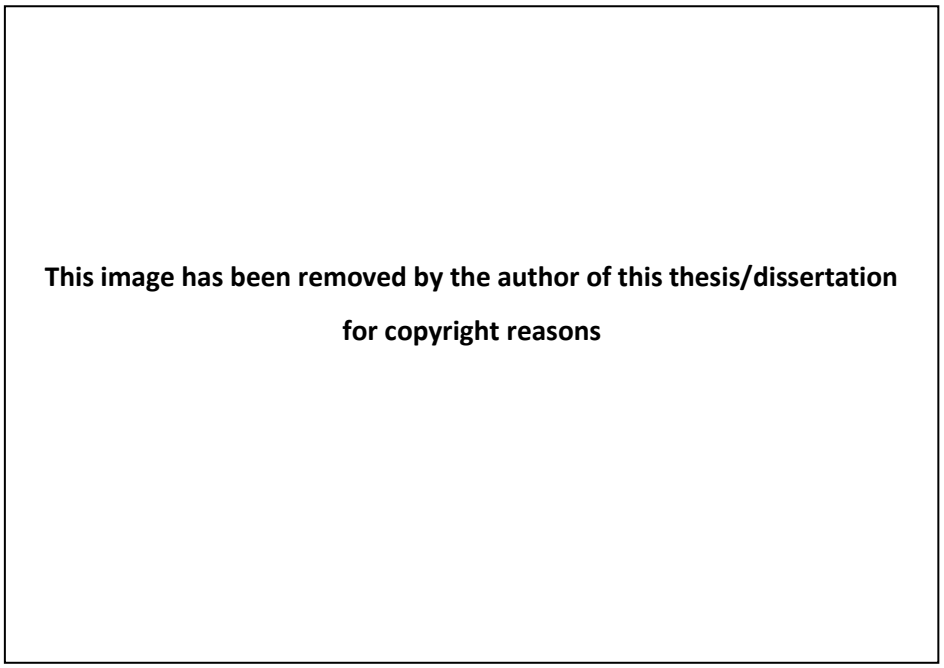


Figure 3. Schematic showing the traditional selective (antibiotic concentration >MIC of the susceptible strain) and sub-inhibitory selective (antibiotic concentration <MIC susceptible strain) windows. The MSC is defined as the lowest antibiotic concentration where the growth rate of the susceptible strain is reduced, to be the same as the growth rate of the resistant strain.

### 2.1.3. The environmental aspect

Environmental concentrations of antibiotics are very low, generally ranging from low ng/L to low µg/L (Homem and Santos, 2011). Therefore, it was generally accepted that measured environmental concentrations of antibiotics (MECs) were too low to be selective, and therefore there was no danger of selection occurring in the environment (*in situ*). Now, it is recognised environmental risk assessment of antibiotics is incomplete as it currently does not consider the selective potential of the compound. This has led to a call for MSC data (to be generated with a similar assay, or novel assays) to determine if MECs could indeed be selective (Ashbolt et al., 2013, Brandt et al., 2015).

However, single species, *in vitro* assays are not likely to be a good representation of the complex interactions occurring in a natural community (Berglund, 2015). A more recent paper (Lundstrom et al., 2016) approached sub-inhibitory selection from an environmental perspective and attempted to improve upon the single species evolution experiments by Gullberg *et al.*, by using a

natural complex community. Waste water bacterial communities and tetracycline were passed over glass slides allowing biofilms in an attempt to determine the MSC. Several methods for determining effect concentrations were compared, including qPCR, metagenomics, species diversity (through 16S rRNA amplicon sequencing) and pollution-induced community tolerance assays (PICT). QPCR was assigned as the most sensitive method, showing differences in tetracycline resistance gene copy number at concentrations as low as 1 µg/L.

However, as noted by the authors, this is not an MSC in the strictest sense (Lundstrom et al., 2016) as the biofilms were constantly exposed to potentially resistant organisms through the continual addition of treated waste water. Additionally, as the MSC was based upon qPCR data which simply compared prevalence of resistance genes at the end of the experiment without comparing this to prevalence in the original inoculum; it is entirely possible the differences observed were in fact increased *persistence* of resistance genes, as opposed to actual *positive selection* of resistance genes. In other words, without using the resistance gene prevalence at time zero, it is unknown whether the total resistance gene prevalence increased over time with tetracycline exposure compared to the no antibiotic control; or if there was a higher resistance gene prevalence in exposed samples at the end of the experiment, but these had decreased from time zero. To express this as terms of selection coefficients, it is possible the increase in prevalence observed by Lundstrom *et al.* is a result of the cost of resistance decreasing (Figure 4, A), but not to the point where resistance confers advantage over the susceptible bacteria (Figure 4, B and C).

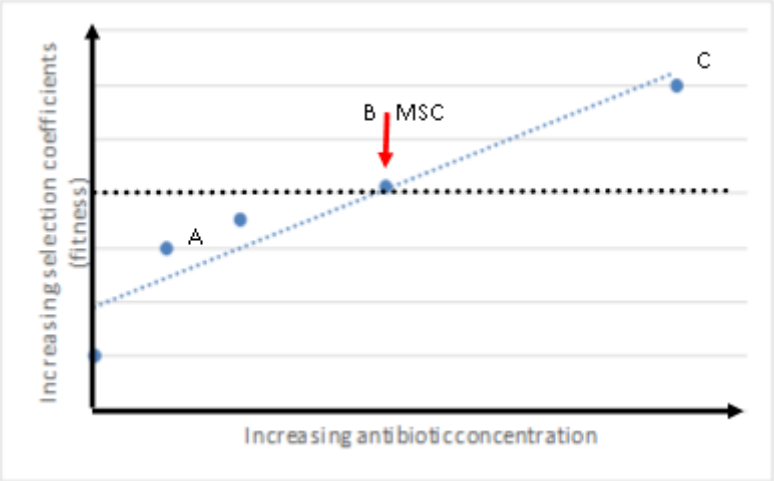


Figure 4. Theoretical selection coefficient graph, the black dotted line is the x-axis which crosses the y axis at 0 (i.e. a selection coefficient of 0, meaning any trait with a selection coefficient of exactly 0 is perfectly neutral in terms of fitness). A = The cost of resistance is decreasing, but the resistance determinant is not yet conferring a fitness advantage (selection coefficients are still <0, i.e. gene prevalence is decreasing over time). B = Where the line of best fit crosses the x axis is the MSC – this is also where the first selection coefficient is >0, meaning at this point, the resistance determinant is conferring at slight fitness advantage at that antibiotic concentration. C = at even higher antibiotic concentrations, the resistance determinant is now conferring a significant fitness advantage (selection coefficient much >0, i.e. gene prevalence is increasing over time).

Inappropriate statistical methods also increased the significance of their findings: a one-tailed T-test was used to determine if there was a significant difference between treatment and the no-antibiotic control – however, it is equally feasible there could be a decrease in the numbers of resistance genes following antibiotic exposure as an increase. It can therefore be argued the first actual MSC in a complex community yet remains to be determined.

### 2.1.4. Indirect selection

Indirect selection can be defined as selection for a susceptible bacterium at antibiotic concentrations exceeding its own MIC, due to presence of an antibiotic degrader (e.g. an extended spectrum β-lactamase (ESBL) producing bacterium)

which degrades extracellular antibiotic, thereby benefitting all bacteria in that population regardless of susceptibility / resistance profile.

Several recent studies have investigated β-lactamases in terms of indirect selection. For example, by noting satellite colonies of susceptible bacteria which grow around a β-lactamase producer on antibiotic plates, it was suggested that bacteriostatic antibiotics can allow the 'persistence' of susceptible cells which can grow once the antibiotic is 'detoxified' below a certain level (Medaney et al., 2015). Additionally, in competition assays in liquid culture it was shown β-lactamase producers protect susceptible bacteria to well above their own MIC, reaching an equilibrium fraction irrespective of the starting ration of resistant to susceptible bacteria. This fraction is also proportional to the antibiotic concentration divided by the cell density (Yurtsev et al., 2013). This phenomenon is confined to resistance mechanisms which degrade the antibiotic (Nicoloff and Andersson, 2016), and can also occur for resistance mechanisms which act only intracellularly (Sorg et al., 2016).

This has clear implications for antibiotic therapy, where colonisation with a β-lactamase producer could render treatment of a susceptible pathogen ineffective. What remains unclear from these studies is the extent to which producers can 'protect' (indirectly select for) susceptible bacteria, and how this affects selection for resistance at sub-lethal and clinical levels.

## 2.2. This study

The *bla*$_{\text{CTX-M}}$ genes encode ESBLs, which cleave the β-lactam ring of β-lactam antibiotics, rendering them inactive (Livermore, 1995). This gene class was chosen as the selective marker in this study as ESBL-producing *Enterobacteriaceae* pose a 'serious' threat to the clinic according to the Centre for Disease Control (CDC, 2013), but they have also been found in several environmental compartments including surface waters and soil (Sidrach-Cardona et al., 2014, Hartmann et al., 2012). Most importantly, they have been shown to be more prevalent downstream of WWTPs than upstream (Amos et al., 2014a), suggesting heavy loads of CTX-M-producing bacteria in influent; and/or selection within the WWTP itself. Indeed, though the vast majority of ESBL-producing *E. coli* can be removed by treatment, it has been estimated that 6 billion ESBL-

producing *E. coli* can be released into downstream rivers daily (Brechet et al., 2014).

Cefotaxime was chosen as the selective agent, as it is a World Health Organisation (WHO) designated 'essential' human medicine (WHO, 2011, WHO, 2015). As a third generation cephalosporin β-lactam, cefotaxime's mode of action is to prevent peptidoglycan cross-linkage by interacting with penicillin binding proteins (PBPs), thereby preventing cell wall synthesis; and at higher concentrations, causing bacterial cell lysis (Reygaert, 2011). MECs of cefotaxime range from 0.001 µg/L in surface water (UmweltBundesamt, 2016), to 1.1 µg/L in WWTP influent (Gulkowska et al., 2008), and up to a maximum of 150 µg/L in hospital effluent (Gomez et al., 2007). The lowest, estimated predicted no effect concentration for resistance ($PNEC^R$) for cefotaxime is 0.125 µg/L (Bengtsson-Palme and Larsson, 2016), indicating all of these environments are likely to experience *in situ* selection. However, experimentally defined lowest observed effect concentrations (LOECs) have yet to be determined.

In this study, a natural, complex bacterial community (untreated waste water) was exposed to a range of cefotaxime concentrations. The untreated waste water was selected to be representative of both the human gut, hospital effluent and WWTP influent; all of which will face a range of sub-inhibitory antibiotic concentrations. Experimental evolution microcosms were passaged daily for 8 days and qPCR at the beginning and end of the experiment determined prevalence of $bla_{CTX\text{-}M}$ genes, and the two most common groups within this class ($bla_{CTX\text{-}M\text{-}1}$- and $bla_{CTX\text{-}M\text{-}9}$), which are found worldwide (Canton and Coque, 2006).

Numbers of *E. coli* and other enterics were enumerated by qPCR to identify any potential trends in $bla_{CTX\text{-}M}$ host abundance. Phenotypic clinical resistance to cefotaxime was also quantified to see if this correlated with $bla_{CTX\text{-}M}$ prevalence, and chemical quantification was performed at selected time points to evaluate the stability of cefotaxime during the experiment and in the presence and absence of the bacterial community, and to highlight any disparity between expected (nominal) assay antibiotic concentration, and actual measured antibiotic concentration. Metagenomic analyses were performed on communities evolved at selected concentrations at the end of the experiment. qPCR has been shown as the most sensitive method for determining MSCs previously (Lundstrom et al., 2016), but metagenomics was also performed to primarily

determine if any other resistance genes were being co-selected; and to quantify other gene classes which also confer 3GC resistance.

Alongside the complex community experiment, a single species competition experiment between laboratory strain J53 and the multi-resistant, non-conjugative plasmid pEK499 containing variant (NCTC 13451 strain, publically available from Public Health England) was conducted to elucidate if single species assays could be useful for extrapolating to complex community scenarios.

Based on the data generated by the single species experiment, a final experiment was conducted that investigated indirect selection, in terms of the protective effect of excreted ESBLs. Briefly, the susceptible J53 strain was grown with different volumes of supernatant from an overnight culture of the ESBL producer NCTC 13451, at several cefotaxime concentrations. This simultaneously determined the minimum amount of excreted ESBL required for indirect selection; and the maximum antibiotic concentration at which indirect selection was observed.

### 2.2.1. Primary aims of this study

- Determine if a group of clinically relevant antibiotic resistance genes (*bla*$_{\text{CTX-M}}$) can be selected for at low concentrations of a clinically important antibiotic in a complex community.
- Test whether community and single species MSCs are comparable.
- Determine if cefotaxime can co-select for resistance at low concentrations.

### 2.2.2. Secondary aims of this study

- Assess the importance of chemical quantification during MSC determination.

## 2.3. Author contribution

I designed the final experimental evolution protocol for the complex community and single species experiments. I designed the ESBL supernatant experiment. I

performed all the experiments, and collected and analysed all data. Chemical extraction protocols were designed by Dr Maciek Trzndael and Dr Malcolm Hetheridge (University of Exeter), and LC-MS analyses and data collection were performed by Dr Maciek Trznadel. I analysed the chemical data. Metagenomics analysis in the ARGS-OAP pipeline was performed by Xiaole Yin in the research group of Dr Tong Zhang of the University of Hong Kong, I performed all subsequent data analysis.

## 2.4. Materials and Methods

### 2.4.1. Sample collection, storage and preparation

Sewage influent from Falmouth, Cornwall (UK), was collected on the morning of 27$^{th}$ October 2015. This WWTP serves a population size of around 43,000 and treats primarily domestic sewage but also a small outpatient hospital. Single use aliquots were mixed in a 1:1 ratio with 20 % glycerol, vortexed and stored at -80 °C until use. Before use, samples were spun down at full speed for 10 minutes, the supernatant removed, and the pellet resuspended in equal volume of 0.85 % NaCl twice to prevent nutrient/chemical carry over.

### 2.4.2. Strains

NCTC 13451 (the *E. coli* lab strain J53 plus plasmid pEK499) was ordered from Public Health England and cultured according to instructions, with 4 mg/L cefotaxime as the selective agent. J53 for the non-resistant control was grown under the same conditions, but without cefotaxime.

### 2.4.3. Minimum Inhibitory Concentration (MIC) determination

The MIC of cefotaxime for J53 and NCTC 13451 was determined in a broth microdilution assay. Overnight cultures of each strain were diluted to an optical density (OD, 600 nm) of 0.01 in Iso-sensitest (Sigma) broth with cefotaxime at different concentrations in a 96 well plate. There were 4 replicates for each treatment for each antibiotic, with an antibiotic free control, and a blank broth

assay control. The plate was incubated in a Varisokan Flash plate reader for 24 hours, at 37 °C, with background shaking at 160 rpm at 3 mm. The MIC was considered as the lowest concentration at which no growth was visible after 24 hours (Andrews, 2001).

### 2.4.4. Antibiotics

Cefotaxime (Molekular) stocks were prepared in autoclaved and filtered, deionised water. Single-use aliquots were stored at -80 °C until use to prevent degradation by repeated freeze-thawing.

### 2.4.5. Pilot experiments

#### 2.4.5.1. Experimental evolution experiment

Firstly, the appropriate amount of inoculum to use in the week long assay was determined. In this initial pilot experiment, a 96 well plate was inoculated with Iso-sensitest Broth (SigmaAldrich) containing one of the following; no bacteria (sterile 0.85 % NaCl added as a control), overnight culture of a positive control (*bla*$_{\text{CTX-M}}$ bearing *E.coli*) strain diluted 100x, or waste water diluted 10x or 100x in sterile 0.85 % NaCl, to a total volume of 200 µl. Antibiotic concentrations were 100,000, 32,000, 10,000, 2000, 1000, 500, 250, 125, 62.5, 32.25L, 15.62 and 0 µg/L for each different inoculum. The plate was incubated in a Varisokan Flash plate reader at 37 °C for 24hours, with background shaking at 160 rpm at 3 mm, with OD readings at 600 nm every hour.

Following results from the pilot experiment, the 10x diluted waste water inoculum was selected for use in the week long assay and for the cefotaxime 24 hour degradation experiment (alongside degradation in the absence of bacteria) due to the fact it had the most standard growth curve with the least variation (see Appendix).

#### 2.4.5.2. Cefotaxime 24 hour degradation

A pilot experiment for the 24 hour degradation experiment was performed, with chemical quantification and growth (OD readings) taken at 0, 4, 8, and 24 hours. Results indicated more frequent sampling time points were required.

### 2.4.6. Supernatant experiment

Two pilot experiments were carried out to optimise conditions of the supernatant experiment. In the first, it was verified the supernatant was sterile, and that it could confer protection of the susceptible strain to a cefotaxime concentration exceeding its own MIC. Briefly, J53 and NCTC 13451 were grown overnight at 37°C, shaking at 180 rpm in Iso-sensitest broth (supplemented with 2 mg/L cefotaxime for NCTC 13451). This concentration was chosen on the basis it was greater than the J53 MIC (see 2.5. Results), and that it would be fully degraded in an ESBL producing community (as shown from the degradation experiment).

The supernatant from both overnight cultures was spun down at full speed for 2 minutes twice, and then filtered through a 0.22 µM filter. J53 was then inoculated at a starting optical density of 0.01 into fresh Iso-sensitest broth and antibiotic / supernatant was added as appropriate. A blank control (to check general aseptic technique), broth with each supernatant control (to verify the supernatant was sterile); and J53 in broth both with and without antibiotic (to deduce effects of nutrient dilution) were included in all plates as controls. The assay comprised of J53 in broth with 100, 75, 50 and 25 µl of either J53 or NCTC 13451 supernatant, in both the presence and absence of cefotaxime at a final concentration of 1 mg/L.

The second pilot plate was as above, but 20, 10, 5 and 1 µl of each supernatant was added, to determine the lowest volume of supernatant which indirectly selected for J53.

### 2.4.7. Cefotaxime 24 hour degradation experiment

For this experiment, washed, untreated waste water was diluted 10x in 25ml Iso-sensitest broth aliquots spiked with cefotaxime concentrations of 0, 15.625, 31.25, 62.5, 125, 250, 500 or 2000 µg/L. These were incubated at 37 °C, 180 rpm shaking in between sampling. Chemical extractions (see below) and destructive

sampling for OD readings were performed at time 0, then every 3 hours for 24 hours. OD measurements were carried out in a spectrophotometer (Jenway) at the same time points at 600 nm. Any OD readings with a value greater than 1 were diluted 10x in Iso-sensitest broth and then remeasured.

### 2.4.8. Waste water (complex community) microcosm experiment

Iso-sensitest broth was inoculated with a 10x dilution of washed, untreated waste water. This was separated into 30ml aliquots and appropriate amounts of cefotaxime stocks were added. This was then separated further into 5ml aliquots of 5 replicates for each of the cefotaxime assay concentrations: 2000, 1000, 500, 250, 125, 62.5, 32.25, 15.625 and 0 µg/L.

All replicates were immediately sampled for the day 0 sampling time point: 2 x 1 ml of each was spun down at full speed for 3 minutes, the supernatant removed and pellet resuspended in 500 µl 20 % glycerol followed by storage at -80 °C. All other samples for DNA extraction were taken after incubation overnight at 37 °C, 180 rpm shaking, as above but 500 µl was sampled.

After each incubation, 50 µl of each microcosm was introduced into 5 ml fresh media with fresh antibiotic, and samples were taken as above, every day for total of 8 days. Remnants of the assay after the eighth day were spun down and stored as above.

A second complex community assay was then conducted, as above, but with higher cefotaxime concentrations. The concentrations used were 0, 2, 4, 8, 16, 32, 64 and 128 mg/L.

### 2.4.9. Single species microcosm experiment

Overnight cultures of NCTC 13451 and J53 from a single colony were combined into a 1:1 ratio based on OD600 readings. A 1000x dilution of this (as in Gullberg et al. (2011)) was added to Iso-sensitest broth, and 1000x dilutions thereafter. The same cefotaxime concentrations in two separate experiments as the complex community assays were used (see 2.4.8. Waste water (complex community) microcosm experiment). These samples were processed as above

except samples were only collected at day 0 and day 8, and 1 ml was spun down at day 8.

### 2.4.10. Chemical extraction

Waste water microcosms were sampled at day 0, after the first 24 hours, at the beginning of day 7, and at the end of the experiment (day 8). Antibiotic stocks were also analysed at the beginning and the end of the experiment. The single species microcosms were only sampled at the beginning of day 8, alongside the stocks.

The extraction procedure was as follows: 400 µl of culture was mixed with 400 µl HiPerChromosolv Acetonitrile in a 2 ml 96 well plate, and spun at 3500 rpm for 30 minutes. 100 µl of this supernatant was then mixed with 900 µl of 1:4 Acetonitrile to HPLC-grade water in a fresh plate, and stored in the fridge. Antibiotic stocks were diluted to a final concentration of 100 ng/L in 1:4 Acetonitrile. Extractions were kept at -20 °C or 4 °C until processing.

### 2.4.11. Chemical analysis

Each concentration had a minimum of two chemical replicates from at least two of the biological replicates. Stocks were single replicate only. Chemical quantification was performed at the University of Exeter Streatham Campus by Maciek Trnzadel.

### 2.4.12. QPCR

Frozen samples / untreated waste water were thawed and DNA extracted using the MBio UltraClean DNA extraction Kit according to instructions; with initial spinning time elongated to 3 minutes. DNA was stored at -20 °C.

GBlock synthetic genes (IDTDNA - Table 1) were used as standards for the complex community experiment; these were resuspended in TE buffer according to the manufacturer's instructions and were stored at -80 °C. For the single species experiment, 1 ml of overnight culture of NCTC 13451 underwent DNA extraction as above, and was used as the standards for both 16S and *bla*$_{\text{CTX-M}}$ qPCRs for the single species experiment. For 16S, the cfu/ml was multiplied by

7 to account for the multiple copies present in *E. coli*, whereas *bla*$_{CTX-M}$ copy number was left unchanged.

All DNA standards were 10x serially diluted in TE buffer and stored at -20 °C before use. Every PCR plate was always run with 5 serial dilutions of standards in duplicate (and a duplicate negative control). Provided the efficiency for the reaction was between 90 % and 110 %, the average CT's for the duplicate technical replicates for each sample was used to calculate the copy number based on a 'gold standard' standard dilution series, where the DNA concentration had been quantified by QuBit and the copy number per µl quantified immediately prior to cycling.

Standards were first verified using qPCR conditions described below, with a melt curve in SYBR assays. Brilliant qPCR SYBR Green reagents (Agilent) were used in 20 µl reactions comprising of 10 µl master mix, 2 µl primer pair (10 µM for 16S, *Enterobacteriaceae* and *E. coli* primers, 18 µM for *bla*$_{CTX-M}$ primers), 0.2 µl BSA (20 mg/ml), 0.4 µl dye (20 µM), 5 µl diluted DNA template and filtered, sterilised water to a total volume of 20 µl. The qPCR programme for all SYBR reactions was 95 °C for 3 minutes, followed by 40 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds, except *bla*$_{CTX-M}$ which was 58 °C for 1 minute. Relative numbers of *bla*$_{CTX-M}$ copies were divided by cell number (16S) to determine numbers of *bla*$_{CTX-M}$ per copy number of 16S (a molecular 'prevalence').

To determine relative numbers of *bla*$_{CTX-M}$ groups 1 and 9, a TaqMan assay was performed. 20 µl reactions containing 10 µl Life Technologies TaqMan master mix for environmental samples, 2 µl CTX-M consensus primer pair (18 µM), 0.2 µl BSA (20 mg/ml), 1 µl CTX-M1/9 probe (4 or 2 µM, respectively), 5 µl diluted DNA template and filtered, sterilised water to a total volume of 20 µl. Primer sequences and original references are shown in Table 1.

Table 1. Sequences and length of the different primers, probes and gBlocks used in this study, with original references or the accession number used where applicable.

| Name | Sequence | Size (bp) | Accession number | Reference |
|---|---|---|---|---|
| 16S gBlock | ACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAACCG | 144 | - | This study |

| | | | | |
|---|---|---|---|---|
| CTX-M gBlock | GATGTGCAGCACCAGTAAAGTGATGGCCGCGGCCGC GGTGCTGAAGAAAAGTGAAAGCGAACCGAATCTGTTA AATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTTAA CTATAATCCGATTGCGGAAAAGCACGTCAATGGGACG ATGTCACTGGCTGAGCTTAGCGCGGCCGCGCTACAGT ACAGCGATAACGTGGCGATGAATAAGCTGATTGCTCA CGTTGGCGGCCCGGCTAGCGTCACCGCGTTCGCCCG ACAGCTGGGAGACGAAACGTTCCGTCTCGACCGTACC GAGCCGACGTTAAACACCGCCATTCCGGGCGATCCG CGTGATA | 338 | - | This study |
| Group 1 CTX-M gBlock | ACTGGGTGTGGCATTGATTAACACAGCAGATAATTCG CAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGTG CAGCACCAGTAAAGTGATGGCCGTGGCCGCGGTGCT GAAGAAAAGTGAAAGCGAACCGAATCTGT TAAATCAGCGAGTTGAGATCAAAAAATCTGACTTGGTT AACTATAATCCGATTGCGGAAAAGCACGTCGA TGGGACGATGTCACTGGCTGAGCTTAGCGCGGCCGC GCTACAGTACAGCGATAACGTGGCGATGAATAAG CTGATTTCTCACGTTGGCGGCCCGGCTAGCGTCACCG CGTTCGCCCGACAGCTGGGAGACGAAACGTTCC GTCTCGACCGTACCGAGCCGACGTTAAACACCGCCAT TCCGGGCGATCCGCGTGATACCACTTCACCTCG GGCAATGGCGCAAACTCTGCGTAATCTGACGCTGGGT AAAGCATTGGGTGACAGCCAACGGGCGCAGCTG | 490 | KJ484641 .1 | This study |
| Group 9 CTX-M gBlock | GCTGGGCGTCGCGCTCATCGATACCGCAGATAATACG CAGGTGCTTTATCGCGGTGATGAACGCTTTCCA ATGTGCAGTACCAGTAAAGTTATGGCGGCCGCGGCG GTGCTTAAGCAGAGTGAAACGCAAAAGCAGCTGC TTAATCAGCCTGTCGAGATCAAGCCTGCCGATCTGGT TAACTACAATCCGATTGCCGAAAAACACGTCAA CGGCACAATGACGCTGGCAGAACTGAGCGCGGCCGC GTTGCAGTACAGCGACAATACCGCCATGAACAAA TTGATTGCCCAGCTCGGTGGCCCGGGAGGCGTGACG GCTTTTGCCCGCGCGATCGGCGATGAGACGTTTC GTCTGGATCGCACTGAACCTACGCTGAATACCGCCAT TCCCGGCGACCCGAGAGACACCACCACGCCGCG GGCGATGGCGCAGACGTTGCGTCAGCTTACGCTGGG TCATGCGCTGGGCGAAACCCAGCGGGCGCAGTTG | 490 | HF545433 .1 | This study |
| *E. coli* gBlock | GCAGTCTTACTTCCATGATTTCTTTAACTATGCCGGGA TCCATCGCAGCGTAATGCTCTACACCACGCCGAACAC CTGGGTGGACGATATCACCGTGGTGACGCATGTCGC GCAAGACTGTAACCACGCGTCTGTTGACTGGCAGGTG GTGGCCAATGGTGATGTCAGCGTTGAACTGCGTGATG CGGATCAACAGGTGGTTGCAACTGGACAAGGCACTAG CGGGACTTTGCAAGTGGTGAATCCGCACCTCTGGCAA CCGGGTGAAGGTTATCTCTATGAACTGTGCGTCACAG CCAAAAGCCAGACAGAGTGTGATATCTACCCGCTTCG CGTCGGCATCCGGTCAGTGGCAGTGAAGGGCGAACA GTTCCTGATTAACCACAAACCGTTCTACTTTACTGGCT TTGGTCGTCATGAAGATGCGGACTTGCGTGGCAAAGG ATTCGATAACGTGCTGATGGTGCACGACCACGCATTA ATGGACTGGATTGGGGCCAACTCCTACCGTACCTCGC ATTA | 522 | NC_0009 13.3 | This study |
| Enteric 23s rRNA gBlock | TGCCGTAACTTCGGGAGAAGGCACGCTGATATGTAGG TGAAGTCCCTCGCGGATGGAGCTGAAATCAGTCGAAG ATACCAGCTGGCTGCAACTGTTTATTAAAAACACAGCA CTGTGCAAACACGAAAGTGGCAGTATCAGGTGTGACA CCTGCCCGGTGCCGGAAGGTTAATTGATGGGGTTAGC GGTAACGCGAAGCTCTTGATCGAAGCCCCGGTAAACG GCGGCCGTAACTATAACGGTCCTAAGGTAGCGAAATT CCTTGTCGGGTAAGTTCCGACCTGCACGAATGGCGTA ATGATGGCCAGGCTGTCTCCACCCGAGACTCAGTGAA ATTGAACTGCGTGTGAAGATCGAGTGTACCCGCGGCA AGACGGAAAGACCCCGTGAACCTTTACTATAGCTTGA CACTGACACTTGACGATTGA | 428 | - | This study |
| 16S F (1396F) | CGGTGAATACGTTCYCGG | 142 | - | (Suzuki et al., 2000) |

| | | | | |
|---|---|---|---|---|
| 16S R (1492R) | GGWTACCTTGTTACGACT | 142 | - | |
| CTX-M consensus F | ATG TGC AGY ACC AGT AAR GTK ATG GC | ~300 | - | (Birkett et al., 2007) |
| CTX-M consensus R | ATC ACK CGG RTC GCC XGG RAT | ~300 | - | |
| Group 1 probe | MGB-CCCGACAGCTGGGAGACGAAACGT-FAM | ~300 | - | |
| Group 9 probe | MGB-CTGGATCGCACTGAACCTACGCTGA-VIC | ~300 | - | |
| E.coli F (YccTR-F) | GCATCGTGACCACCTTGA | 522 | - | (Clifford et al., 2012) |
| E.coli R (YccTRT-R) | CAGCGTGGTGGCAAAA | 522 | - | |
| Enteric F (En-ISu3F) | TGCCGTAACTTCGGGAGAAGGCA | 428 | - | (Matsuda et al., 2007) |
| Enteric R (En-Isu3'R) | TCAAGGCTCAATGTTCAGTGTC | 428 | | |

### 2.4.13. Minimal Selective Concentration (MSC) determination

The LOECs were determined statistically (see 2.4.16. Statistics), and MSCs by using selection coefficients, as previously (Gullberg et al., 2014, Gullberg et al., 2011). Selection coefficients were determined with the following equation: "[ln(R(*t*)/R(0))]/[*t*]"; where R = the ratio of resistant to susceptible (i.e. resistance gene prevalence) at the beginning ('R(0)') or end ('R(t)') of the experiment and *t* = time (i.e. length of the assay, in days). All 5 selection coefficients representing each biological replicate for each antibiotic concentration were averaged. These were then plotted against antibiotic concentration (a maximum of 6 antibiotic concentrations, as used previously (Gullberg et al., 2014, Gullberg et al., 2011)) with a line of best fit. Where this line crosses the x-axis is the MSC.

### 2.4.14. Plating for phenotypic resistance

The complex community assay was also plated at the beginning and end of both high and low concentration experiments on Chromocult Enhanced Selectivity agar (Merck), with and without cefotaxime at the clinical breakpoint concentration for *Enterobacteriaceae* ((EUCAST, 2014), equating to 2 mg/L). Equal volumes of all biological replicates were combined and diluted in 0.85 % saline solution. Plating was performed in duplicate or triplicate for each tenfold dilution (for the

low and high concentration assays, respectively). Higher replication was possible for the higher concentration experiment as there was a smaller concentration range. This enabled enumeration of average total numbers of resistant presumptive *E. coli*, other enterics, and other Gram negatives based on colour. The percentage of each group of bacteria that was resistant was also determined for day 0 and day 8 of the higher concentration experiment, and at day 0 only for the lower concentration experiment (plates without cefotaxime were contaminated at day 8).

### 2.4.15. Metagenome analyses

Three replicates were chosen at random from the no antibiotic, 125, 500 and 2000 µg/L treatment at day 8 to undergo sequencing on the MiSeq2 v2 platform at University of Exeter Sequencing Service (ESS). These concentrations were selected based on the qPCR results (see 2.5.2. Results) and corresponded to the LOEC, *bla*$_{\text{CTX-M}}$ prevalence peak, and clinical breakpoint concentrations.

DNA was extracted from 1 ml of frozen overnight culture using the MoBio extraction kit according to manufacturer's instructions. DNA was cleaned and concentrated using Ampure™ beads. Firstly, 2 µl of 20 mg/ml RNAse A (Qiagen) was added to 50 µl DNA and incubated for 10 minutes at 37 °C. 50 µl of Ampure™ beads were mixed with the DNA / RNAse solution gently by pipetting, then incubated at room temperature for 5 – 10 minutes. Following pulse centrifugation to collect droplets, tubes were placed on a magnetic stand and left until all beads had precipitated to the side of the tube. Supernatant was removed and beads were washed two times with 300 µl freshly prepared 80 % ethanol. Beads were air dried briefly (1 – 2 minutes), resupsended in 10 µl 10 mM Tris-HCL and then incubated for another 10 minutes at 50 °C. Following pulse centrifugation and bead precipitation, DNA was transferred into a fresh tube and stored at -20 °C until library preparation and sequencing.

The 12 Nextera Library preparations, quality control, sequencing and primary sequencing analysis (including trimming reads of the barcodes) was performed by ESS. Data was then run through the "online analysis pipeline for antibiotic resistance genes detection from metagenomic data using an integrated structured antibiotic resistance gene database", namely the ARGs-OAP (Yang et al., 2016) pipeline by Xiaole Yin in the research group of Dr Tong Zhang. This

provides the abundance of different resistance gene classes and subtypes within these groups normalised by parts per million, 16S copy number, and per cell. For all subsequent analysis, data normalised by 16S copy number was used to allow comparison with qPCR data. The negative control sequencing sample had no hits for any resistance genes.

Heat maps were generated in using python packages matplotlib, pandas and seaborn (Hunter, 2007, McKinney, 2010, Waskom, 2016) for resistance gene class and β-lactam resistance gene subtype.

### 2.4.16. Statistics

All statistics were performed in RStudio (2015). A Kruskal Wallis was used to compare: total 16S, *bla*$_{\text{CTX-M}}$ gene copy number and *bla*$_{\text{CTX-M}}$ gene prevalence at day 0 (to verify replicates were not significantly different at day 0), and at day 8. For determining LOECs, Dunn's test was used to determine if there was a significant difference between treatment (with cefotaxime) and the no antibiotic control.

Spearman's rank was performed to determine if there was a significant, monotonic relationship between antibiotic concentration and average *bla*$_{\text{CTX-M}}$ prevalence for both the complex community and single species experiments. This was for all concentrations across both experiments, using the no antibiotic control and the 2 mg/L treatment from the lower concentration experiment.

## 2.5. Results

### 2.5.1. *Bla*$_{\text{CTX-M}}$ genes are selected for at low cefotaxime concentrations in a complex community

These results relate to the primary hypothesis that clinically important resistance genes are selected for in a complex community at low antibiotic concentrations. This was investigated with qPCR by tracking resistance gene prevalence (total *bla*$_{\text{CTX-M}}$, *bla*$_{\text{CTX-M}}$ group 1, *bla*$_{\text{CTX-M}}$ group 9 and 16S rRNA copy numbers) over time at different cefotaxime concentrations, to determine MSCs (Minimal Selective Concentrations, with the selection coefficient method) and LOECs (Lowest Observed Effect Concentrations, using a statistical approach).

There was no significant statistical difference in *bla*$_{CTX-M}$ prevalence between any of the treatments at day 0 ($p$ > 0.05, Kruskal Wallis, Figure 5). However, at day 8 there was a significant statistical difference in *bla*$_{CTX-M}$ prevalence at concentration 125 µg/L, and all the treatments above compared to the no antibiotic control ($p$ < 0.05, Dunn's Test). This is termed the LOEC. However, the increase in *bla*$_{CTX-M}$ prevalence was not monotonic (i.e. there was no dose response relationship between prevalence and cefotaxime concentration, Spearman's rank, $r_s$ = 0.48, $p$ > 0.05).

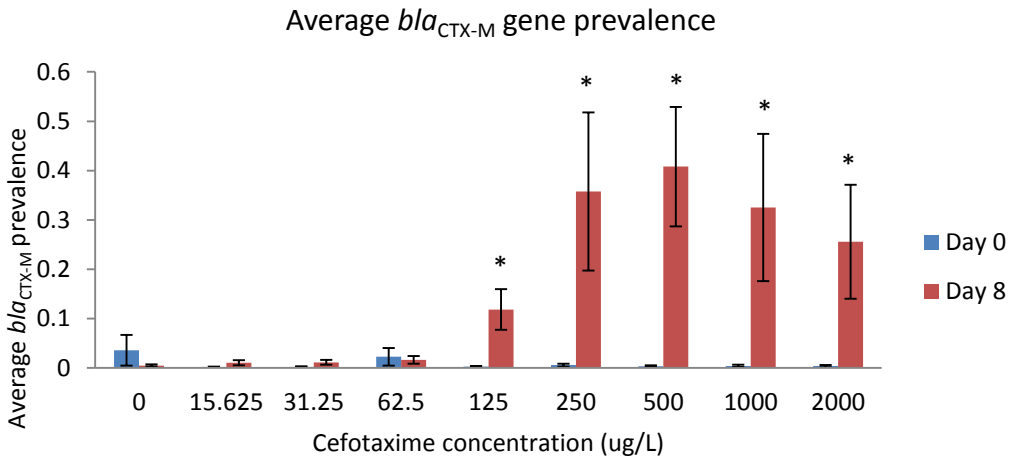


Figure 5. Average (biological replicate n=5, qPCR replicate n=2) *bla*$_{CTX-M}$ gene prevalence at the beginning and end of the lower cefotaxime concentration complex community experiment, shown with standard error bars. Prevalence calculated by dividing *bla*$_{CTX-M}$ gene copy number by 16S copy number for each biological replicate. There is a significant increase in *bla*$_{CTX-M}$ gene prevalence from 125 µg/L, and all treatments above (p < 0.05, Dunn's test '*').

The selection coefficient method for MSC determination was also used (Figure 6), as previously (Gullberg et al., 2014, Gullberg et al., 2011). The MSC was calculated as 0.4 µg/L.

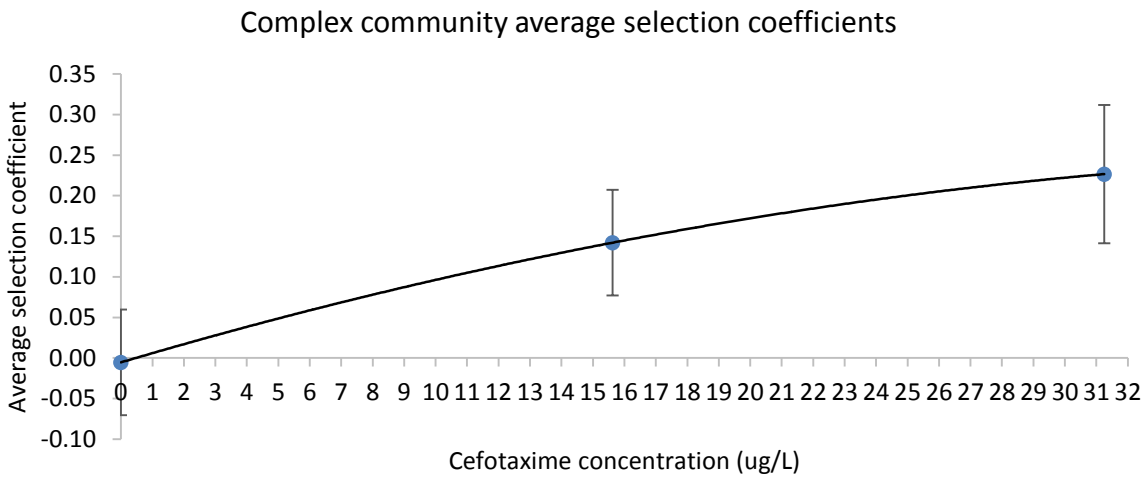


Figure 6. Average (biological replicate n=5, qPCR replicate n=2) selection coefficients based on the qPCR *bla*$_{CTX-M}$ prevalence data at day 0 and day 8, shown with standard error bars and polynomial (order 2) line of best fit. The MSC is where the line crosses the x-axis - at 0.4 µg/L.

There was no statistical difference between any of the treatments at day 0 or day 8 for 16S copy number ($p$ > 0.05, ANOVA and Kruskal Wallis, respectively), allowing for analysis of the *bla*$_{CTX-M}$ gene copy number (which would not be biased by changes in overall community numbers). The data is presented in Figure 7 but the statistical difference still emerged at the 125 µg/L treatment.

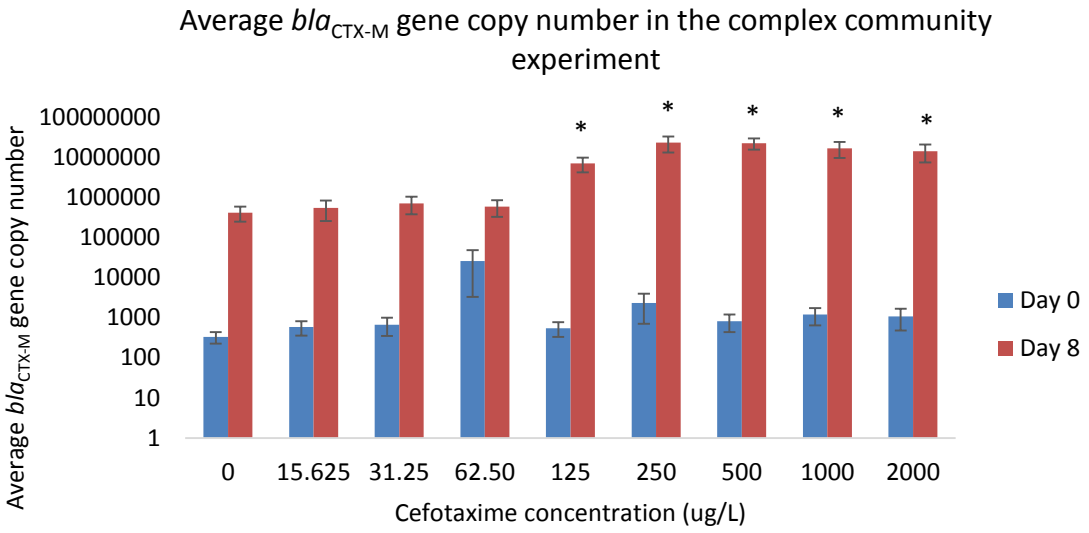


Figure 7. Average (biological replicate n=5, qPCR replicate n=2) *bla*$_{CTX-M}$ gene copy number at day 0 and day 8 in the lower cefotaxime concentration complex community experiment. The 125 µg/L treatment and all treatments above were significantly different to the no antibiotic control ($p < 0.05$, Dunn's test '*').

The *bla*$_{CTX-M}$ group specific TaqMan assay (Figure 8) showed that group 1 *bla*$_{CTX-M}$ genes were always more prevalent than group 9, even in the no antibiotic control; however, there was a more pronounced increase in group 9 *bla*$_{CTX-M}$ genes when exposed to cefotaxime (though this was still not a significant difference). Again, a significant difference to the no antibiotic control was observed at 125 µg/L and all the concentrations above for both *bla*$_{CTX-M}$ group 1 and group 9. There was no significant difference observed between treatments for group 1 and group 9 *bla*$_{CTX-M}$ genes at the beginning of the experiment (Kruskal Wallis tests, $p > 0.05$, data not shown).

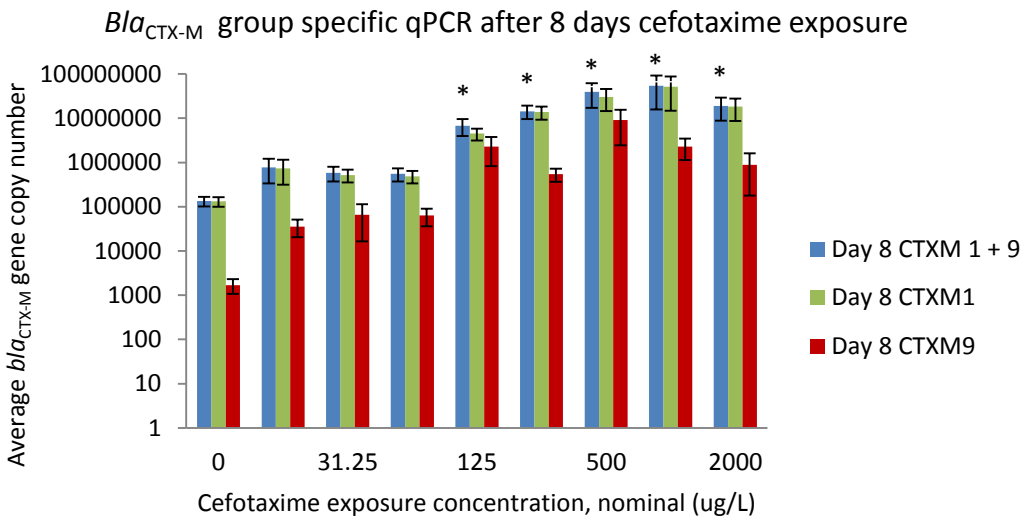


Figure 8. Average (biological replicate n=5, qPCR replicate n=2) copy number for group 1 and group 9 *bla*$_{CTX-M}$ genes following 8 days cefotaxime exposure. Shown with standard error bars, on a logged y axis. The 125 µg/L treatment and all treatments above were significantly different to the no antibiotic control ($p < 0.05$, Dunn's test '*').

Additional qPCR data was also generated for the day 1 and day 4 time points, to evaluate the change in *bla*$_{CTX-M}$ prevalence over time (Figure 9). For all concentrations except 1000 and 2000 µg/L, average *bla*$_{CTX-M}$ prevalence increased steadily overtime. The LOEC was determined as 125 µg/L at day 1, and at 250 µg/L at day 4 (Dunn's test, $p < 0.05$).

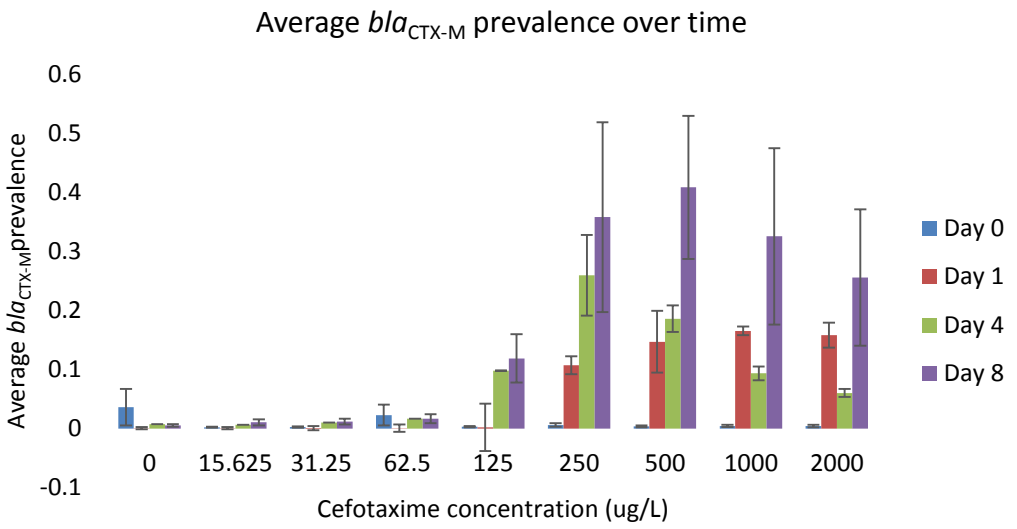


Figure 9. Average (biological replicate n=5, qPCR replicate n=2) *bla*$_{CTX-M}$ prevalence at day 0, 1, 4 and 8 of the lower cefotaxime concentration complex community experiment.

### 2.5.2. The increase in *bla*$_{CTX-M}$ genes is not due to an enrichment for *Enterobacteriaceae*

An *E. coli* and enteric specific qPCR was used to determine if their respective percentages in the community changed following cefotaxime exposure. Results showed that there was no significant difference in the numbers of *E. coli* or total enterics across the treatments, indicating *bla*$_{CTX-M}$ genes are not confined only to these bacterial groups (Figure 10).

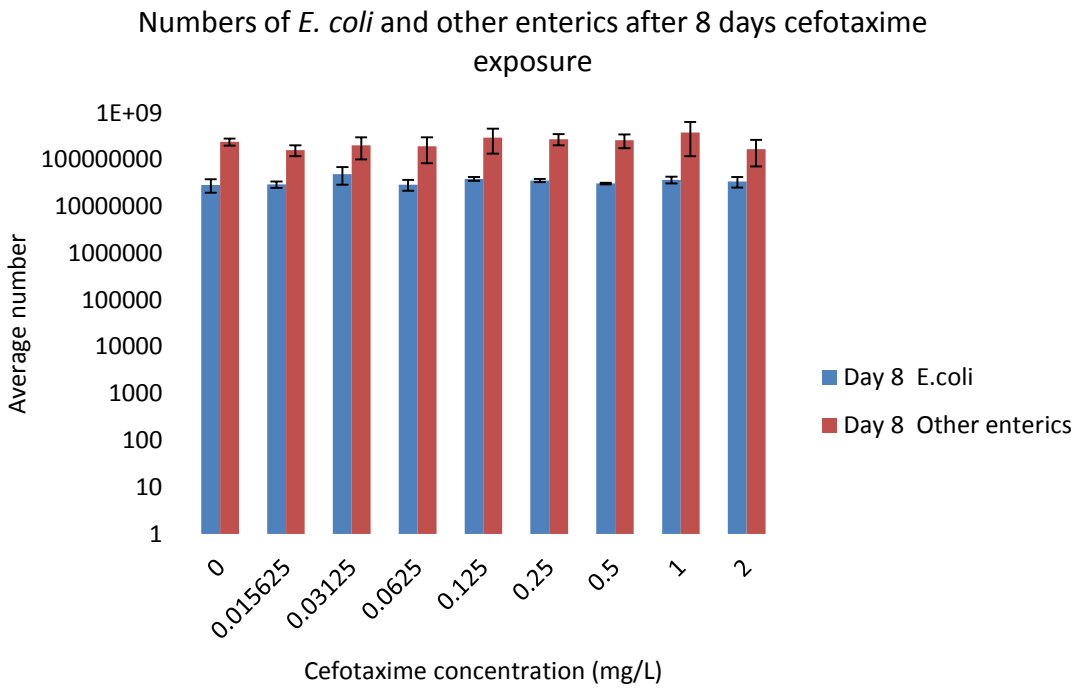


Figure 10. Average (biological replicate n=5, qPCR replicate n=2) numbers of *E. coli* and enterics in the complex community following 8 days cefotaxime exposure, using SYBR green qPCR. Shown with standard error bars.

### 2.5.3. *Bla*$_{CTX-M}$ prevalence does not reach 1 even at extremely high cefotaxime concentrations

Following the plateau observed in *bla*$_{CTX-M}$ prevalence at 500 µg/L and above, an additional experiment was performed at higher cefotaxime concentrations (Figure 11). The *bla*$_{CTX-M}$ gene prevalence for all cefotaxime treatments were significantly different at day 8 compared to the no antibiotic control ($p < 0.05$, Dunn's test). The highest average *bla*$_{CTX-M}$ prevalence of 0.79 was at 64 mg/L; then *bla*$_{CTX-M}$ prevalence decreased at the highest assay concentration of 128 mg/L. The prevalence data for time 0 and at day 8 for the no antibiotic control and the 2 mg/L treatment were highly comparable with the prevalence data obtained for these treatments in the lower cefotaxime concentration experiment. Both data sets were combined to test for monotonic increase in *bla*$_{CTX-M}$ gene prevalence with antibiotic concentration, which was not the case ($r_s = 0.48$, $p > 0.05$).

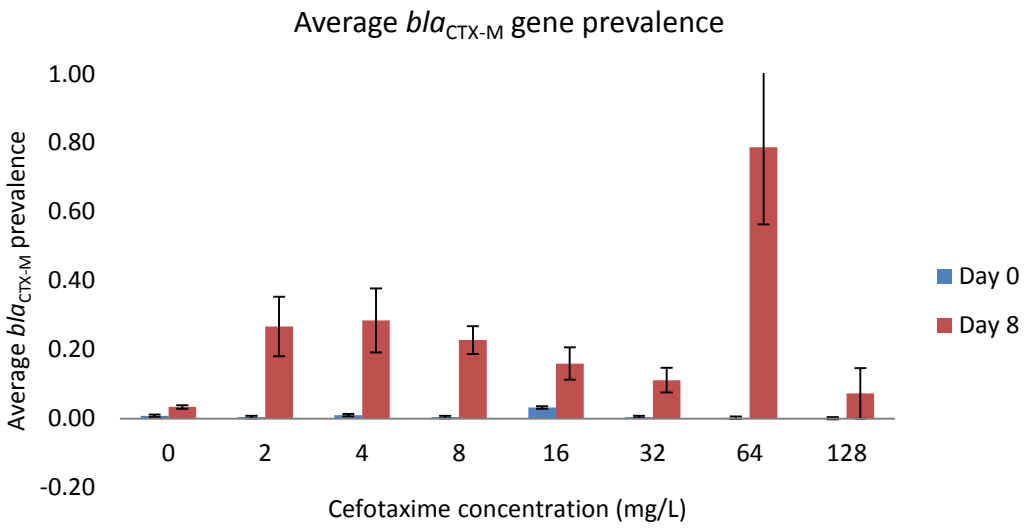


Figure 11. Average (biological replicate n=5, qPCR replicate n=2) *bla*$_{CTX-M}$ gene prevalence in the complex community before and after exposure to the higher cefotaxime concentrations. Shown with standard error bars. *Bla*$_{CTX-M}$ gene prevalence calculated by dividing the *bla*$_{CTX-M}$ gene copy number by 16S copy number for each biological replicate.

The *bla*$_{CTX-M}$ gene group numbers varied at the end of the experiment following exposure to higher cefotaxime concentrations (Figure 12). Group 9 *bla*$_{CTX-M}$ genes were more prevalent at 2, 4 and 16 mg/L, but were absent (or below detection limit) at 32, 64 and 128 mg/L.

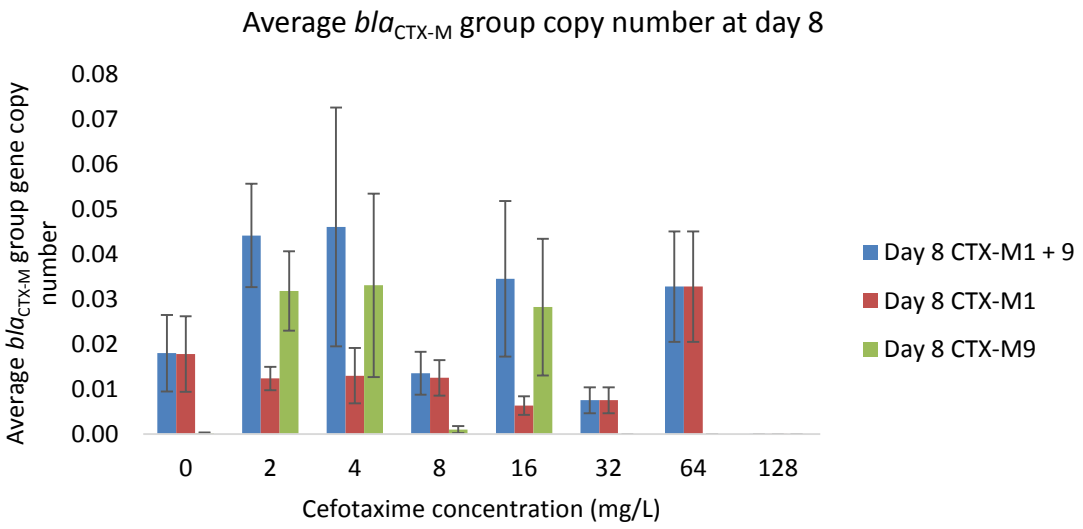


Figure 12. Average (biological replicate n=5, qPCR replicate n=2) $bla_{CTX-M}$ gene copy number for group 1 and group 9 $bla_{CTX-M}$ genes. Shown with standard error bars.

### 2.5.4. Phenotypic clinical resistance in *Enterobacteriaceae* agrees with LOEC

As in a previous study (Lundstrom et al., 2016), phenotypic and qPCR selective endpoints were compared. Figure 13 shows the average (n=2) numbers of resistant (MIC > 2 mg/L) *E. coli*, enterics and other Gram negatives in the original sewage inoculum ('Sewage') and following 8 days cefotaxime exposure in the low concentration experiment. The cfu/ml of resistant *E. coli* plus enterics remains high for all cefotaxime concentrations, but increases significantly at 125 µg/L ($p < 0.05$, Dunn's test) and at 1000 µg/L, with a trend for significance ($p = 0.09$) at 2000 µg/L. Interim concentrations are not significantly different to the no antibiotic control. Numbers of resistant enterics are absent in the no antibiotic treatment, which is not observed in the higher concentration experiment (Figure 14). Numbers of resistant other Gram negatives decrease drastically compared to the numbers in the original inoculum, again not observed in the higher concentration experiment (Figure 15).

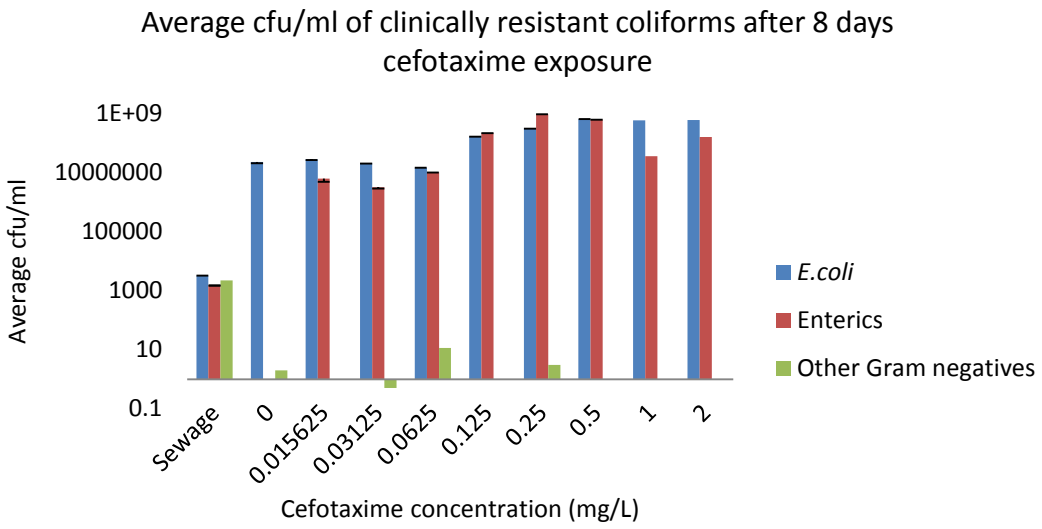


Figure 13. The average (biological replicate n=5 combined, plating replicate n=2) colony forming units (cfu) per ml of presumptive *E. coli*, other enterics and other Gram negatives quantified on Chromocult Coliform Enhanced Selectivity agar supplemented with the clinical breakpoint concentration of cefotaxime (2000 µg/L, (EUCAST, 2014)), at the end of the lower cefotaxime concentration, complex community experiment. Shown with standard error bars.

In the higher cefotaxime concentration experiment, again, the total numbers of resistant *Enterobacteriaceae* for all cefotaxime concentrations were significantly different to the no antibiotic control as with the genotypic data ($p < 0.05$, Dunn's test) at day 8 (Figure 14). However, average (plating replicates n=3) numbers of resistant Gram negatives and enterics remained constant throughout, until resistant enterics disappeared at 32 mg/L and all concentrations above. In terms of percentage of clinical resistance (Figure 15), all enterics present at 2 mg/L and above were resistant (MIC >2 mg/L). At 64 and 128 mg/L, 100 % of *E. coli* and other Gram negatives were resistant. The increase in percentage of resistance for all three types of coliforms was dramatic, with the no antibiotic control having less than 5 % of each group resistant, and the lowest percent resistance for cefotaxime exposure treatments being for *E. coli* at 8 mg/L, at 40 %.

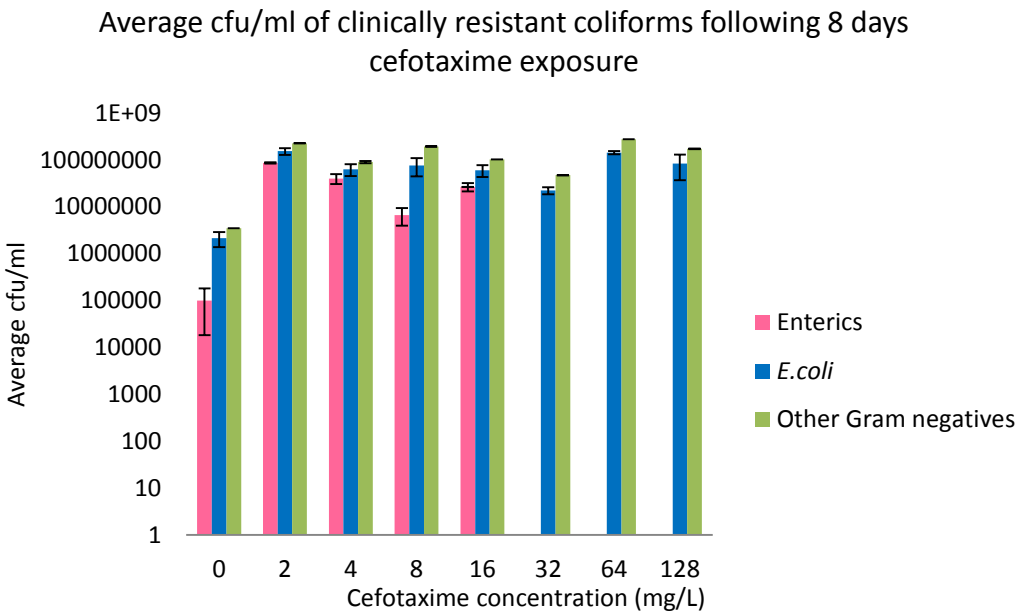


Figure 14. Average (biological replicate n=5 combined, plating replicate n=3) colony forming units (cfu) per ml of presumptive *E. coli*, other enterics and other Gram negatives quantified on Chromocult Coliform Enhanced Selectivity agar supplemented with the clinical breakpoint concentration of cefotaxime (2 mg/L, (EUCAST, 2014)), at the end of the higher cefotaxime concentration, complex community experiment. Shown with standard error bars.

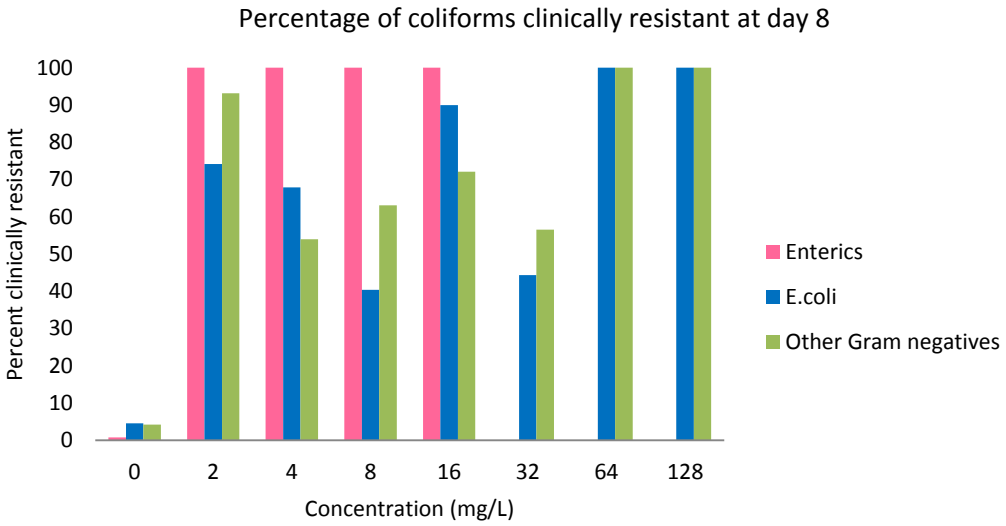


Figure 15. Percentage of coliforms at the end of the higher cefotaxime concentration, complex community experiment that were resistant (MIC > 2 mg/L)

to cefotaxime. Percentage calculated by taking the average numbers of resistant bacteria (Figure 14) and dividing by the average total coliform count, multiplied by 100.

### 2.5.5. Co-selection for different gene classes occurred

To assess the co-selective potential of cefotaxime, metagenome analyses using the ARGS-OAP pipeline was performed on three randomly selected replicates from day 8 from the no antibiotic control, LOEC, *bla*$_{CTX-M}$ prevalence peak and clinical breakpoint concentrations. These analyses showed several resistance gene classes were enriched alongside the β-lactam resistance gene class (Figure 16), namely genes conferring aminoglycoside, sulphonamide, trimethroprim, tetracycline, macrolide and (to a lesser extent) vancomycin resistance. Concurrently, some resistance gene class abundances decreased, such as those for chloramphenicol, fosfomycin and fosmidomycin. Intriguingly, quinolone resistance was enriched at 500 µg/L but then lost again at 2000 µg/L. Resistance genes for carbomycin, fusaric-acid, fusidic-acid, promycin, rifamycin, spectinomycin and tetracenomycin-C were undetected at all antibiotic concentrations.

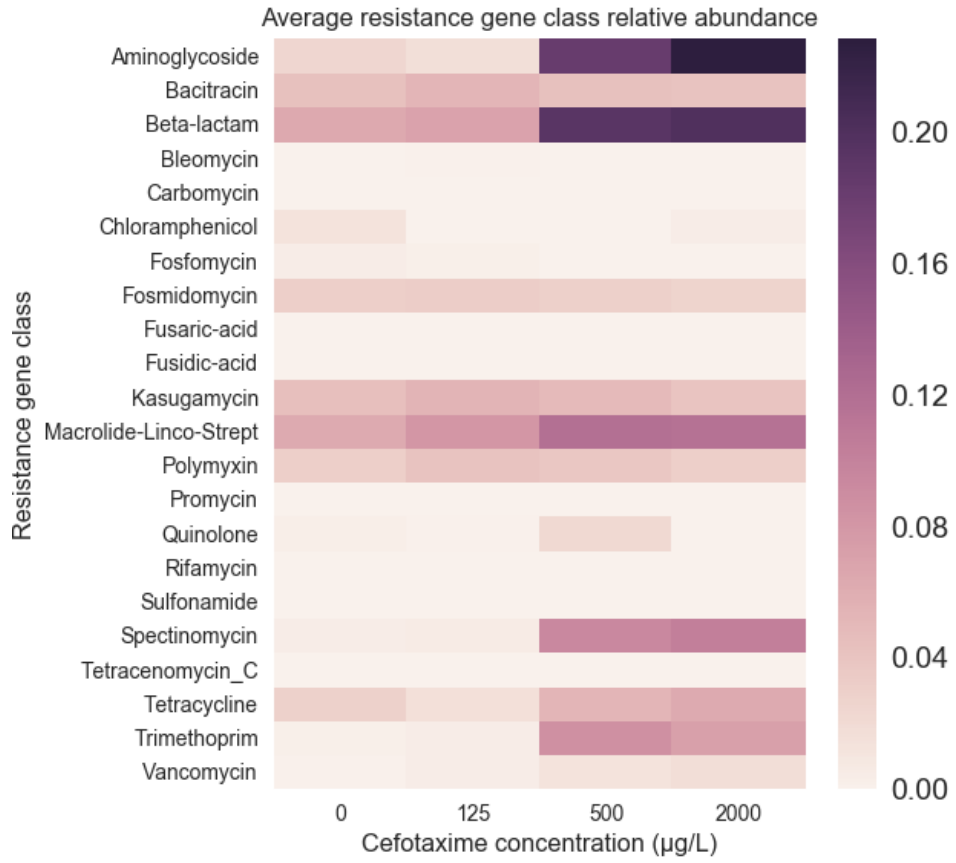


Figure 16. Average (biological replicate n=3) resistance gene class abundance, normalised by 16S copy number.

The sum of different resistance gene subtypes within the β-lactam resistance gene class were also analysed (Figure 17). Almost all major β-lactamases were enriched by cefotaxime exposure, including *bla*$_{CTX-M}$, *bla*$_{OXA}$, *bla*$_{TEM}$, and other Class A and Class C β-lactamases. The *bla*$_{CTX-M}$ gene class was enriched the most, with an increase in abundance of over 70 times in the 2000 µg/L treatment compared to the no antibiotic control, meanwhile the next highest increase in abundance was for *bla*$_{OXA}$ and *bla*$_{TEM}$, which both increased by over 8 times.

Class B β-lactamases were present in the no antibiotic control at a very low abundance, but were undetectable in the cefotaxime treatments. *Bla*$_{SHV}$ β-lactamases were present in the 0 and 125 µg/L treatments at relatively low abundance, but were absent at higher cefotaxime concentrations. Other Class D β-lactamases and metallo-β-lactamases were undetected in all of the treatments,

including the no antibiotic control. There was no noticeable increase, and most frequently there was a decrease, in the abundance of other resistance gene subtypes such as PBPs (penicillin binding proteins). A variety of other β-lactamases were detected at varying abundances (Figure 17).

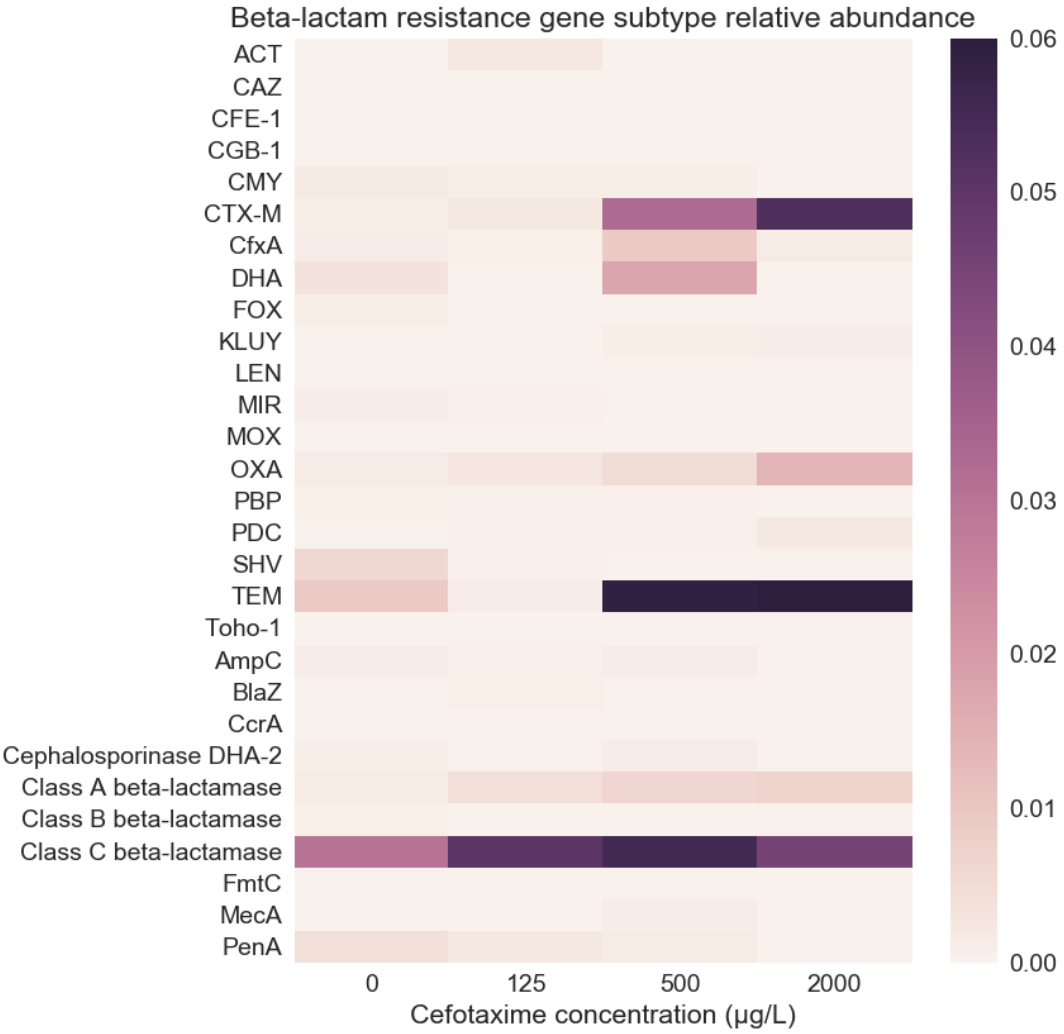


Figure 17. Average (biological replicate n=3) β-lactam resistance gene subtype abundance, normalised by 16S copy number. Only detected resistance gene subtypes are shown. Resistance gene key for encoded enzymes: ACT = AmpC type β-lactamase, CAZ = ceftazidimase, CFE-1 = AmpC type β-lactamase, CGB-1 = Ambler Class B β-lactamase, CMY = Ambler Class C β-lactamase, CTX-M = ESBL, CfxA = Divergent Ambler Class A β-lactamase, DHA = AmpC type β-lactamase, FOX = AmpC type β-lactamase, KLUY = chromosomally-encoded β-lactamase, LEN = chromosomally-encoded β-lactamase, MIR = AmpC type β-lactamase, MOX = AmpC type β-lactamase, OXA = Ambler Class D β-lactamase, PBP = penicillin binding protein, PDC = AmpC type β-lactamase, SHV = Ambler Class A β-lactamase or ESBL, TEM = Ambler Class A β-lactamase or ESBL,

Toho-1 = Ambler Class A mutant β-lactamase, AmpC = Ambler Class C β-lactamase, blaZ = *Staphylococcus aureus* β-lactamase, CcrA = Ambler Class B metallo-β-lactamase, FmtC = membrane-associated protein, MecA = penicillin binding protein, PenA = Ambler Class A secreted β-lactamase.

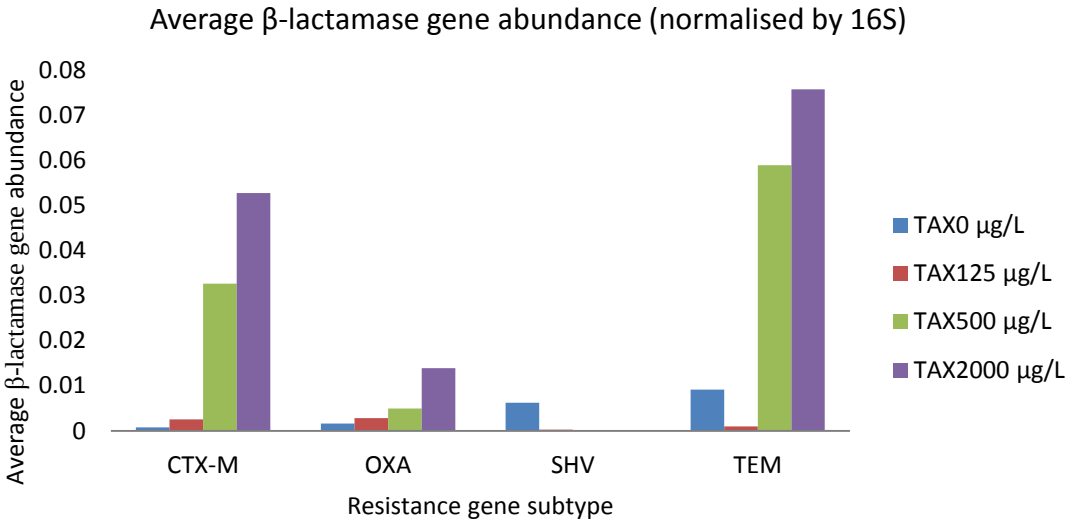


Figure 18. The change in average major β-lactamase gene abundance (normalised by 16S copy number) for each of the cefotaxime ('TAX') concentrations, in µg/L. On average, *bla*$_{TEM}$ genes were most prevalent in the 2000 µg/L treatment, though the increase from 0 to 2000 µg/L was largest for the *bla*$_{CTX-M}$ genes.

On average, *bla*$_{TEM}$ genes were most prevalent in the 2000 µg/L treatment, though the increase from 0 to 2000 µg/L was largest for the *bla*$_{CTX-M}$ genes (Figure 18). The *bla*$_{CTX-M}$ prevalence determined by metagenomics was an order of magnitude lower than that determined by qPCR. *Bla*$_{SHV}$ genes were the only β-lactamase genes detected which decreased in relative abundance with increasing antibiotic concentration. Both *bla*$_{CTX-M}$ and *bla*$_{TEM}$ gene abundances significantly increased with cefotaxime exposure at 500 µg/L.

### 2.5.6. Single species assays are not representative of complex communities

To assess the utility of single species assays for determining MSCs in complex communities, a single species competition experiment was performed in the same experimental system. The MIC of J53 was > 0.25 mg/L and < 0.5 mg/L, and for strain NCTC 13451 it was > 256 mg/L and < 512 mg/L. The single species assay using susceptible J53 and J53 bearing the multidrug resistance plasmid pEK499 was performed in two separate low and high cefotaxime concentration experiments. There were no significant differences between any of the treatments for either experiment at day 0 for 16S gene copy number, *bla*$_{CTX-M}$ gene copy number or *bla*$_{CTX-M}$ gene prevalence.

There were no significant differences in *bla*$_{CTX-M}$ prevalence between treatments at the beginning or the end of the experiment (Figure 19). The overall prevalence was much lower than in the complex community experiment, with a peak of 0.4.

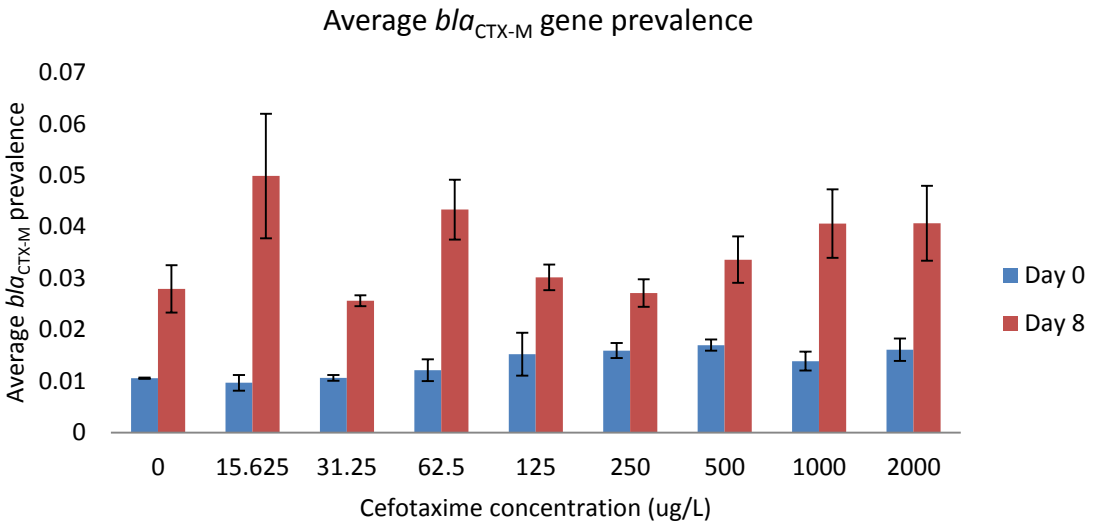


Figure 19. Average (biological replicate n=3, qPCR replicate n=2) *bla*$_{CTX-M}$ gene prevalence at the beginning and end of the lower cefotaxime concentration single species experiment, shown with standard error bars. Prevalence calculated by dividing *bla*$_{CTX-M}$ gene copy by 16S copy number. There are no significant differences between treatments.

The higher cefotaxime concentration experiment (Figure 20) showed a dose-dependent increase (Spearman's rank, $r_s$ = 0.57, $p$ = 0.02) in *bla*$_{CTX-M}$ gene prevalence from 8 mg/L to 64 mg/L, which peaks at a prevalence of 0.18. The prevalence at all these concentrations were also significantly different to the no antibiotic control ($p$ <0.05, Dunn's test). The prevalence decreased at 128 mg/L. This decrease was due to a decrease in *bla*$_{CTX-M}$ gene copy number (Figure 21), not in 16S copy number (which remained constant, with no significant difference between treatments at day 8, Kruskal Wallis).

There was no selection coefficient MSC determined for the J53 competition experiment as all selection coefficients were >0, meaning the line of best fit never crossed the x axis (Figure 22). Therefore the *bla*$_{CTX-M}$ gene (resistance plasmid) was under positive selection even in the absence of a selective pressure (cefotaxime exposure).

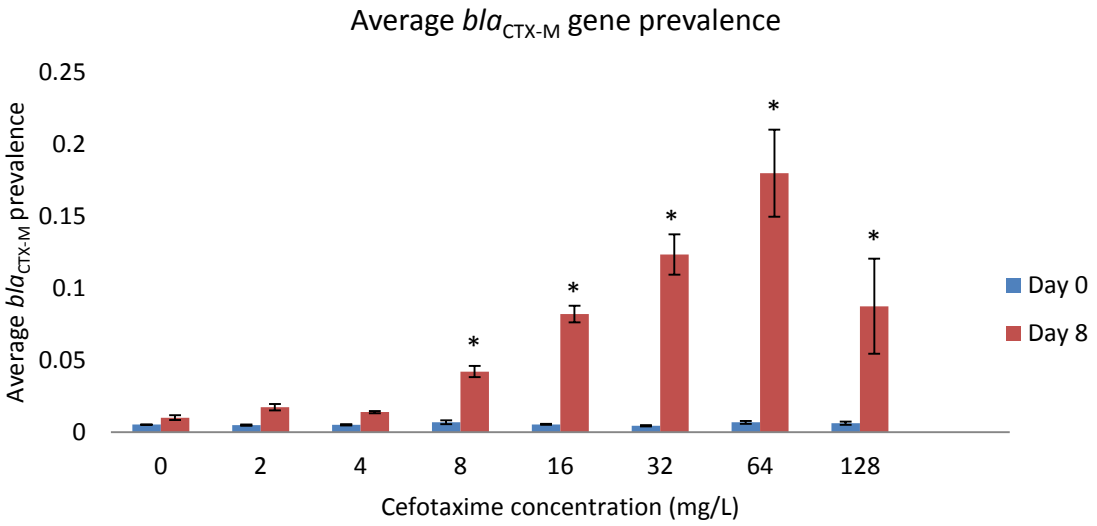


Figure 20. Average (biological replicate n=3, qPCR replicate n=2) *bla*$_{CTX-M}$ gene prevalence at the beginning and end of the higher cefotaxime concentration single species experiment, shown with standard error bars. Prevalence calculated by dividing *bla*$_{CTX-M}$ gene copy by 16S copy number. There were significant differences between 8 mg/L and all concentrations above compared to the not antibiotic control ($p$ < 0.05, Dunn's test).

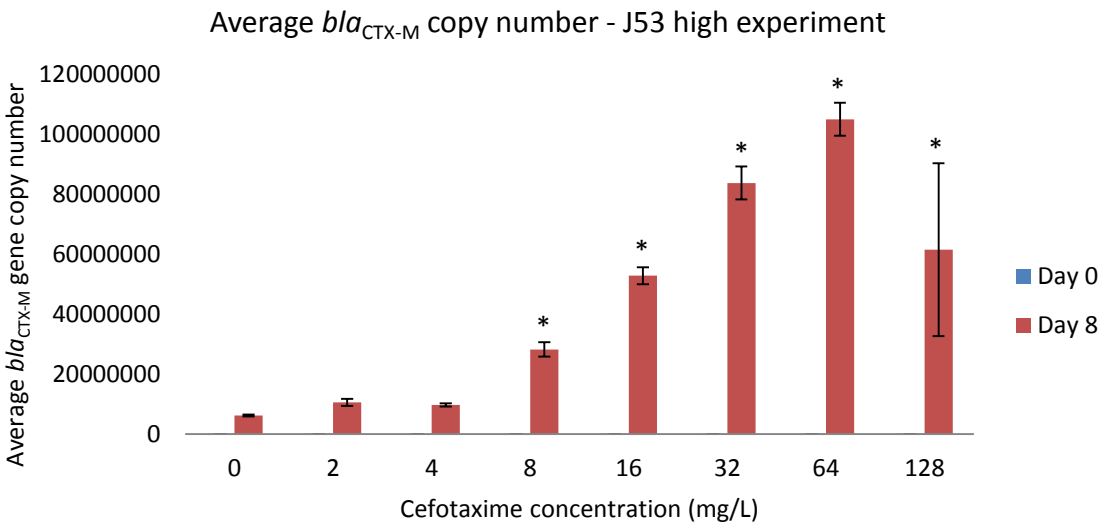


Figure 21. Average (biological replicate n=3, qPCR replicate n=2) *bla*$_{CTX-M}$ gene copy number at day 0 and day 8 in the higher cefotaxime concentration single species experiment. The 8 mg/L treatment and all treatments above were significantly different to the no antibiotic control ($p < 0.05$, Dunn's test).

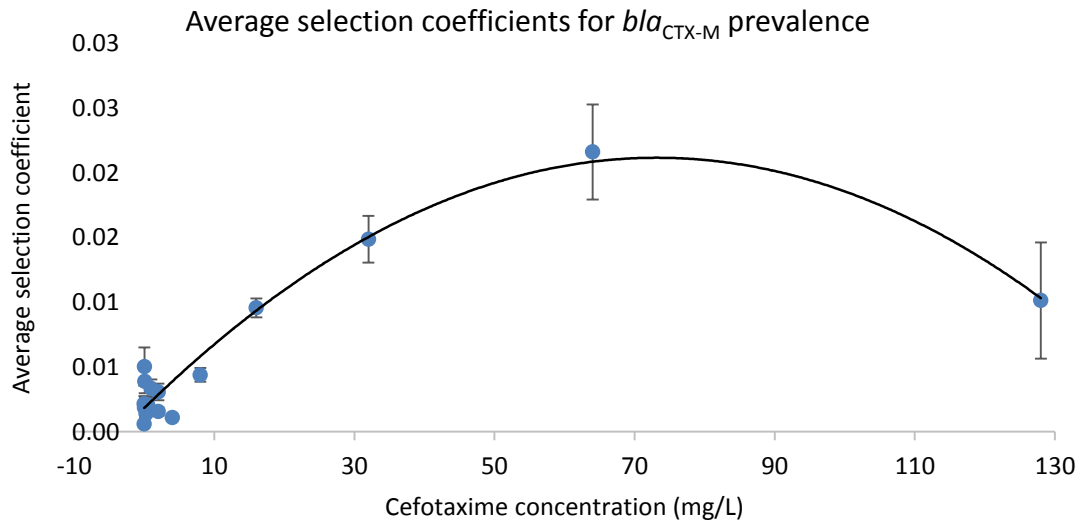


Figure 22. Average (biological replicate n=5, qPCR replicate n=2) selection coefficients based on day 0 and day 8 *bla*$_{CTX-M}$ prevalence data in the low and high cefotaxime concentration single species selection experiment, using both data points for the no antibiotic control and 2 mg/L treatments. All selection coefficients are positive, so no MSC was determined.

### 2.5.7. Cefotaxime is readily degraded, mostly via biodegradation

To assess the need for chemical quantification to determine accurate MSCs, stability of cefotaxime during the complex community was assessed. Table 2 shows the nominal (expected) cefotaxime assay concentrations and the measured concentration at the beginning of the day and after 24 hours (i.e., upon transfer into fresh medium and antibiotic). Measured assay concentrations were consistently lower than expected, with the lowest assay concentration of 15.625 µg/L being below the detection limit. After 24 hours, almost all the cefotaxime was degraded for nearly all the assay concentrations, even the very highest.

Table 2. The expected (nominal) cefotaxime concentration, actual measured cefotaxime concentration immediately sampled and following 24 hours incubation in the complex community assay. Averages of 2 biological replicates (chosen at random) and 2 chemical replicates. The two cefotaxime stock concentrations were also quantified.

| Nominal Concentration (µg/L) | Measured concentration (µg/L) | Measured concentration after 24hours (µg/L) |
|---|---|---|
| **15.625** | 0 | 0 |
| **31.25** | 25.5 | 0 |
| **62.5** | 26.75 | 6 |
| **125** | 46.25 | 0 |
| **250** | 205 | 0 |
| **500** | 438 | 2.6 |
| **1000** | 830 | 0 |
| **2000** | 1686 | 4 |
| | | |
| **Stock 1 = 1250** | 1272 | - |
| **Stock 2 = 78.125** | 51.56 | - |

This prompted further experiments to analyse the degradative capacity of the complex community compared to degradation in the experimental system (i.e. lability). Figure 23 shows the degradation of cefotaxime over 24 hours in the presence and absence of the complex community. Here, all cefotaxime is completely degraded after 24 hours in the presence of the community. Around 40 % of cefotaxime is degraded abiotically in the absence of the community.

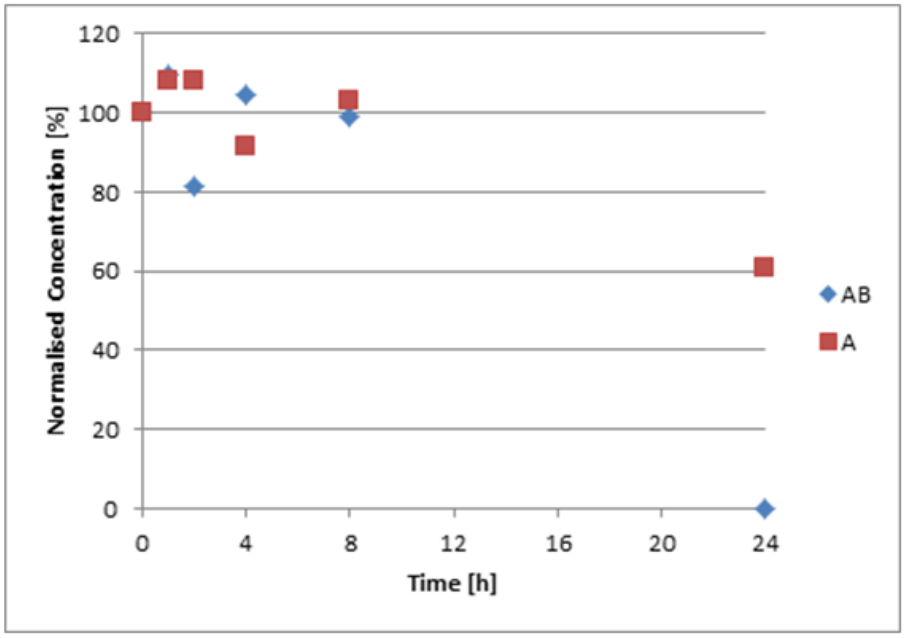


Figure 23. Single biological replicate, duplicate chemical replicate of cefotaxime degradation over 24 hours, sampling at 0, 4, 8 and 24 hours, in the presence (AB) and absence (A) of the complex community.

A final experiment increased the number of sampling points over the 24 hour period and compared this to growth of the complex community (measured by optical density (OD)) – Figure 24 A and B respectively. Degradation for all assay concentrations coincided with the exponential growth phase of the complex community.

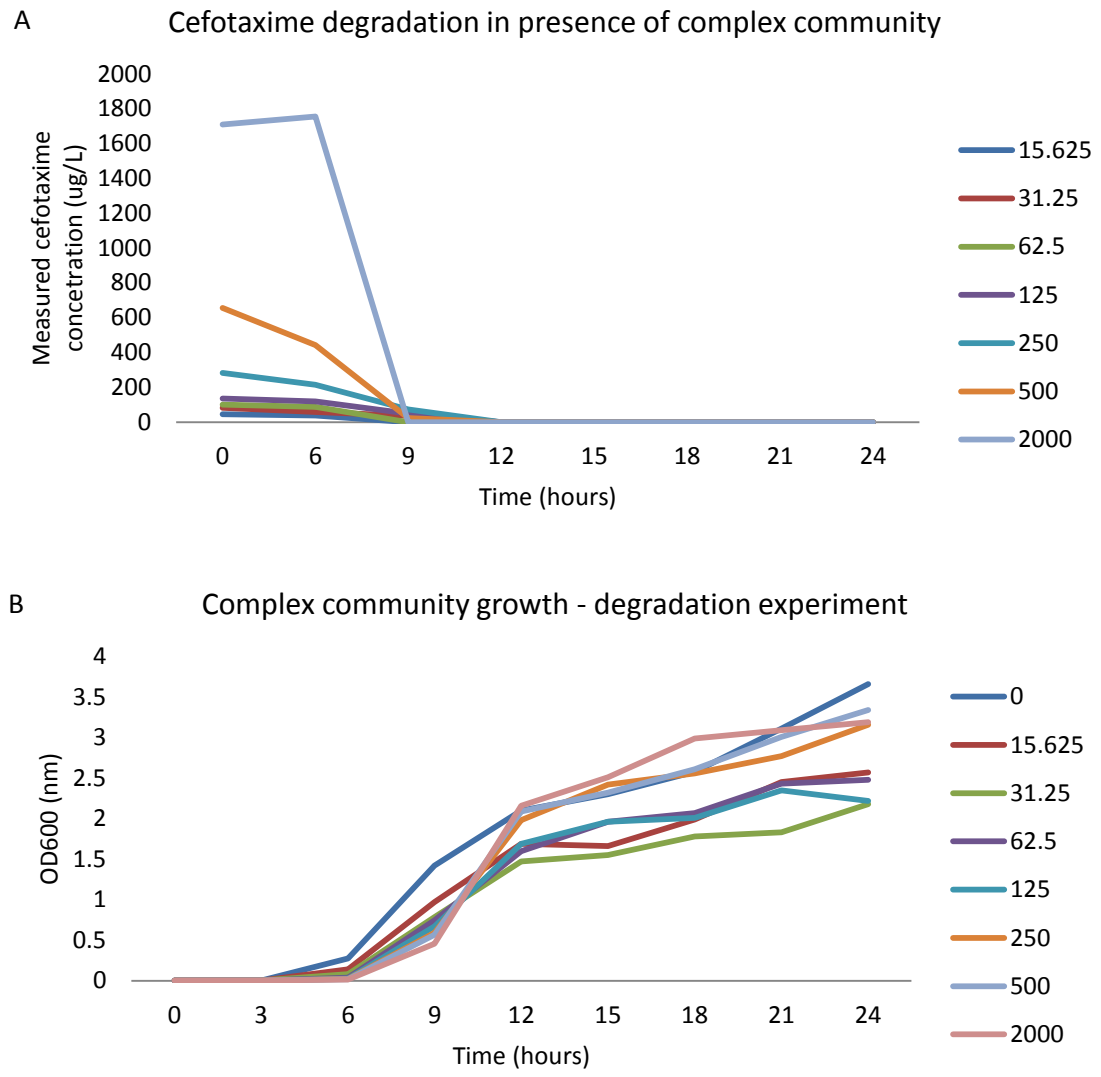


Figure 24. Single biological replicate, duplicate chemical replicate chemical quantification of cefotaxime ('Measured cefotaxime concentration µg/L) every 3 hours for 24 hours at different concentrations (µg/L) in the presence of the complex community (A). (B) The growth (optical density measured at 600 nm) from the same sampling time points, single replicate only.

As an aside, aliquots of the sample were frozen down for all concentrations at 6, 9 and 12 hours. Cultures were analysed with qPCR to determine *bla*$_{CTX-M}$ gene copy number (Figure 25), 16S copy number (Figure 26), and *bla*$_{CTX-M}$ prevalence (Figure 27). Please note all these experiments are based on single biological replicates. Increases in *bla*$_{CTX-M}$ gene copy number and *bla*$_{CTX-M}$ gene prevalence increase over time, although not concurrently. Meanwhile, 16S gene copy number appears to stabilise at 12 hours.

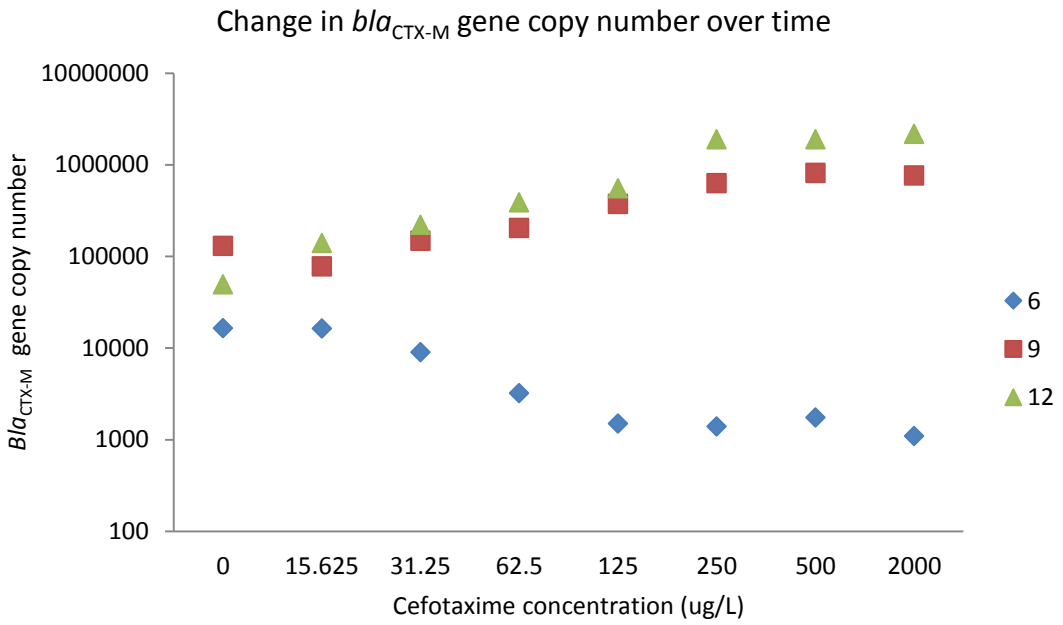


Figure 25. *Bla*CTX-M gene copy number at different time points (6, 9 and 12 hours) during the cefotaxime 24 hour degradation experiment. Single biological replicate only, qPCR replicate n=2.

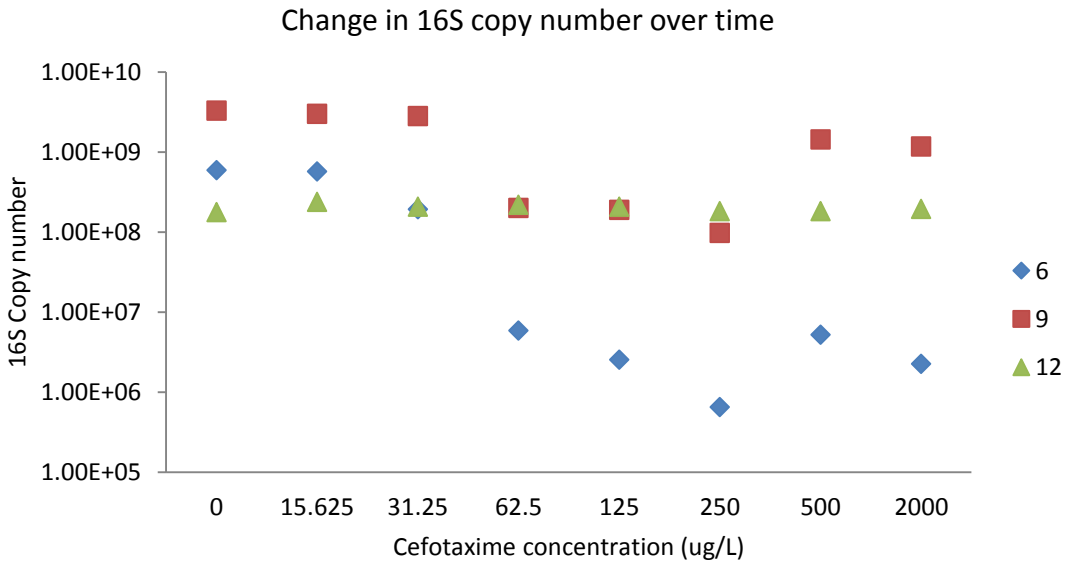


Figure 26. 16S gene copy number at different time points during the cefotaxime 24 hour degradation experiment. Single biological replicate only, qPCR replicate n=2.

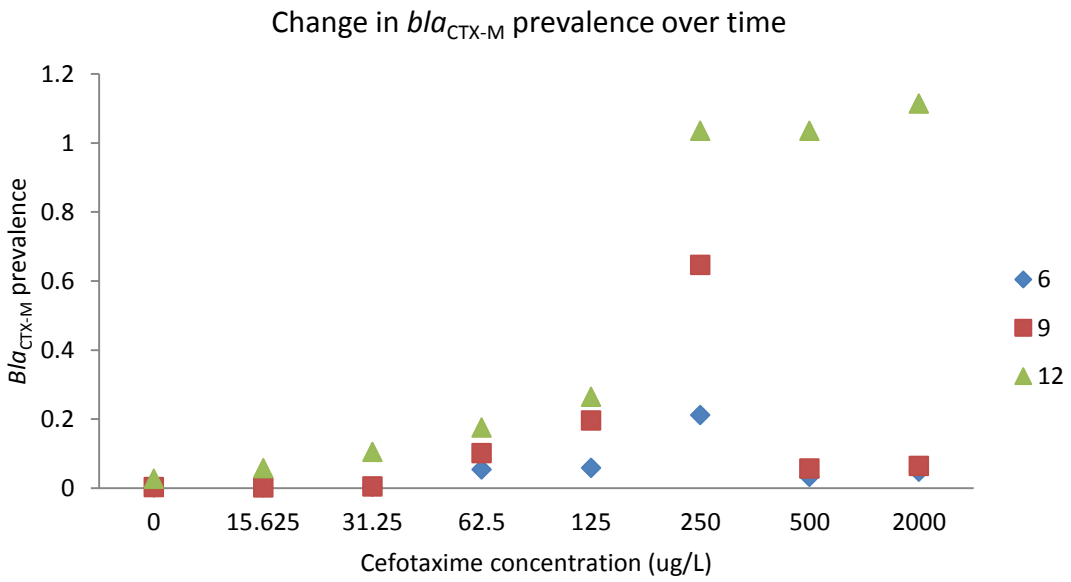


Figure 27. *Bla*CTX-M gene prevalence (*bla*CTX-M gene copy number/16S copy number) at different time points during the cefotaxime 24 hour degradation experiment. Single biological replicate only, qPCR replicate n=2.

### 2.5.8. Relatively small amounts of ESBLs can protect susceptible bacteria to very high antibiotic concentrations

The relatively low *bla*CTX-M prevalence in the single species assays (compared to the complex community assays), and the confirmation that the majority of cefotaxime is biodegraded, and that this degradation coincides with exponential growth lead to the assumption that biodegradation (performed by extra-cellular β-lactamases) benefits the community as a whole (see 2.6. Discussion). A simple experiment quantified the amount of supernatant (i.e. β-lactamase) that could protect (i.e. indirectly select) for susceptible bacteria at cefotaxime concentrations above the susceptible bacterium's MIC.

Figure 28 shows the growth of susceptible J53 strain at a range of cefotaxime concentrations exceeding J53 MIC (< 0.25 mg/L), incubated with 25 µl (equating to 12.5 % of the final volume) of supernatant taken from the overnight culture of the pEK499 bearing (i.e. β-lactamase producing) J53 resistant strain. This allowed J53 to grow up to 32 mg/L without a significant difference in final OD (i.e. at 24 hours) compared to the no antibiotic control, also containing the NCTC 13451 supernatant ($p$ >0.05, Kruskal Wallis test).

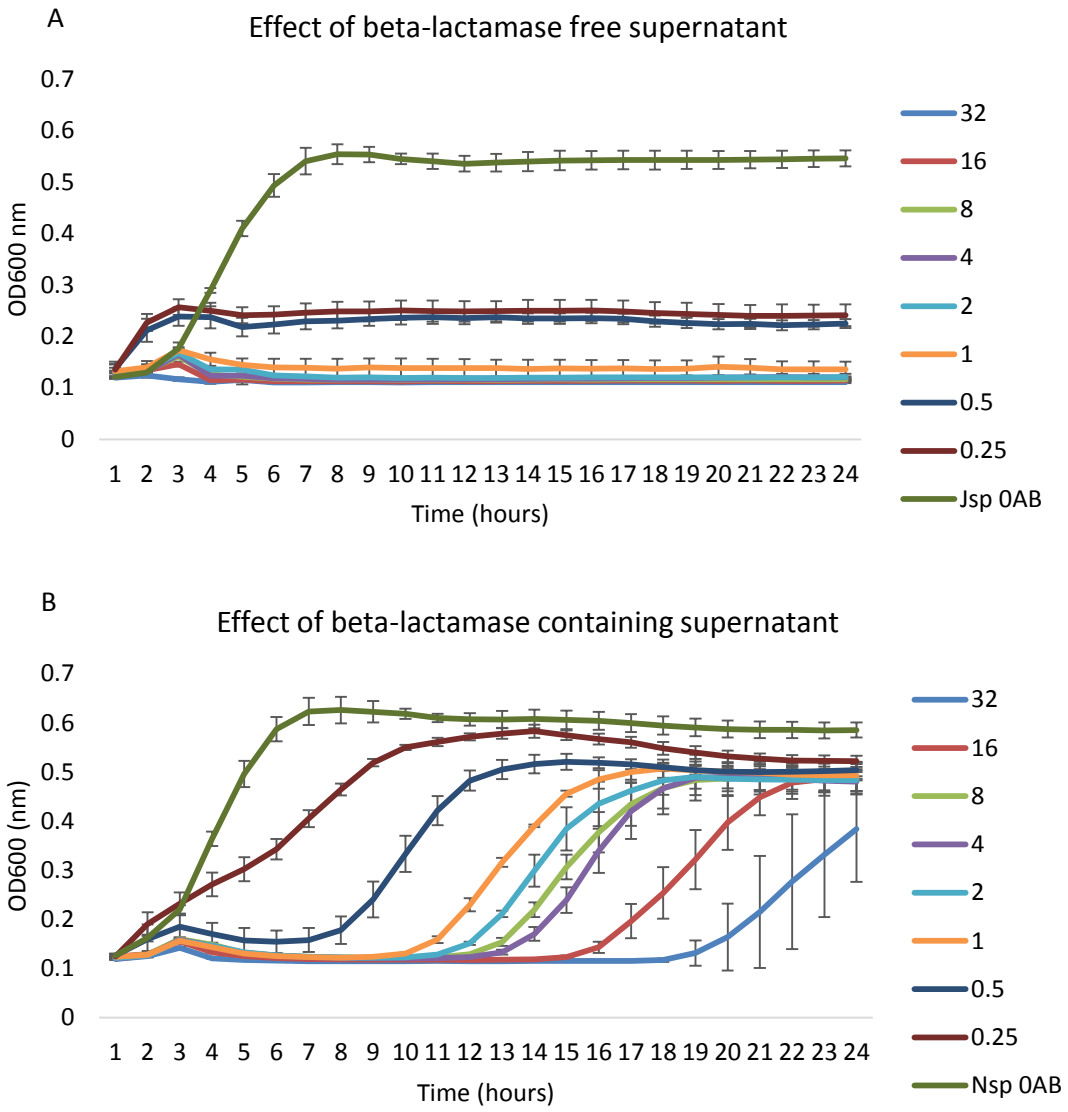


Figure 28. Average (n=4) growth of susceptible J53 at different cefotaxime concentrations, without (A) and with (B) ESBL-containing supernatant (i.e. supernatant from susceptible J53 and resistant J53, respectively) at different cefotaxime concentrations. “Jsp0AB” or “Nsp 0AB” represents the no antibiotic control bearing the ESBL free (J53) and ESBL-containing (NCTC 13451) supernatant respectively. Shown with standard deviation bars.

## 2.6. Discussion

In this study the first ever MSC in a complex community was determined using the worldwide, clinically important resistance gene group *bla*$_{\text{CTX-M}}$, as the

endpoint. QPCR methods have been used previously to determine the MSC in a community biofilm (Lundstrom et al., 2016), but this was not strictly an MSC as discussed in the introduction.

The MSC of cefotaxime, the only antibiotic tested in this chapter (see chapter three for further work), was very low at 0.4µg/L. More replication would increase confidence in the estimated MSC, but qPCR analysis is costly and time consuming (see chapter three for a new method). Whilst the MSC of 0.4 µg/L (nominal) exceeds the majority of cefotaxime MECs (UmweltBundesamt, 2016), there are isolated cases of MECs exceeding this concentration in certain environmental compartments, such as hospital effluent (Gomez et al., 2007). This indicates selection for resistance may be occurring *in situ* in certain hotspots, and that appropriate monitoring practices may be required to identify such hotspots and ensure MECs do not exceed the MSC determined in this study (see chapter three for more details).

For the most part, *bla*$_{\text{CTX-M}}$ prevalence increased over time except for the 1000 µg/L and 2000 µg/L treatments, where prevalence decreased between day 1 and 4. This is similar to the 'overshoot' observed in the resistant fraction from the paper by Yurtsev *et al.* (2013). However, unlike their single species experiment, in the complex community the prevalence then increased further, rather than continuing to decrease to a stable level (termed the 'equilibrium fraction'). The gradual increase over time for all other treatments suggests that if the experiment were continued over a longer time frame the MSC would decrease, as selection has a longer time to act on gene prevalence. It would be interesting to continue the experiment indefinitely to determine if resistance prevalence would reach an 'equilibrium fraction', and what this fraction may be at different concentrations; or if eventually carriage could reach 100 % at even low concentrations.

This research also demonstrated that MSCs derived in single species competition assays like those conducted previously (Gullberg et al., 2014, Gullberg et al., 2011) are, as expected, unrepresentative of the MSC determined in a community (Brandt et al., 2015, Berglund, 2015). This is the first time single species and complex community experiments have been directly compared in the same experimental system, and indicates that for real world applicability, any assays developed for environmental risk assessment must include a community aspect.

Indeed, in the case of cefotaxime, the LOEC in the single species assay was 20,000x greater than the MSC in the complex community. Though it is difficult to compare the LOEC and MSC as the former is a statistical approach and the latter an estimate, it is not possible to compare both MSCs as even in the absence of antibiotic treatment, the resistant strain (or resistance plasmid) conferred a small fitness advantage. This is a prime example of how the genetic and community context of a resistance gene can influence its fitness.

The pEK499 plasmid, though large (> 100 kb) and non-conjugative, was not fully outcompeted by the plasmid free strain most likely due to the five different maintenance systems encoded on pEK499, which was hypothesised to result in maintenance even in the absence of selection (Woodford et al., 2009). This seems to be such a case where the plasmid, rather than the resistance gene itself, is responsible for positive selection (Carattoli, 2013). This raises concerns of whether plasmids such as pEK499 could be selected for in patients and then persist even after antibiotic treatment is ceased. An additional concern is that plasmids carrying ESBLs have been shown to increase the virulence of their host (Schaufler et al., 2016). Were pEK499 conjugative, it is likely the MSC would decrease due to mobilisation to the susceptible strain. This is because firstly, the plasmid has a fitness benefit in the absence of selection; secondly, the presence of resistance genes will provide an additional benefit to the recipient host in the presence of antibiotic; and third because in general, HGT may be induced by sub-inhibitory concentrations (Kim et al., 2014b, Moon et al., 2005).

As well as the LOEC in the single species assay being much higher than in the complex community, the prevalence of *bla*$_{\text{CTX-M}}$ at each concentration (peaking at 0.18 at 64 mg/L) was much lower than in the complex community assay (peaking at 0.79 at 64 mg/L). It can be hypothesised that this is due to indirect selection for susceptible bacteria (see 2.1.4. Indirect selection), which has been shown to occur solely for resistance mechanisms which are degradative in nature (Nicoloff and Andersson, 2016). To verify this and determine the level to which susceptible bacteria could be 'protected' (indirectly selected for), supernatant experiments were performed to first validate that: 1.) the resistant (plasmid bearing) strain excreted active β-lactamases, and 2.), relatively low volumes (which supports the low *bla*$_{\text{CTX-M}}$ prevalence observed in the single species experiment) could protect the susceptible bacteria.

The final supernatant experiment showed that while J53 lag phases extended with increasing cefotaxime concentration, growth at 24 hours (the endpoint typically used clinically when determining susceptibility profiles), even up to 32 mg/L, was not significantly different to the no antibiotic control. The fact that growth inhibition of J53 (in the form of an extended lag phase) was observed at even the lowest concentration tested, calls into question current MIC determination methods for particularly labile compounds. Here, 40 % of cefotaxime was shown to be degraded in sterile culture over 24 hours. Therefore, it is likely the dose response relationship observed between J53 growth and cefotaxime concentrations is due to abiotic degradation of cefotaxime to a sub-inhibitory level. However, by considering the endpoint only (i.e. OD at 24 hours), the MIC is determined to be 500 µg/L which is much higher than the MSC of 0.4 µg/L determined in the complex community experiment. This is likely to be similar for MICs determined on solid media, though these may have even greater disparity between expected and actual antibiotic concentration due to complexing with the agar. It may be that a significant increase in length of lag phase compared to the no antibiotic control, or continual addition of antibiotic to ensure a constant exposure concentration, would be improvements to the current MIC microdilution method.

In the supernatant experiment, a difference was observed between the no antibiotic controls with and without supernatant. This is most likely a nutrient effect, reducing growth as the growth medium was diluted with nutrient-depleted medium (i.e. the supernatant). It is also proposed that while susceptible bacteria can be indirectly selected for, it is plausible that bacteria resistant to antibiotics other than those the population are exposed to may be indirectly selected for as well. I have termed this ‘protective co-selection’, another mechanism by which antibiotic resistance can be co-selected for (Figure 29). To prevent indirect selection during antibiotic treatment, clinical assays similar to the microarray developed to quickly screen for presence of a panel of resistance genes from saliva and faeces (Card et al., 2014), should be used to identify patients most at risk (i.e. those colonised with β-lactamase producing bacteria).

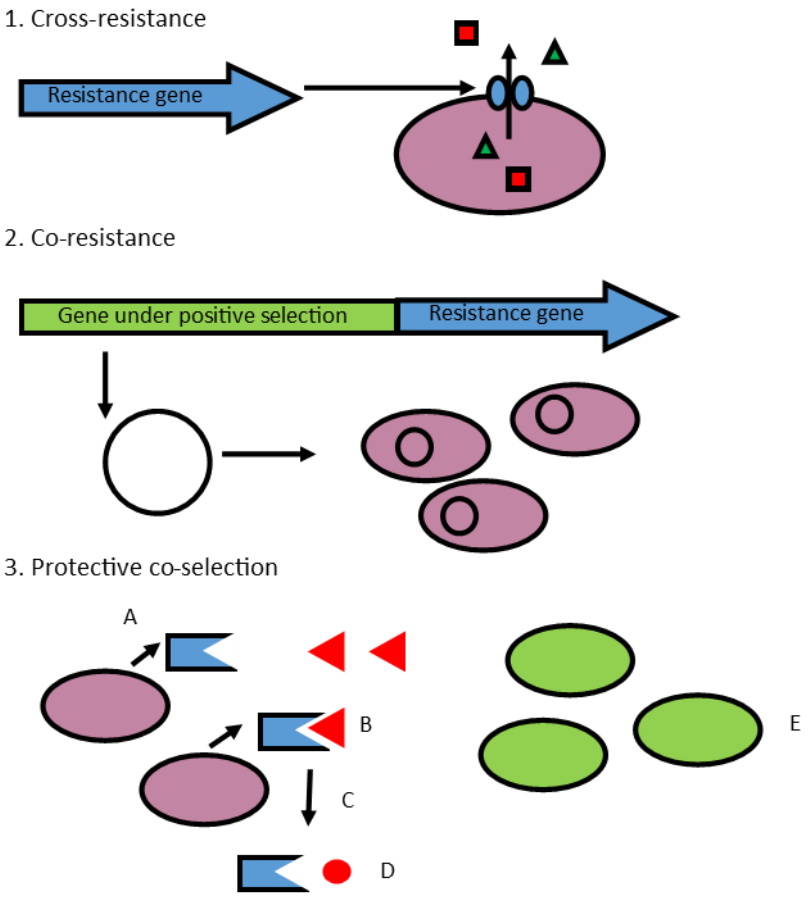


Figure 29. The three mechanisms of co-selection. 1. Cross-resistance - when a single gene confers resistance to multiple compounds, e.g. a multi-drug efflux pump. 2 Co-resistance - selection for one or more genes which are genetically linked (e.g. on a plasmid). Only one gene need be under positive selection for the plasmid and therefore all genes to be selected for. 3. Protective co-selection – this occurs when resistant bacteria (pink) produce a degradative (blue) enzyme (A), which binds to the (red triangle) antibiotic (B). Following antibiotic degradation (C), the antibiotic (red circle) is inactive (D), allowing both susceptible bacteria and susceptible bacteria carrying different resistant mechanisms (green) to grow (E).

The use of β-lactamase inhibitors (βLIs) has counteracted indirect selection in the clinic to an extent, though resistance to βLIs has also emerged (Drawz and Bonomo, 2010). Even more concerning is that it has been shown experimentally that inclusion of βLIs at suboptimal levels can select for β-lactam resistance (Yurtsev et al., 2013). This is intuitive, as β-lactamases are found both intracellularly and extracellularly (Livermore, 1995), and it has been hypothesised that there must be some fitness benefit to being a producer, otherwise producers

would go extinct in the absence of a selective pressure – however this is not the case when such interactions are modelled (Dugatkin et al., 2003); nor was it the case in this study. If βLIs do not fully saturate all the active sites of the β-lactamases, and as it is likely that the majority of saturation would occur in the media (in extracellular β-lactamases), this means intracellular β-lactamases remain more active compared to extracellular β-lactamases. This would then allow growth of the resistant strain whilst simultaneously preventing growth of the susceptible strain, which is still being inhibited (as extracellular ESBLs are βLI-bound and unable to degrade the β-lactam).

Cefotaxime concentrations in different body compartments greatly exceed the MSC determined in the complex community in this study, but are within the concentration range shown to indirectly select for susceptible bacteria. For example, blood serum concentrations for intramuscular injections of 1g of cefotaxime are 20 – 25 mg/L (FDA, 2007). Therefore, extrapolating the results from this study, blood serum concentrations of cefotaxime would not be sufficiently high to prevent indirect selection (shown here to occur at 32 mg/L), let alone diffuse concentrations in different body compartments.

It should also be noted, however, that the MSCs quoted throughout this discussion are based on the nominal (i.e. expected) cefotaxime concentrations. In reality, all cefotaxime was degraded after 24 hours in the presence of the complex community in most of the treatments which were chemically quantified. This degradation coincided with the exponential growth phase of the bacterial community, meaning that for over half of the treatment time during each day (including part of the exponential growth phase) the bacteria were not exposed to any cefotaxime at all.

The actual exposure concentrations during the complex community experiment were much lower than the nominal concentrations. Therefore, when taking this into account, the predicted MSC of 0.4 µg/L is likely to be much lower. Based on measured concentrations at the beginning of the experiment, the LOEC would actually be 46.25 µg/L. Together, these suggest cefotaxime may pose a risk of selection for resistance evolving *in situ* due to high similarity to MECs (UmweltBundesamt, 2016). Clearly more work should investigate the best way to determine the MSC or LOEC of labile antibiotics, but currently the selection coefficient method using measured assay concentrations seems the most protective approach.

The qPCR data from this degradation experiment, while based on data from a single replicate only, would inform any future experiments. Most interestingly, a change in *bla*$_{CTX-M}$ gene copy number and prevalence was observed over time, meanwhile the 16S copy number reached what could be an equilibrium at 12 hours, which coincided with the end of the exponential growth phase (Figures 25 and 27). At 12 hours *bla*$_{CTX-M}$ prevalence increased dramatically at 250 µg/L, compared to the LOEC in the long term experiment, which was 125 µg/L at day 1 suggesting selection occurs beyond the exponential growth phase. The dose-response relationship observed between exponential growth and cefotaxime concentration whilst collecting the data led to investigation of indirect selection, and the supernatant experiment, as well as the basis for chapter three. More replicates would be a necessity to perform statistical analyses, and generate selection coefficients for selection over 24 hours as opposed to over several days. If in agreement, this could allow more rapid generation of MSC data.

The metagenome data was the least sensitive of all methods used for determining MSCs, as shown previously (Lundstrom et al., 2016). Selection for *bla*$_{CTX-M}$ genes was statistically significant at 125 µg/L using qPCR data, but a significant increase in *bla*$_{CTX-M}$ relative abundance was only observed at 500 µg/L in the metagenome data. Sequencing of the metagenome is still useful however, as it highlights other resistance gene classes under co-selection.

A major concern arising from this study from both a clinical and environmental perspective is the co-selection for resistance to other antibiotic classes. Relative abundance of genes conferring resistance to aminoglycosides, sulphonamides and trimethoprim all increased under cefotaxime exposure, in a dose-dependent manner. Genes conferring resistance to these antibiotic classes are all commonly found on integrons (Partridge et al., 2009) (mobile genetic elements which harbour and express gene cassette arrays often comprised of antibiotic resistance genes, see chapters one and four) which may have been enriched by cefotaxime exposure, facilitating further selection for these genes and others. It would be useful if integron prevalence were quantified alongside resistance gene classes in the ARGS-OAP pipeline, as integrons have been suggested, on numerous occasions, as excellent indicators of selection for resistance and anthropogenic impact ((Amos et al., 2015, Rizzo et al., 2013, Gillings et al., 2015, Berglund, 2015), and see chapter three and chapter four).

As integrons also often carry other antimicrobial resistance genes such as the multi-drug *qac* efflux pump gene variants (Partridge et al., 2009), co-selection by sub-inhibitory cefotaxime concentrations could also reduce the efficacy of other antimicrobials, such as biocides. However, selection for *qacA, qacB and qacG* (the only *qac* genes quantified in the ARGS-OAP pipeline) were absent, or below the limit of detection.

There was also co-selection for other resistance gene classes not commonly associated with integrons, such as those conferring macrolide resistance. This demonstrates for the first time the magnitude of the co-selective potential of sub-inhibitory concentrations of cefotaxime in a complex microbial community. A number of macrolides have recently been added to the first ever Watch List of Hazardous Compounds by the European Commission as part of the Environmental Quality Standards Water Framework Directive (Carvalho et al., 2015), as MECs are often found to exceed PNECs. However, this work also demonstrates the importance of considering selection for resistance more holistically, as selection for resistance to one compound does not necessarily depend on the MEC of the compound it confers resistance to.

Most interestingly, in terms of β-lactam resistance, the metagenome analyses showed that of all the resistance mechanisms quantified, β-lactamases were enriched the most, even outcompeting some other resistance mechanisms (e.g. PBPs), which decreased with cefotaxime exposure. *Bla*$_{\text{CTX-M}}$ abundance increased the most of all the β-lactamases under sub-MIC selection, which may explain the rapid dissemination of *bla*$_{\text{CTX-M}}$ genes across the globe (Canton and Coque, 2006), particularly if they are preferentially selected for in the environment at (Gaze et al., 2005, Gaze et al., 2011, Sidrach-Cardona et al., 2014, Hartmann et al., 2012) low antibiotic concentrations or in the human or animal gut (Hammerum et al., 2014, Woerther et al., 2013). However, there was significant variation between the three sequenced replicates (for both gene subtype and resistance gene class), which might be expected due to the inherent variability in a natural, complex system. More research is required to confirm that *bla*$_{\text{CTX-M}}$ genes are enriched over all other β-lactamases, and to determine if it is the case for all β-lactam antibiotics; or even all 3$^{rd}$ generation cephalosporins. Reassuringly, the strong selection for *bla*$_{\text{CTX-M}}$ supports its choice as the appropriate resistance gene to be used for the qPCR endpoints, used here for MSC determination.

The ARGS-OAP pipeline can identify the resistance gene subtype with high confidence, but not the exact genes within each subtype (T. Zhang, *pers. comm.*). The TaqMan qPCR assay provided information on the dynamics of *bla*$_{CTX-M}$ group selection in the complex community, indicating very similar genes can be differentially selected at different antibiotic concentrations. For example, group 1 *bla*$_{CTX-M}$ were most prevalent at the lower concentrations, but group 9 *bla*$_{CTX-M}$ increased the most overall. Conversely at higher concentrations, group 9 *bla*$_{CTX-M}$ were more abundant until the 32 mg/L concentration and above - at which point they were lost (or were at numbers below the limit of detection). However, there was some difficulty with reproducibility of the TaqMan assay, despite repeated optimisation, which can be seen in the disparity between effects at 2 mg/L in the lower and higher concentration experiments. This seems unlikely to be an artefact of different inocula, as the SYBR assay data were very similar. Therefore, these findings should be interpreted with caution, and preferably the experiments should be repeated.

The phenotypic data at the end of the complex community experiments was less sensitive than qPCR for predicting MSC, which has also been shown previously (Lundstrom et al., 2016). However, phenotypic and qPCR LOECs were in agreement. This is unsurprising as clinical resistance was quantified in coliforms only. Though these were assumed to be the most prevalent family present in the community due to both the experimental conditions and the high prevalence of *bla*$_{CTX-M}$ genes in Gram negatives, *E. coli* and enteric qPCR showed these species only made up a very small percentage of total bacterial population. This data also showed the experimental system was not biased towards proliferation of *E. coli* and other enterics.

## 2.7. Conclusions

### 2.7.1. The clinical implications

The MSC of cefotaxime determined in this study indicates that when antibiotic concentrations reach sub-inhibitory levels in the body (as discussed is sometimes the case), selection for CTX-Ms, other β-lactamases and resistance to other antibiotic classes may occur *in vivo.* The selection was cumulative over time,

highlighting the importance in minimising the % *T* < MIC during antibiotic treatment.

The extent to which *bla*$_{CTX-M}$ genes are selected for at low concentrations may partly explain their rapid dissemination worldwide, and persistence both in the gut and in the environment. These results agree with others (Bottery et al., 2016) in that the resistance mechanism could be key for predicting MSC depending on gene sociality.

Excreted substances from resistant bacteria (presumed to be β-lactamases), were shown to allow growth of susceptible bacteria well above their own MICs. This 'protective effect' (indirect selection) could extend not only to bacteria that are fully susceptible, but also bacteria harbouring other resistance mechanisms to other selective compounds - a new mechanism of co-selection termed protective co-selection (Figure 29). Indirect selection was observed up to very high antibiotic concentrations. This is concerning, as theoretically, the higher the antibiotic concentration, the longer the window of opportunity exists for the resistant bacteria to grow and overtake the population. Blood plasma concentrations of patients treated with cefotaxime are within the concentration window for indirect selection according to this study.

Current antibiotic treatment strategies should be further refined, to ensure sufficiently high concentration to prevent sub-inhibitory selection and co-selection.

### 2.7.2. The environmental implications

This research confirms that selection for *bla*$_{CTX-M}$ genes are likely to occur at concentrations in the same order of magnitude as MECs (low µg/L). The disparity between LOECs and selection coefficient based MSCs can be explained by the large variability between replicates, expected in a natural system. Increasing the number of replicates may increase the power of the statistical tests.

Cefotaxime is known to be readily degradable, and this is why MECs are extremely low (UmweltBundesamt, 2016). In this experimental system, cefotaxime was fully degraded after 24 hours with all degradation occurring within the first 12 hours. This, combined with lower initial measured concentrations than expected, indicates that the average exposure concentrations during the experiment were actually much lower than the nominal values presented. This

demonstrates the absolute necessity of chemical quantification in MSC assays, if combining with MECs to determine a Risk Quotient.

The complex community inoculum contained coliforms resistant at the clinical breakpoint concentration of cefotaxime. As coliforms are used as water quality measures (Michael et al., 2012), this assay could be applicable for assessing the potential risk for selection of resistance in waste water treatment plants. This experiment could feasibly be modified to assess risk of selection in other aquatic systems simply by changing the culturing conditions to a minimal media, and lowering the temperature.

### 2.7.3. Final comments

This work indicates that *bla*$_{\text{CTX-M}}$ genes, other β-lactamase genes and genes conferring resistance to several other antibiotic classes are selected and co-selected for at low concentrations of a clinically important antibiotic. Therefore, selection for multiple resistance genotypes in the environment and in the body is likely. Additionally, there is a risk for indirect selection during antibiotic therapy. These findings should be verified as reproducible with different inocula and under a wider range of experimental conditions to more confidently identify the risk to the environment and human health.

# Chapter three: Development of a simple assay to detect effect concentrations of antimicrobials

## 3.1. Introduction

This chapter directly addresses the thesis title: “Development of a novel assay to detect effect concentrations of micropollutants”. The introduction is framed around this objective.

### 3.1.1. Development of a novel assay

Current environmental risk assessment (ERA) guidelines dictate that the toxic effects of antibiotics (both human and veterinary) on microbes should be determined using three standardised tests. The soil nitrogen transformation test (used to assess the effect of antibiotics on nitrification in soil-dwelling bacteria) and growth inhibition of cyanobacteria test are both required for human and veterinary antibiotics. Additionally, human antibiotics are further assessed using the activated sludge respiration inhibition test (ASRIT), to assess the risk antibiotics may pose when undergoing the waste water treatment process (Brandt et al., 2015).

Whilst using microbial endpoints is a necessity when assessing the risk of antibiotics (as bacteria are the target species), none of the current tests currently assess the ‘selective potential’ of a compound - i.e. its ability to select for antimicrobial resistance. In light of the sub-inhibitory selection first observed by Gullberg et al. (2011), this now appears to be a significant oversight in antibiotic ERA. Therefore, an immediate need has been identified for a novel assay which can be used for this purpose (Ashbolt et al., 2013, Brandt et al., 2015, Berglund, 2015). This is so sufficient data can be generated which can be used in the ERA of antibiotics, to determine whether antibiotics pose a ‘selective hazard’ or ‘selective risk’ (i.e. select for resistance) in the environment.

Recently, predicted no effect concentrations (PNECs) for resistance ($PNEC^{R}$s) were estimated using the publically available EUCAST MIC data (Bengtsson-Palme and Larsson, 2016). This produced a large data set that could be used to inform environmental regulation, and indicated that PNECs generated

from current ecotoxicological tests were often not protective of the estimated $PNEC^R$. However, as noted by the authors, generation of experimental data is still required (Bengtsson-Palme and Larsson, 2016), both to verify these predicted $PNEC^R$s and to continue to inform regulatory practice.

Indeed, there is a scarcity of experimental, minimal selective concentration (MSC) data, with only three data papers published to date. These were discussed in chapter two (Lundstrom et al., 2016, Gullberg et al., 2014, Gullberg et al., 2011). Only the single species experiments designed by Gullberg et al. (2011, 2014), which determined MSCs based on selection coefficients (increase in numbers of resistant bacteria overtime) have been used more than once. This method proved suitable for MSC determination for both chromosomally-encoded resistance mutations, and entire resistance genes encoded on a clinically-isolated, multi-drug resistance plasmid. However, as single species experiments they have little predictive power for determining MSCs in the natural environment. Chapter two discussed indirect selection, which is just one of the many possible community interactions which makes selection for resistance much more complex in a natural community than in a single species system. Additionally, the quantification of resistant bacteria used fluorescently activated cell sorting (FACS), which although offers a high level of accuracy, is not well-suited to inter-laboratory testing, as it is not a standard piece of lab equipment and requires fluorescent protein tagging of experimental strains.

The paper by Lundstrom *et al.* (2016) did use a natural complex community, but was subject to other issues discussed more thoroughly in chapter two. In addition, the experimental conditions were complex and therefore not easily replicable. The set-up of multiple open system microcosms, and the harvesting of multiple biofilm replicates, does not lend itself to the rapid generation of data, or again to inter-laboratory replication. This would make validation of this method according to OECD guidelines difficult (OECD, 2005). Additionally, as discussed in chapter two, chemical quantification is necessary for determining truly accurate MSCs. Biofilm-based microcosms would be a difficult matrix from which to extract the compounds for quantification. However, the study did investigate several different endpoints for assessing selection for resistance, including qPCR, metagenomics, phenotypic data and pollution-induced community tolerance (PICT) assays. QPCR was determined as the most sensitive, which is the method used primarily in this chapter and chapter two.

This study used selection coefficients determined by qPCR as the 'gold standard' to which the novel assay (based on difference in growth) is compared, though statistical derivation of LOECs (Lowest Observed Effect Concentrations) is also explored. The different methods used to estimate MSC (i.e. selection coefficient approach) or LOEC (i.e. statistical approach) were compared statistically using Bland-Altman plots (Bland and Altman, 1986). These assess the level of agreement between measurements derived from the two methods; as opposed to a correlation which, though commonly used, simply assesses the strength of the relationship between two measurements, and not how much they may differ (Giavarina, 2015).

### 3.1.2. Effect concentrations of micropollutants (antimicrobials)

The antimicrobials used in the long term experiments forming this study were the β-lactam antibiotic cefotaxime (TAX), the macrolide clarithromycin (CLA), trimethoprim (TRMP), and the fluoroquinolone ciprofloxacin (CIP). Compounds were chosen to assess a range of antibiotic classes, which have been detected at varying concentrations in the environment; which are widely prescribed and which have clinically relevant resistance genes present in the environment. All the antibiotics described below have been included as 'essential' medicines by the WHO (WHO, 2017). The in-depth rationale for selecting these compounds is described subsequently, as well as a selection of available PNECs and MSCs (Table 3).

The growth rate experiments were also used to estimate the MSC of azithromycin (AZ) and erythromycin (ERY), on the basis that long term data would soon be available for these compounds (forming part of the thesis of another student). The quaternary ammonium compound (QAC) benzalkonium chloride (BKC) was also chosen as single example to ascertain if this assay could also be used to determine MSCs for biocides, or other antimicrobials.

### 3.1.3. Clarithromycin (CLA)

The macrolides AZ, CLA and ERY, as a group, were recently included amongst the 10 compounds posing the highest risk in the first ever Watch List developed

by the Environmental Quality Standards Directive (Carvalho et al., 2015). This is because the predicted environmental concentration (PEC) exceeds the PNEC for these compounds, resulting in a risk quotient (RQ) of > 1 (derived by PEC/PNEC). However, this data is based on the standard ecotoxicological tests, and so it is unknown if these will be protective of resistance selection. Additionally, the PEC was based solely on human consumption. Therefore, it is likely that the actual measured environmental concentration (MEC) will be much greater (Carvalho et al., 2015) as ERY is also used extensively in agriculture (Pyörälä et al., 2014) which will increase load in aquatic systems through, for example, run-off of animal excreta from farm land. The only MSCs determined so far for any of these compounds was for ERY in a competition experiment between a susceptible *E. coli* lab strain and an isogenic, resistance-plasmid bearer (Gullberg et al., 2014).

The macrolides have been used for decades against primarily Gram positive bacteria, with some efficacy against intracellular Gram negatives and Gram negative cocci. There are three main pathways to resistance: through modification of drug target (the ribosome), for example the *erm* genes; drug efflux, for example by the *msr* or *mef* genes; and drug inactivation, by for example the *mph* and *lin* genes (Leclercq, 2002). In this study, *ermF* was selected as the gene target, as a recommended antibiotic resistance gene marker to assess resistance selection *in situ* (Berendonk et al., 2015).

### 3.1.4. Ciprofloxacin (CIP)

CIP is a fluoroquinolone, and the only other antibiotic to be included on the Watch List by the Environment Quality Standards Directive (Carvalho et al., 2015). The fluoroquinolones are broad spectrum, with great potency against both Gram positive and Gram negative bacteria, primarily targeting topoisomerase IV and DNA gyrase, respectively; and interfering with the otherwise highly regulated DNA supercoiling and uncoiling. The result of inhibition of either enzyme is that DNA replication is severely impaired at lower concentrations, and at higher concentrations the result is cell death (Redgrave et al., 2014).

CIP can increase intra-chromosomal recombination in *E. coli*, which can in turn increase the potential for novel resistance determinants arising (Lopez et al., 2007); or increase rates of HGT (Guerin et al., 2008, Guerin et al., 2010, Guerin et al., 2009, Beaber et al., 2004). CIP is the most frequently prescribed

fluoroquinolone (Pico and Andreu, 2007). It has greater microbial inhibition in water than in soil (due to sorption) but can remain biologically active in soil over time (Girardi et al., 2011) as it is not readily biodegradable (Cabello, 2006, Kummerer, 2009a). CIP MSCs have been determined (Table 3) for chromosomal point mutations previously (Gullberg et al., 2011).

Like macrolide resistance, fluoroquinolone resistance is mediated by target modification (e.g. *gyr* genes), or efflux (of which there are many examples, including but not limited to NorA). In addition, fluoroquinolone resistance can be conferred by reduced cell wall permeability, or carriage of plasmid-mediated quinolone resistance genes (PMQRs), such as the *qnr* genes. The Qnr proteins bind to topoisomerase IV and prevent fluoroquinolone binding (Redgrave et al., 2014). Initially, *qnrS* was selected for quantification as it is mobile (Redgrave et al., 2014), has been found on numerous occasions in the environment, and is also the second most common *qnr* gene quantified in clinical *Enterobacteriaceae* (Strahilevitz et al., 2009).

However, following suboptimal results using the *qnrS* target (3.3.2.3. Results and 3.4. Discussion), the *intI1* gene was used to determine CIP MSC and LOEC as *qnr* genes can be associated with these genetic elements (Partridge et al., 2009). The *intI1* gene encodes the class 1 integrase, the gene in class 1 integrons which facilitates homologous recombination of gene cassettes. Class 1 integrons are common to both clinical and natural environments and often harbour a selection of antibiotic resistance genes in gene cassette arrays which can be readily interchanged; and as such, they are frequently cited as being good indicators for both anthropogenic impact and presence of antimicrobial resistance genes (Berglund et al., 2014, Kotlarska et al., 2014, Gillings et al., 2009, Gaze WH, 2011, Gaze et al., 2005, Abella et al., 2015b, Gillings MR, 2008, Gillings et al., 2015, Amos et al., 2015, Jechalke et al., 2013). In this context, targeting *intI1* for MSC determination can determine not only the selective potential of a compound but the co-selective potential as well; as numerous other resistance genes are likely to be harboured on a single integron.

### 3.1.5. Trimethoprim (TRMP)

TRMP is most often used in combination with the sulphonamide antibiotic sulfamethoxazole, as the compound co-trimoxazole. This is not fully metabolised

in the body (Yang et al., 2011), and so is commonly used to treat urinary tract infections. TRMP's target is dihydrofolate reductase, and resistance arises due to alteration of this enzyme encoded by the *dfr* genes. 30 *dfr* genes have been recorded, and they are frequently associated with class 1 and class 2 integrons. Following the increase in resistance over recent years and consequent increasingly ineffective treatment, a study investigated the effects on TRMP resistance by reducing TRMP use over two years. Despite this, *dfr* gene prevalence did not decrease, suggesting the genes confer a low fitness cost and or these genes are readily co-selected (Brolund et al., 2010). Importantly, TRMP is excreted unchanged in urine (Brolund et al., 2010) and can reach concentrations of up to 162 µg/L in WWTP influent and 100 µg/L in surface water (UmweltBundesamt, 2016). It also cannot be biodegraded (Sirtori et al., 2010) which may make it a significant driver of antibiotic resistance in the environment.

Due to the many *dfr* variants and their association with integrons, the *intI1* gene was used as the target for the long term selection experiments.

### 3.1.6. Cefotaxime (TAX)

TAX is a third generation cephalosporin, and a β-lactam antibiotic. β-lactam antibiotics prevent cell membrane synthesis, resulting in cell lysis, by binding membrane-bound transpeptidases (or 'penicillin-binding proteins': PBPs) which cross-link peptidoglycan. Resistance mechanisms include modified PBPs, changes in outer membrane permeability, efflux pumps (for example, MexXY in *Pseudomonas aeruginosa*) and enzymes (β-lactamases) which degrade the antibiotic by hydrolysing the β-lactam ring (Reygaert, 2011).

There are over 1000 β-lactamases described to date, and they are common in pathogens (Walsh, 2013b). They can be divided into classes A and B, which are found in Gram positive and Gram negative bacteria respectively (Reygaert, 2011); and 2 groups, the first comprising of three classes of serine β-lactamases, and the second including 2 groups of metallo-β lactamases which require a bivalent metal ion catalyst (Bush, 1998). The *bla*$_{\text{CTX-M}}$ genes encode for extended spectrum β-lactamases (ESBLs), and can be associated with a range of MGE's, including plasmids, IS's, IS*CR*'s and integrons. *Bla*$_{\text{CTX-M}}$ containing plasmids are often multi-drug resistant. These features may account for their

rapid dissemination worldwide, with the *bla*$_{\text{CTX-M-15}}$ gene being the most common (Canton and Coque, 2006).

Used extensively in the clinic for treating problematic multidrug resistant Gram negative pathogens, TAX is readily degradable and as such the parent compound is found only at very low concentrations in the environment, ranging from 1.1 µg/L in untreated WWTP influent, to as low as 0.001 µg/L in surface water (UmweltBundesamt, 2016). However, TAX concentrations have been measured as high as 150 µg/L in hospital effluent (Gomez et al., 2007). Despite this, *bla*$_{\text{CTX-M-15}}$ genes have been found in a variety of environmental compartments including soil (Hartmann et al., 2012), river water, river sediment (Dhanji et al., 2011, Marti et al., 2013, Sidrach-Cardona et al., 2014, Amos et al., 2014a), and even in recreational waters (Blaak et al., 2014). This disparity between environmental concentration and presence of resistance genes in the environment makes TAX an interesting selective agent, as it prompts deeper investigation of fitness effects at the gene level and potential co-selective effects of other more persistent compounds.

Table 3. The compounds used in this study, with a selection of available PNECs and all available MSCs, with corresponding references. Concentrations with a * are toxicity data, i.e. with a safety factor added (normally 10 for chronic and 1000 for acute (Straub, 2013)).

| Compound | PNEC(s) (µg/L) | Reference(s) | MSC(s) (µg/L) | Reference(s) |
|---|---|---|---|---|
| Azithromycin (AZ) | 0.09<br><br>0.15 | (Carvalho et al., 2015)<br>(Kümmerer and Henninger, 2003) | *No data* | |
| Cefotaxime (TAX) | 0.04 | (Kümmerer and Henninger, 2003) | 0.4 | Chapter One |
| Ciprofloxacin (CIP) | 0.089<br><br>0.02 | (Carvalho et al., 2015)<br>(Kümmerer and Henninger, 2003) | 0.1<br><br>2.5 | (Gullberg et al., 2011)<br>(Gullberg et al., 2011) |
| Clarithromycin (CLA) | 0.013<br>0.04 | (Carvalho et al., 2015)<br>(Kümmerer and Henninger, 2003) | *No data* | |
| Erythromycin (ERY) | 0.2<br><br>0.04 | (Carvalho et al., 2015)<br>(Kümmerer and Henninger, 2003) | 3000 | (Gullberg et al., 2014) |
| Trimethoprim (TRMP) | 1<br><br>5.1* (acute)<br>240* (chronic) | (Kümmerer and Henninger, 2003)<br>(Straub, 2013)<br>(Straub, 2013) | 33 | (Gullberg et al., 2014) |
| Benzalkonium chloride (BKC) | 3.9<br><br><br>0.032* (chronic) | (EnvironmentCanada and HealthCanada, 2009)<br>(Guo et al., 2016) | 500 | *Lihong Zhang, pers. comms* |

| | | | | |
|---|---|---|---|---|
| | 0.28* (acute) | (Guo et al., 2016) | | |

### 3.1.7. Primary aims of the study

- Determine selective endpoints (MSCs and / or LOECs) using long term experiments and the growth experiment, for several antibiotics.
- Compare these data to assess the reliability of the growth assay for predicting LOECs generated using the long term assay.
- Compare these MSC and LOEC data to PNECs and determine if current ecotoxicological endpoints are protective of resistance selection.
- Define the RQs of these antibiotics to determine the risk for resistance selection *in situ*.

MSCs and LOECs are defined in chapter two, but redefined here for clarity. Briefly, MSCs are estimated selective endpoints, determined by calculating selection coefficients based on change of resistance gene prevalence over time. LOECs are selective endpoints defined statistically, by determining the lowest concentration where resistance gene prevalence is significantly different to the no antibiotic control at the end of the experiment.

## 3.2. Materials and Methods

### 3.2.1. Sample collection, storage and preparation

Sewage influent from Falmouth, Cornwall (UK), was collected on the morning of 27$^{th}$ October 2015. Sewage effluent from Camborne, Cornwall (UK) was collected on 3$^{rd}$ February 2017 at the point of entry into the environment (following primary treatment, activated sludge). The waste water treatment plants serve populations of 43,000 and 73,000, respectively.

Single use aliquots were mixed in a 1:1 ratio with 20 % glycerol, vortexed and stored at -80 °C until use. Before use, samples were spun down at full speed for 10 minutes, the supernatant removed, and the pellet resuspended in equal volume of 0.85 % NaCl twice to prevent nutrient/chemical carry over.

### 3.2.2. Antibiotics

TAX (Molekular) and CIP (Sigma) stocks were prepared in autoclaved and filtered, deionised water; TRMP (Sigma) in DMSO, AZ (Sigma) in ethanol, CLA (Molekular) in acetone, ERY (Acros Organics) in ethanol; and BKC (Sigma) in sterile, filtered deionised water. Single-use aliquots were stored at -80 °C until use to prevent degradation by repeated freeze-thawing.

### 3.2.3. Growth rate experiments

These were used to inform the concentration ranges in the long term selection experiments, where appropriate. A broth microdilution assay was performed for each antibiotic, with 6 or 7 twofold antibiotic dilutions. This was in a final volume of 200 µl comprising Iso-Sensitest broth, wastewater (10 % volume) and each antibiotic, starting from the clinical breakpoint (EUCAST, 2014) concentration for *Enterobacteriaceae* (where possible). When the lowest concentration showed a significant difference, the experiment was run again with a new, lower concentration series which included the concentration which showed the significant difference in the previous plate. Only data where decreased growth rate was determined, but not at the lowest assay concentration, are shown. All plates also contained a no antibiotic control, as well as a sterile control. There were 6 replicates for each treatment. Plates were incubated immediately in a Varisokan Flash plate reader, at 37 °C with background shaking at 180 rpm at 3 mm. Optical density (OD) was measured at 600 nm, every hour, for 24 hours.

### 3.2.4. Pilot effluent experiment

The growth rate assay was also performed with effluent. This was used as the complex community inoculum in a pilot growth rate experiment, as above, using sub-inhibitory concentrations of TAX (encompassing the LOECs determined by both growth rate and long term assays; and the MSC as determined by long term experiments - see 3.3. Results). The assay concentrations were 625, 312.5, 156.25, 78.13, 39.06 and 19.53 µg/L, as well as a no antibiotic and sterile controls as previously described. Culturing conditions were as previously described.

### 3.2.5. Pilot low temperature and low nutrient experiment

Influent and effluent were diluted to a 10 % volume / volume of either iso-sensitest broth or M9 buffer, in a total volume of 30 ml. There was one replicate for each. All four samples were incubated at 20 °C, shaking at 180 rpm for several days. Samples were taken intermittently and OD at 600 nm was determined using a Jenway spectrophotometer. When necessary, cultures exceeding an OD of 1 were diluted 10-fold and remeasured. Sampling ceased when the growth curves appeared to reach stationary phase.

### 3.2.6. Long term waste water (complex community) microcosm experiments

The data for TAX is taken directly from chapter two. Methods are as described in chapter two, but with different antibiotics. Iso-sensitest broth was inoculated with a 10x dilution of washed, untreated waste water. This was separated into 25 ml aliquots and appropriate amounts of antibiotic stocks were added, which was then diluted twofold in a further 25 ml broth with waste water for the required number of dilutions.

The concentrations selected for the long term experiments were based on the growth rate data results where appropriate (i.e. where a significant difference growth rate was observed, and 2 concentrations below). Concentration series were two-fold dilutions, with 0 µg/L as a negative (no antibiotic) control. For CIP the concentrations were 1000 µg/L down to 0.98 µg/L; and for TRMP the concentrations were 4000 µg/L down to 7.81 µg/L. For CLA, the concentrations were 0, 100, 250, 500, 750, 1000, 10,000 and 100,000 µg/L.

All replicates (n=5 for TAX, CIP, TRMP and CLA unless otherwise indicated) were immediately sampled for the Day 0 sampling time point: 2 x 1 ml of each was spun down at full speed for 3 minutes, the supernatant removed and pellet resuspended in 500 µl 20 % glycerol followed by storage at -80 °C. The remaining sample was incubated overnight at 37 °C, 180 rpm. Following overnight incubation, 50 µl of each microcosm was introduced into 5 ml fresh media and antibiotic; this was repeated for a total of 7 days (except for the TAX experiment, see chapter two, which was 8 days).

### 3.2.7. QPCR

The qPCR protocol was the same as that used in chapter two: frozen samples / untreated waste water were thawed and DNA extracted using the MBio UltraClean DNA extraction Kit according to instructions; with initial spinning time elongated to 3 minutes. DNA was stored at -20 °C.

gBlock synthetic oligonucelotides (IDTDNA, Table 4) were used as standards; these were resuspended in TE buffer according to the manufacturer's instructions and were stored at -80 °C. All DNA standards were 10x serially diluted in TE buffer and stored at -20 °C before use. Every PCR plate was always run with 5 serial dilutions of standards in duplicate (and a duplicate negative control). For *bla*$_{\text{CTX-M}}$ and 16S gene copy number, provided the efficiency for the reaction was between 90 % and 110 %, the average CTs for the duplicate technical replicates for each sample were used to calculate the copy number based on a 'gold standard' standard dilution series, where the DNA concentration had been quantified by QuBit and the copy number per µl quantified immediately prior to cycling. For all other gene targets, standards within the plate were used for absolute copy number quantification.

Standards were first verified using qPCR conditions described below, with a melt curve in SYBR assays. For the TAX and CLA experiments (see chapter two, and Author Contributions), Brilliant qPCR SYBR Green reagents (Agilent) were used in 20 µl reactions comprising of 10 µl master mix, 2 µl primer pair (10 µM for 16S and *ermF* primers, 18 µM for CTX-M primers), 0.2 µl BSA (20 mg/ml), 0.4 µl dye (20 µM), 5 µl diluted DNA template and filtered, sterilised water to a total volume of 20 µl. The qPCR programme was 95 °C for 3 minutes, followed by 40 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds, except CTX-M which was 58 °C for 1 minute.

For *qnrS* and *intI1* gene targets, PrimerDesign PrecisionPLUS MasterMix with pre-added ROX was used in a 20 µl reactions comprising of 10 µl mastermix, 2 µl primer pair (4.5 µM for both), 0. 2µl BSA (20 mg/ml), 5 µl diluted DNA template and filtered, sterilised water to a total volume of 20 µl. The qPCR programme was 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. This mastermix was compared with the Agilent mastermix, and there was no significant difference between copy numbers determined for the same test template. The PrimerDesign mastermix was used

as it was heavily discounted following my PrimerDesign Silver Student Scholarship award, which ran from January 2015 to January 2016.

The primers and gBlocks used in this chapter are in Table 4. All targeted genes' copy numbers were divided by 16S copy number to determine a molecular 'prevalence' of each resistance gene.

Table 4. Sequences and length of the different primers, probes and gBlocks used in this study, with original references or the accession number used where applicable.

| Name | Sequence | Size (bp) | Accession number | Reference |
|---|---|---|---|---|
| 16S gBlock | ACGGTGAATACGTTCCCGGGCCTTGTAC ACACCGCCCGTCACACCATGGGAGTGGG TTGCAAAAGAAGTAGGTAGCTTAACCTTC GGGAGGGCGCTTACCACTTTGTGATTCA TGACTGGGGTGAAGTCGTAACAAGGTAA CCG | 144 | CP018770.2 | This study |
| CTX-M gBlock | GATGTGCAGCACCAGTAAAGTGATGGCC GCGGCCGCGGTGCTGAAGAAAAGTGAAA GCGAACCGAATCTGTTAAATCAGCGAGTT GAGATCAAAAAATCTGACCTTGTTAACTA TAATCCGATTGCGGAAAAGCACGTCAAT GGGACGATGTCACTGGCTGAGCTTAGCG CGGCCGCGCTACAGTACAGCGATAACGT GGCGATGAATAAGCTGATTGCTCACGTT GGCGGCCCGGCTAGCGTCACCGCGTTC GCCCGACAGCTGGGAGACGAAACGTTCC GTCTCGACCGTACCGAGCCGACGTTAAA CACCGCCATTCCGGGCGATCCGCGTGAT A | 338 | KX452391.1 | This study |
| intI1 gBlock | GGCCTTGATGTTACCCGAGAGCTTGGCA CCCAGCCTGCGCGAGCAGCTGTCGCGT GCACGGGCATGGTGGCTGAAGGACCAG GCCGAGGGCCGCAGCGGCGTTGCGCTT CCCGACGCCCTTGAGCGGAAGTATCCGC GCGCCGGGCATTCCTGGCCGTGGTTCTG GGTTTTTGCGCAGCACACGCATTCGACC GATCC | 198 | CP020934.1 | See author contributions |
| qnrS gBlock | TTCGACGTGCTAACTTGCGTGATACGACA TTCGTCAACTGCAAGTTCATTGAACAGGG TGATATCGAAGGCTGCCACTTTGATGTCG CAGATCTTCGTGATGCAAGTTTCCAACAA TGCCAACTT | 125 | KY421937.1 | See author contributions |
| *ermF* gBlock | TCTGATGCCCGAAATGTTCAAGTTGTCGG TTGTGATTTTAGGAATTTTGCAGTTCCGA ATTTCCTTTCAAAGTGGTGTCAAATATTCT TATGGCATTACTTCCGATATTTTCAAAATC TGATGTTTGAGAGTCTTGGAAATTTTCTG GGAGGTTCCATTGTCCTTCAATTAGAACC TACACAAAAGTTATTTTCGAGGAAGCTTT ACAATCCATATACCGTTTTCTATCATACTT TTTTTGATTTGAAACTTGTCTATGAGGTA GGTCCTGAAAGTTTCTTGCCACCGCCA | 294 | CP021206.1 | See author contributions |
| 16S F (1396F) | CGGTGAATACGTTCYCGG | 142 | - | (Suzuki et al., 2000) |
| 16S R (1492R) | GGWTACCTTGTTACGACT | | - | |

| | | | | |
|---|---|---|---|---|
| CTX-M consensus F | ATG TGC AGY ACC AGT AAR GTK ATG GC | ~300 | - | (Birkett et al., 2007) |
| CTX-M consensus R | ATC ACK CGG RTC GCC XGG RAT | | - | |
| intI1-L1 | GCCTTGATGTTACCCGAGAG | 196 | - | (Barraud et al., 2010) |
| intI1-L5 | GATCGGTCGAATGCGTGT | | - | |
| qnrS UP | CGACGTGCTAACTTGCGTGA | 118 | - | (Colomer-Lluch et al., 2014) |
| qnrs LP | GGCATTGTTGGAAACTTGCA | | - | |
| ermF F | TCTGGGAGGTTCCATTGTCCT | 56 | CP021206.1 | See author contributions |
| ermF R | ACTTTCAGGACCTACCTCATAGA | | CP021206.1 | |

### 3.2.8. Minimal Selective Concentration (MSC) determination

Effect concentrations were determined both statistically (see 3.2.9. Statistics) by generating LOECs and by using selection coefficients to estimate MSCs, as previously (Gullberg et al., 2014, Gullberg et al., 2011, Lundstrom et al., 2016). Details for selection coefficient determination can be found in chapter two.

### 3.2.9. Statistics

All statistics were performed in RStudio (2015). A range of statistical tests were used to investigate the data.

The growth based LOEC was based on the lowest antimicrobial concentration which was significantly different to the no antibiotic control (Dunn's test), at a single time point during exponential growth phase. Spearman's rank was performed to determine if there was a significant, monotonic relationship between antimicrobial concentration and average growth (growth based experiment); and between antimicrobial concentration and average target gene prevalence.

A Kruskal Wallis test was used to compare target gene 'prevalence' at day 0 (to verify replicates were not significantly different at day 0), and at the end of the experiment. For the longer term experiments, two different methods for LOEC determination were used – Dunn's test, and / or a general linearised model (GLM) approach. Details of GLM types were data set specific and are summarised in the results.

Bland-Altman plots were also generated using R using the R package 'Bland-Altman-Leh', to compare the different methods for determining effect concentrations. As the differences between the two end points were non-normally

distributed (according to a Shapiro Wilks test for normality), data were log transformed, to be in keeping with the test assumptions (Bland and Altman, 1986, Giavarina, 2015). Log transformed data were used for the Bland-Altman plots shown in the results.

### 3.2.10. Author Contributions

The idea behind this chapter is based on my observations during the degradation experiments from chapter two. I performed the long term experiments (culturing and qPCR) for TAX and CIP, and all the qPCRs for these and the TRMP long term experiment. Isobel Stanton (fellow PhD student) and two visiting college students Alana Dalton and Jessica Wright performed the TRMP experiment under my direction. Isobel Stanton performed the CLA long term experiment and qPCR, also with my input, as part of her thesis; as well as designing the primers and gBlock for *ermF*. Lihong Zhang designed the gBlock primers for *intI1* and *qnrS* gene targets. I designed and conducted all growth rate experiments, as well as designing and performing all data analysis and statistical analyses (both for long term and growth experiments).

## 3.3. Results

### 3.3.1. Growth based experimental results

The dose-response relationship between complex community growth and antibiotic concentrations observed during the degradation experiment in chapter two lead to the hypothesis that growth assays may be rapid, cheap alternatives to long term experiments for determining selective endpoints. To test this hypothesis, more MSC and LOEC data were generated for additional antibiotics, both by using the growth assay and the long term evolution experiment.

All growth based experiments were run over 24 hours (Figures 31 – 37). During exponential growth phase it was noted that there was a dose-response relationship between antimicrobial concentration and complex community growth (Figure 30), which was most apparent at 6 hours.

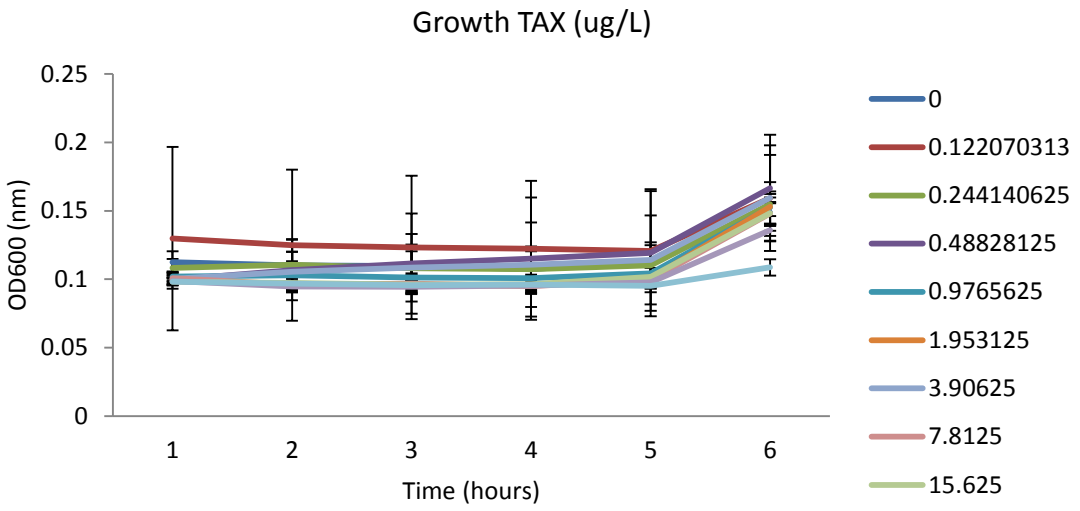


Figure 30. Average (n=7) growth (optical density measured at 600 nm) of the complex community in the presence of different concentrations (µg/L) of cefotaxime, over 6 hours. Shown with standard deviation bars.

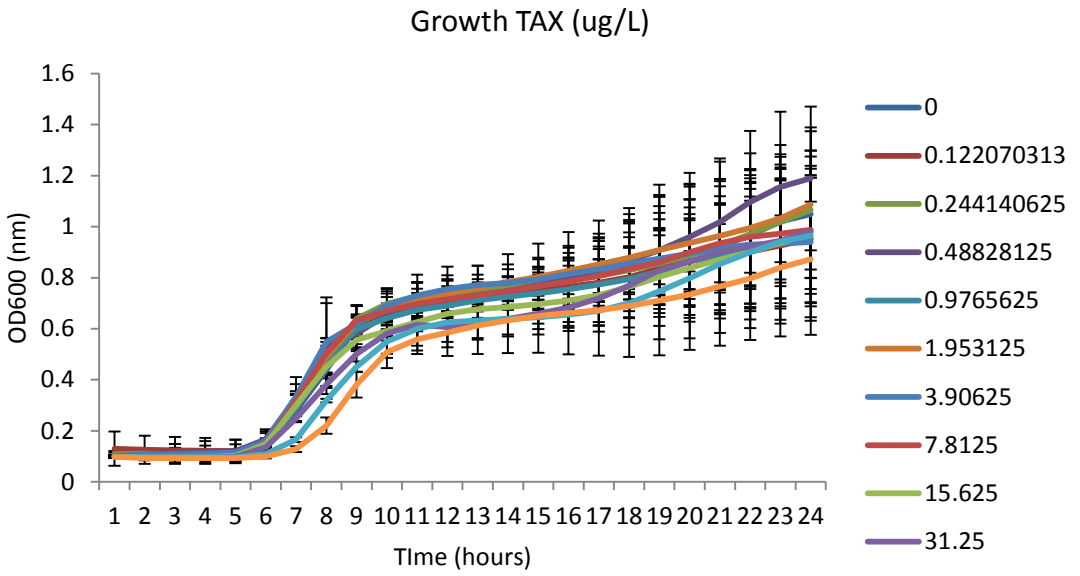


Figure 31. Average (n=7) growth (optical density measured at 600 nm) of the complex community in the presence of different concentrations (µg/L) of cefotaxime, over 24 hours. Shown with standard deviation bars.

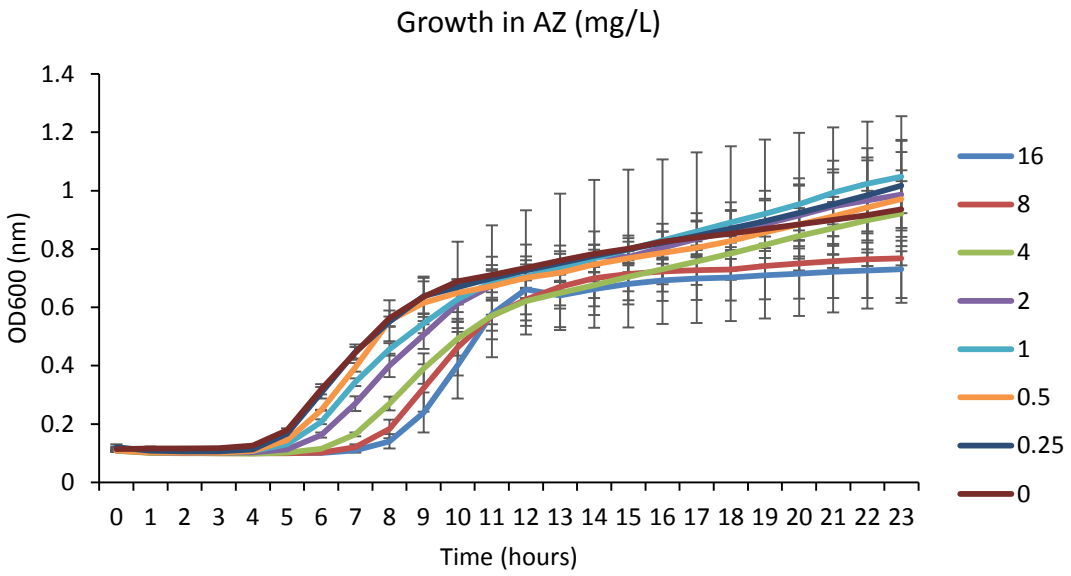


Figure 32. Average (n=6) growth (optical density measured at 600 nm) of the complex community in the presence of different concentrations (mg/L) of azithromycin, over 24 hours. Shown with standard deviation bars.

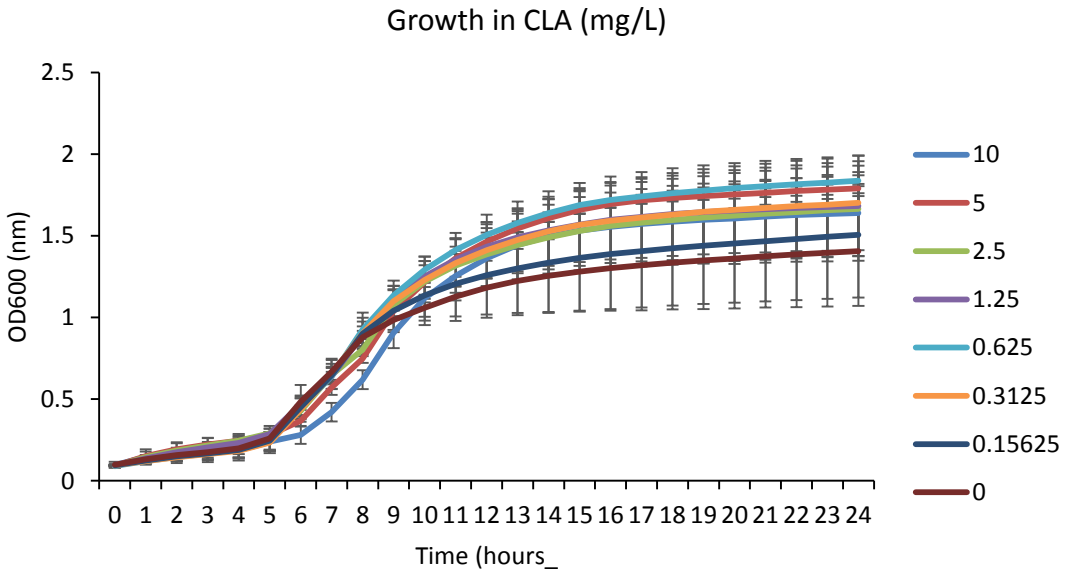


Figure 33. Average (n=6) growth (optical density measured at 600 nm) of the complex community in the presence of different concentrations (mg/L) of clarithromycin, over 24 hours. Shown with standard deviation bars.

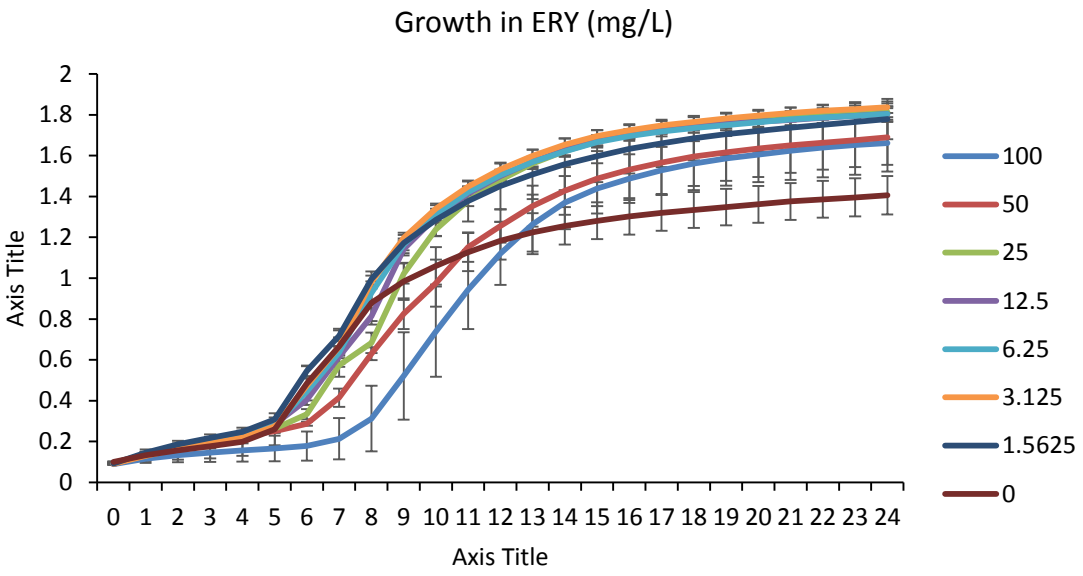


Figure 34. Average (n=6) growth (optical density measured at 600 nm) of the complex community in the presence of different concentrations (mg/L) of erythromycin, over 24 hours. Shown with standard deviation bars.

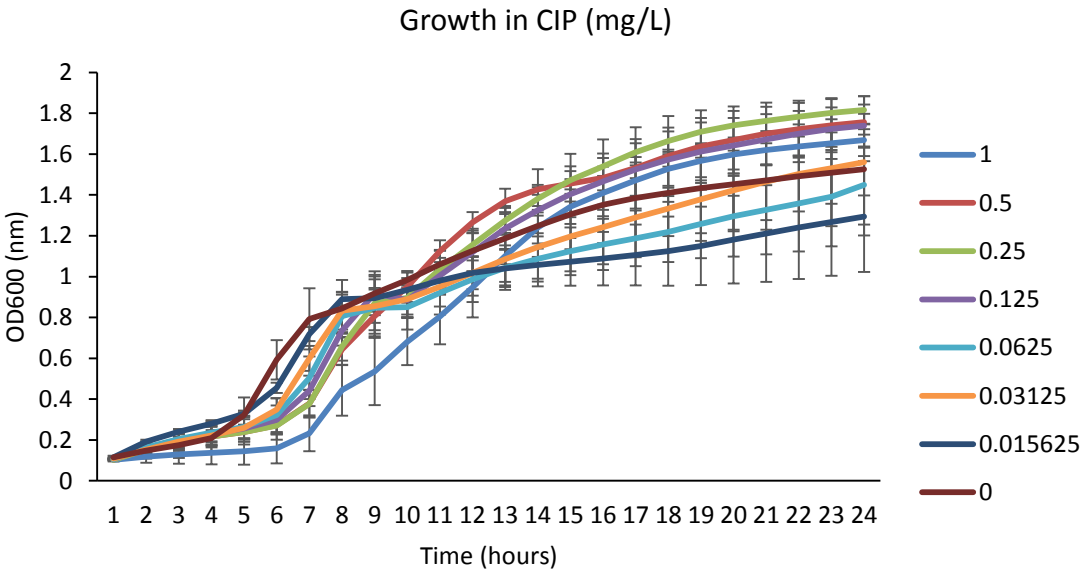


Figure 35. Average (n=6) growth (optical density measured at 600 nm) of the complex community in the presence of different concentrations (mg/L) of ciprofloxacin, over 24 hours. Shown with standard deviation bars.

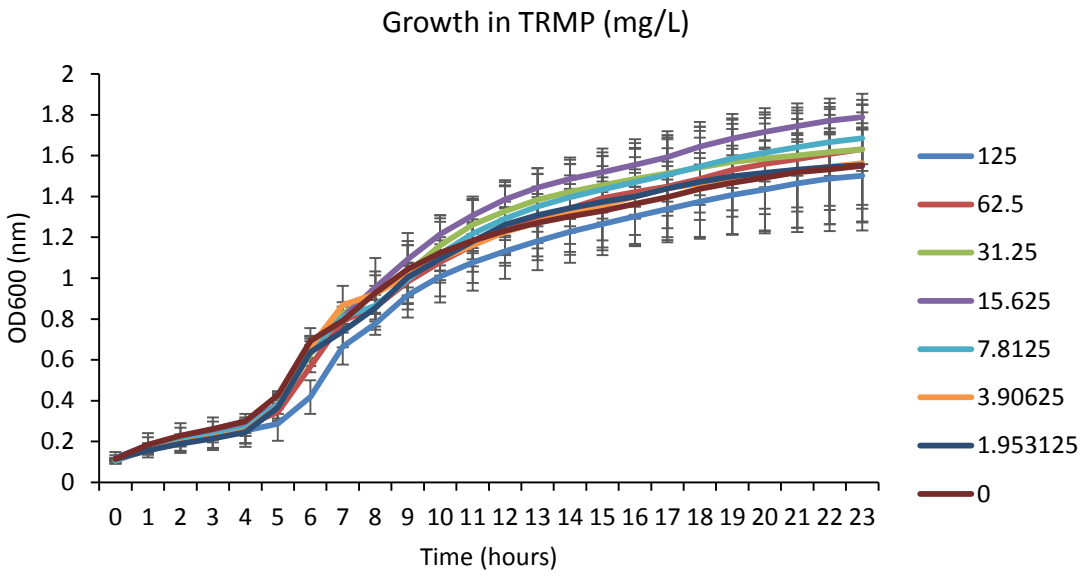


Figure 36. Average (n=6) growth (optical density measured at 600 nm) of the complex community in the presence of different concentrations (mg/L) of trimethoprim, over 24 hours. Shown with standard deviation bars.

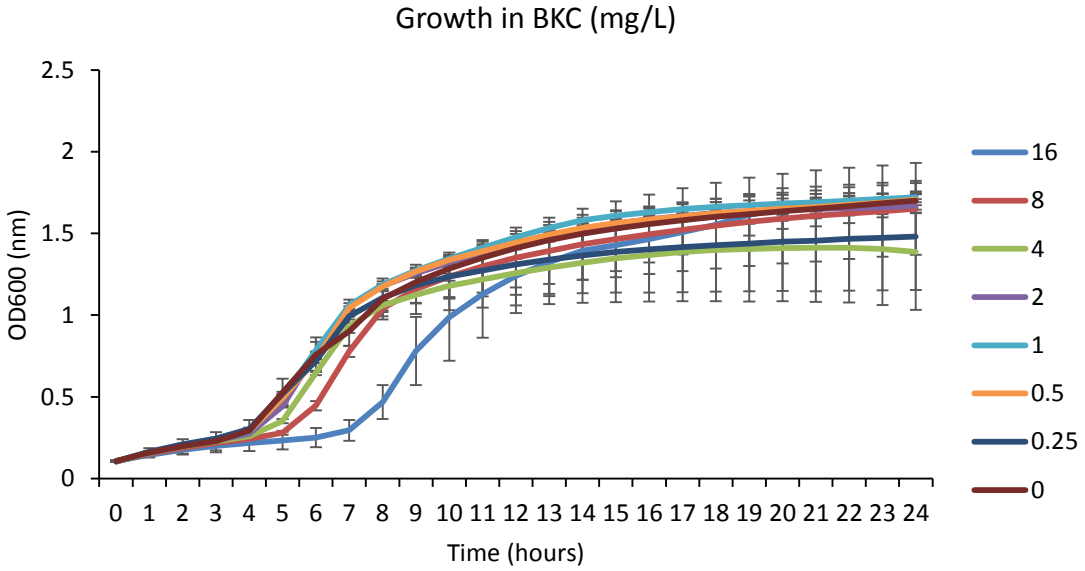


Figure 37. Average (n=6) growth (optical density measured at 600 nm) of the complex community in the presence of different concentrations (mg/L) of benzalkonium-chloride, over 24 hours. Shown with standard deviation bars.

To determine the optimum time point to use for LOEC determination, a range of time points were assessed for the strength of this relationship.

Spearman's rank correlation coefficients were determined by comparing antibiotic concentrations and growth at the 6 hour (beginning of exponential growth phase), 9 hour (end of exponential growth phase), 12 hour (stationary phase) and final time points. This was performed for all antimicrobials, and consistently the 6 hour time point had the highest negative rho ($r_s$) value, and the lowest *p* value – except for TAX which had a higher negative $r_s$ value at 9 and 12 hours; and ERY, which had the highest negative $r_s$ value at 9 hours (Table 5).

The $r_s$ and *p* values were averaged for all antimicrobials at each time point, and the average $r_s$ and *p* values were plotted against the different time points to visualise the decrease in the dose-response relationship (Figure 38). Based on this analysis, the 6 hour time point was used for determining LOECs, using a Dunn's test. The results from this are shown in Table 6.

Table 5. Rho ($r_s$ Spearman's rank) values (in bold) and *p* values (italicised) to test for a dose response relationship between complex community growth and antimicrobial concentration, at different time points during different growth phases.

| Antimicrobial | 6 hours | 9 hours | 12 hours | Final |
|---|---|---|---|---|
| TAX | **-0.4185773**<br>*7.42e-05* | **-0.5249639**<br>2.953e-07 | **-0.4879846**<br>*2.496e-06* | **-0.1565931**<br>*0.1549* |
| AZ | **-0.954791**<br>*< 2.2e-16* | **-0.9404587**<br>*< 2.2e-16* | **-0.4758091**<br>*0.0006307* | **-0.3861504**<br>*0.006711* |
| CLA | **-0.5240322**<br>*0.000132* | **-0.133268**<br>*0.3665* | **0.1855787**<br>*0.2066* | **-f0.005690067**<br>*0.9694* |
| ERY | **-0.756902**<br>*4.84e-10* | **-0.9710926**<br>*< 2.2e-16* | **-0.2742971**<br>*0.05921* | **-0.1471211**<br>*0.3183* |
| CIP | **-0.8451171**<br>*4.23e-14* | **-0.3277629**<br>*0.02296* | **-0.04179557**<br>*0.7779* | **0.08895718**<br>*0.5477* |
| TRMP | **-0.6097578**<br>*1.815e-05* | **-0.1698345**<br>*0.2823* | **-0.2042268**<br>*0.1945* | **-0.2159928**<br>*0.1695* |
| BKC | **-0.6933572**<br>*4.696e-08* | **-0.4785285**<br>*0.000581* | **-0.2395782**<br>*0.101* | **0.136359**<br>*0.3554* |

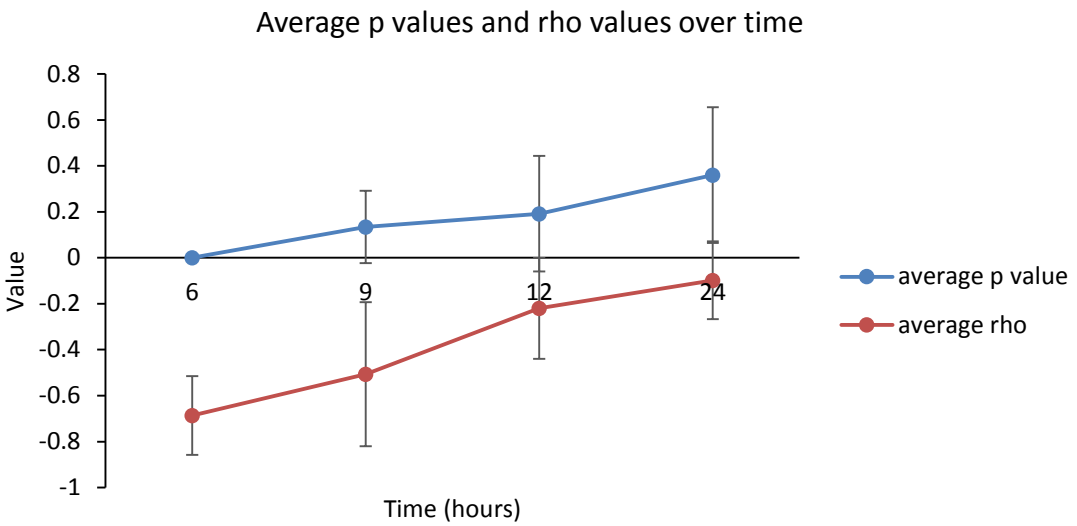


Figure 38. Average (n = 8) *p* values and rho ($r_s$) values from Spearman's rank correlation analysis of growth (optical density) against increasing antibiotic concentration, at 4 different time points. Shown with standard deviation bars.

Table 6. The growth based effect concentration, experimentally derived using average growth data (n=6) at 6 hours growth. Determined using Dunn's test (significance = $p < 0.05$).

| Antimicrobial | Growth based LOEC (µg/L) |
|---|---|
| TAX | 31.25 |
| AZ | 1000 |
| CLA | 5000 |
| ERY | 25,000 |
| CIP | 1.95 |
| TRMP | 31.25 |
| BKC | 4000 |

### 3.3.2. Long term selection experiment results

MSCs and LOECs were determined for several antibiotics using the long term evolution experiment, to allow comparison to growth LOECs and to compare the MSC and LOEC method for determining long term selective endpoints.

#### 3.3.2.1. Cefotaxime (TAX)

The results for the TAX long term experiment (both qPCR graph and selection coefficient graphs) are shown in Figures 5 and 6 in chapter two. The MSC was 0.4 µg/L. There was no dose-response relationship between *bla*$_{CTX-M}$ prevalence at day 8 and cefotaxime concentration.

To make the statistical analyses of the data as comparable as possible, the qPCR data at day 8 was also analysed using a GLM approach. However, despite assessing all possibly error families and links both with the day 8 prevalence data, and the square root transformed day 8 prevalence data, there was not a suitable model which did not significantly violate the assumption that the residuals should be normally distributed (the highest *p* value for the Shapiro Wilks test for residuals was 0.0002, using transformed data).

The Dunn's test was performed on the untransformed data as in chapter two, there was a significant increase ($p < 0.05$) in $bla_{CTX\text{-}M}$ prevalence at 125 µg/L, and all concentrations above. Therefore, the statistically derived LOEC is 125 µg/L.

#### 3.3.2.2. Clarithromycin (CLA)

The qPCR data for the CLA experiment can be seen in Figure 39, and the MSC in Figure 40, which was 65 µg/L. There was no significant difference in day 0 *ermF* prevalence between treatments (ANOVA). There was no significant dose-response relationship between *ermF* prevalence and CLA concentration (Spearman's rank, $p > 0.05$).

As with TAX, multiple GLMs with different error families and link functions were explored; however there was no model, even with transformed data, which did not fail on one or more GLM assumptions. Therefore a Dunn's test was used to find the LOEC (CLA concentration at which *ermF* prevalence was significantly different to that without antibiotic) – in this case, 750 µg/L (Dunn's test, $p < 0.05$).

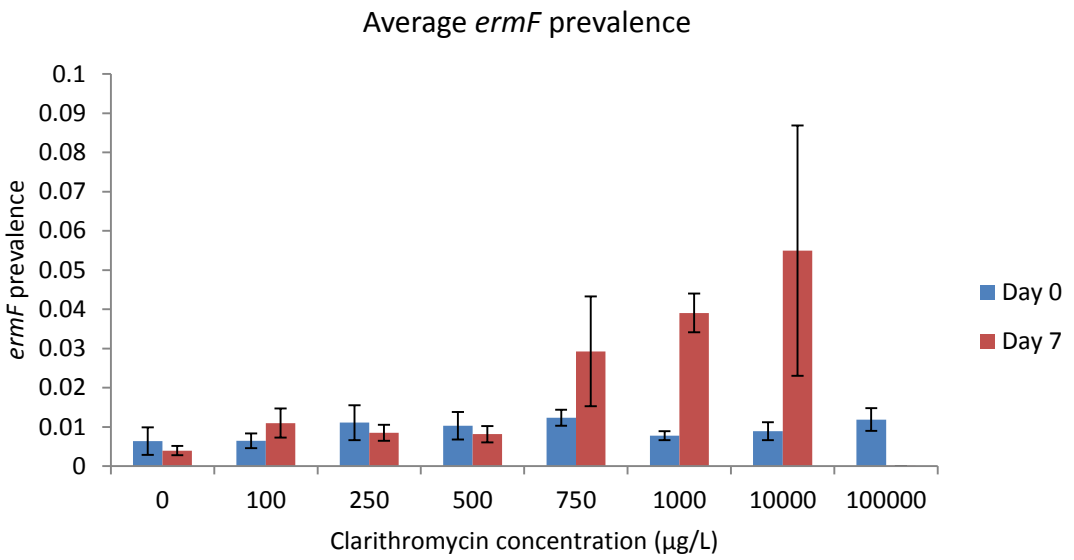


Figure 39. The qPCR data (technical replicate, n=2) for the long term evolution experiment for clarithromycin. Shown is average (n=5, except day 7 250 µg/L n=4) *ermF* prevalence (*ermF* gene copy number divided by 16S copy number) at day 0 and day 7. Shown with standard error bars.

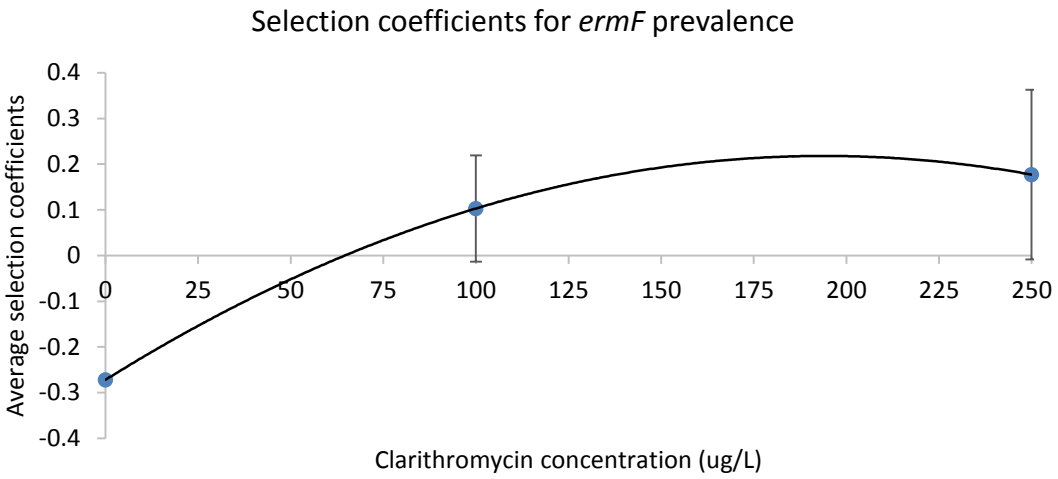


Figure 40. Average (biological replicate n=5 except day 7 250 µg/L n=4, qPCR replicate n=2) selection coefficients based on the qPCR *ermF* gene prevalence data at day 0 and day 7, shown with standard error bars and polynomial (order 2) line of best fit. The MSC is where the line crosses the x-axis – at 65 µg/L.

### 3.3.2.3. Ciprofloxacin (CIP)

Initially, the *qnrS* gene was targeted to determine the MSC of CIP. The qPCR data is shown in Figure 41. There was no clear association of *qnrS* prevalence with CIP concentration – in the majority of treatments *qnrS* prevalence decreased over time, and there was extremely high variability between replicates despite repeated qPCR. For these reasons, the *intI1* gene was targeted.

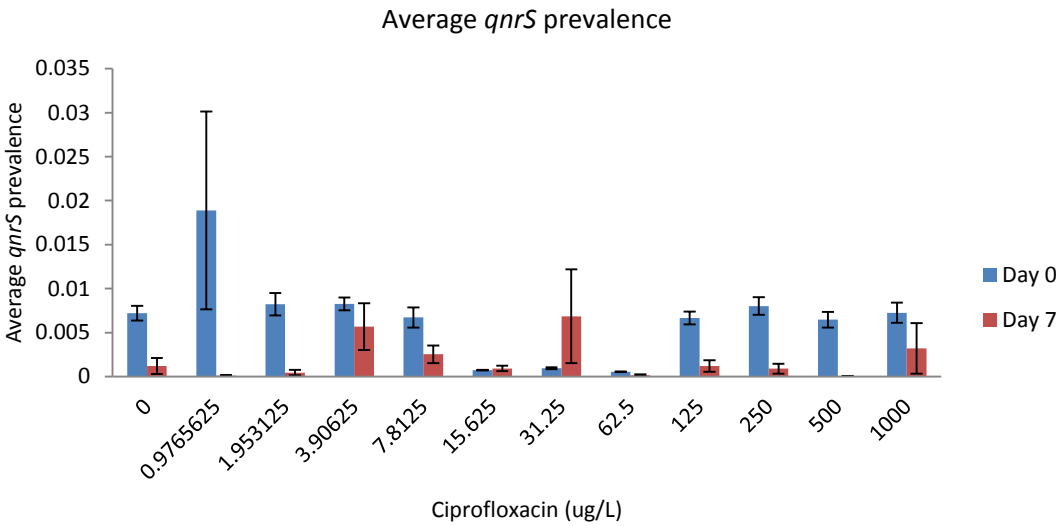


Figure 41. The qPCR data (technical replicate, n=2) for the long term evolution experiment for ciprofloxacin. Shown is average (n=5) *qnrS* prevalence (*qnrS* gene copy number divided by 16S copy number) at day 0 and day 7. Shown with standard error bars.

The *intI1* qPCR data for the ciprofloxacin experiment is shown in Figure 42, and the MSC in Figure 43. The MSC was 1.3 µg/L. Using the qPCR data, there was no significant difference in *intI1* prevalence at day 0 between treatments (Kruskal Wallis). There was a significant dose-response relationship between *intI1* prevalence at day 7 and ciprofloxacin concentration (Spearman's rank, $g$ = 0.7, $p$ = 4.622e-10).

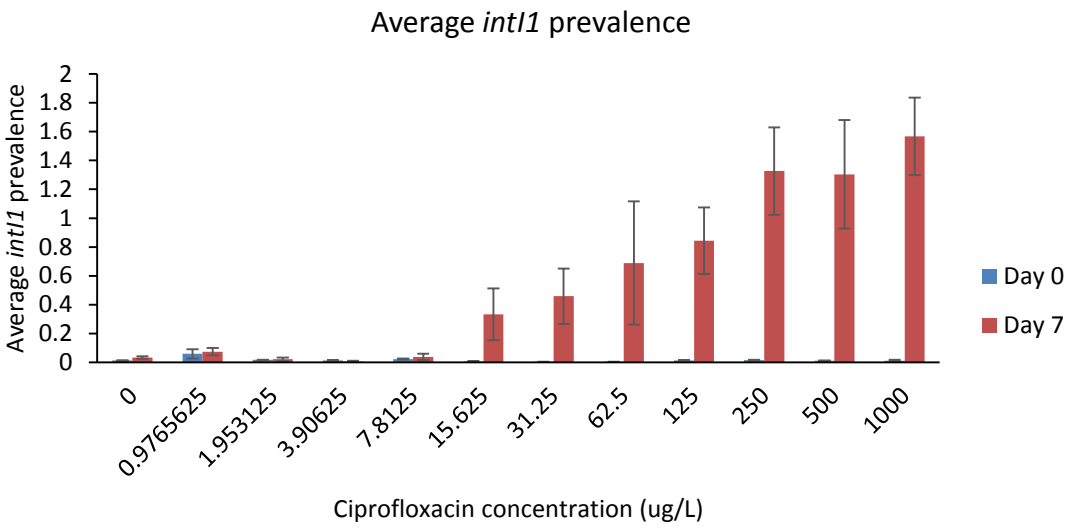


Figure 42. The qPCR data (technical replicate, n=2) for the long term evolution experiment for ciprofloxacin. Shown is average (n=5) *intI1* prevalence (*intI1* gene copy number divided by 16S copy number) at day 0 and day 7. Shown with standard error bars.

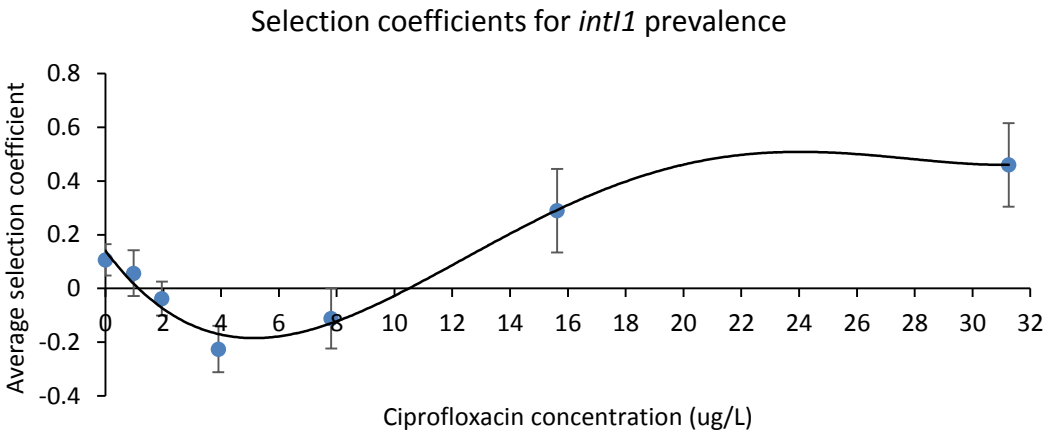


Figure 43. Average (biological replicate n=5, qPCR replicate n=2) selection coefficients based on the qPCR *intI1* gene prevalence data at day 0 and day 7, shown with standard error bars and polynomial (order 4) line of best fit. The MSC is where the line crosses the x-axis - at 10.5 µg/L.

A range of GLM models and respective links were explored using the untransformed, non-normally distributed data. A Gamma GLM and quasi-poisson GLM provided the lowest deviance of residuals. The Gamma model had an

Akaike Information Criterion (AIC) value of -53, indicating an excellent fit, and the residuals were normally distributed (Shapiro Wilks test, $p$ = 0.055). However, the deviance of residuals was 90 and there was slight overdispersion (1.8). The quasi-poisson model indicated a better fit due to the deviance of residuals being 18; however, the residuals were slightly non-normally distributed (Shapiro Wilks test, $p$ = 0.043). The quasi-poisson model also suffered from slight underdispersion (0.37); however this is preferable as it increases the chances of underestimating significance (as opposed to overdispersion, which may lead to overestimation of significance (Thomas et al., 2017)).

To combat the non-normalcy of the data, prevalence at day 7 was transformed (square root). The data was still non-normal following transformation (Shapiro Wilks test $p$ = 1.76e-05 and $p$ = 0.001, respectively), but was used in different GLMs. Again, the Gamma and quasi-poisson GLMs provided the best fit, and using the transformed data reduced the deviance of residuals to 29 and 9, respectively. Concurrently, the AIC for the Gamma model reduced to 14. However, the residuals for the Gamma model were not-normally distributed (Shapiro Wilks test, $p$ = 0.02). Using the transformed data, the residuals for the quasi-poisson were normally distributed (Shapiro Wilks test, $p$ = 0.86); however, there was greater underdispersion (0.18) than with the previous quasi-poisson model.

The ciprofloxacin LOEC (where a significant difference ($p$ <0.05) in *intI1* prevalence was observed) was 15.63 µg/L for the quasi-poisson non-transformed data; however, with the transformed data, there was only a trend for significance at 15.63 µg/L ($p$ < 0.1). This reduction in significance is likely due to the increased underdispersion when using the transformed data. Therefore, based on these findings, the quasi-poisson GLM with square root link function for the untransformed data was chosen to determine the statistically derived LOEC. There was a significant difference ($p$ < 0.05) in *intI1* prevalence at day 7 at the ciprofloxacin concentration of 15.63 µg/L, and all concentrations above (Quasi-poisson GLM, F = 18.26, df = 11, 48, $p$ = 1.415e-13, adjusted $R_2$ = 0.76).

### 3.3.2.4. Trimethoprim (TRMP)

The qPCR data for the TRMP experiment is shown in Figure 44, and the MSC in Figure 45. The MSC was 24 µg/L. Using the qPCR data, there was no significant difference in *intI1* prevalence between treatments at day 0 (Kruskal Wallis). There was a significant dose-response relationship between *intI1* prevalence at day 7 and TRMP concentration (Spearman's rank, $r_s$ = 0.91, $p$ < 2.2e-16).

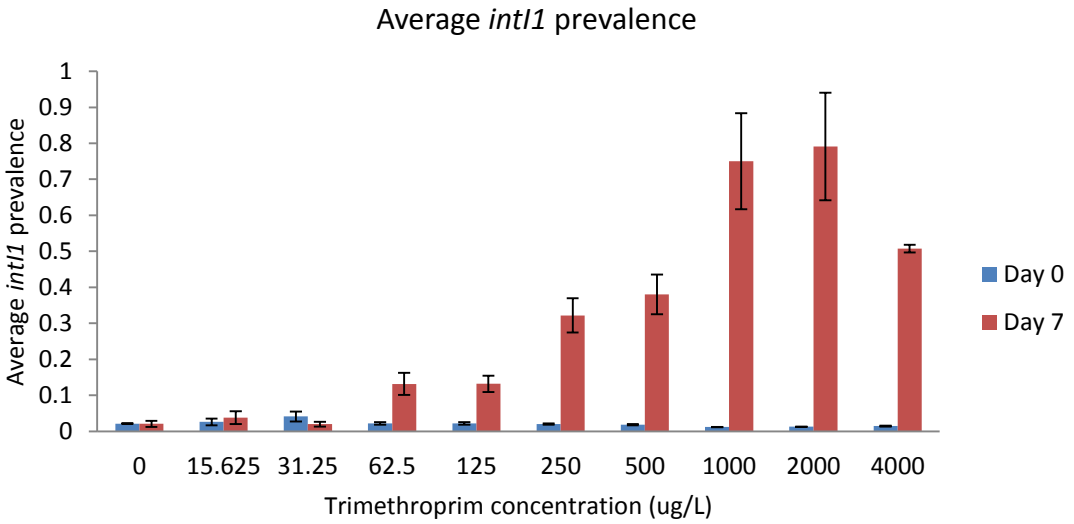


Figure 44. The qPCR data (technical replicate, n=2) for the long term experiment evolution experiment for trimethoprim. Shown is average (n=5, except 4000 µg/L which was n=3) *intI1* prevalence (*intI1* gene copy number divided by 16S copy number) at day 0 and day 7. Shown with standard error bars.

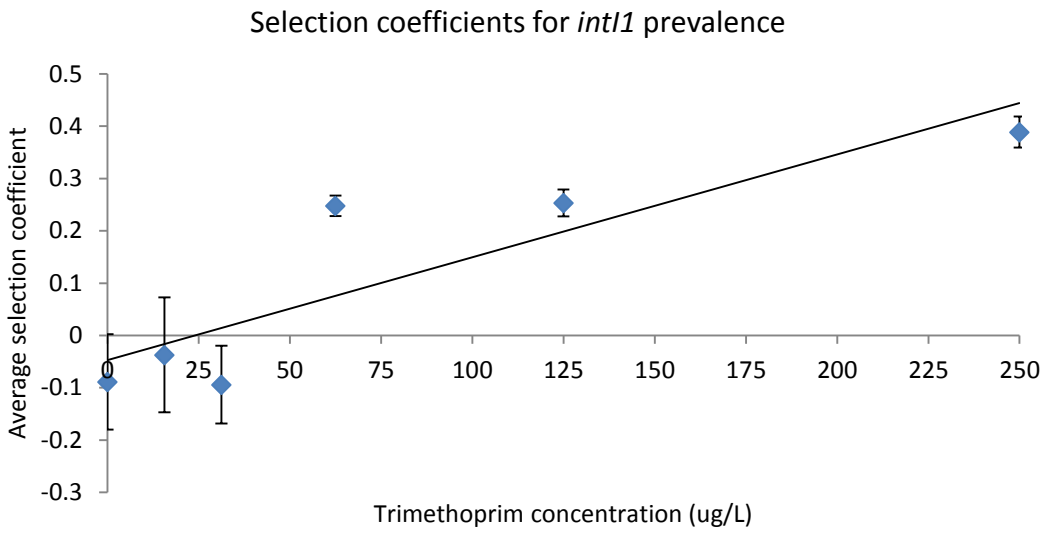


Figure 45. Average (biological replicate n=5, qPCR replicate n=2) selection coefficients based on the qPCR *intI1* gene prevalence data at day 0 and day 7, shown with standard error bars and linear line of best fit. The MSC is where the line crosses the x axis, here at 24 µg/L.

A range of GLM models were fit to the *intI1* prevalence data at day 7. The best was a Gamma GLM model with square root link, using transformed data (square root). Residuals were normally distributed (Shapiro Wilks test, $p = 0.20$), it had the lowest deviance of residuals of all models tested, and the lowest AIC value (-34) of all models tested (where applicable). Residuals were also homoscedastic. Using this GLM, day 7 *intI1* prevalence was significantly different ($p < 0.0001$) to 0 at the trimethoprim concentration of 62.5 µg/L and above (Gamma GLM, F = 26.85, df = 9, 38, $p = 7.939e\text{-}14$, adjusted $R_2 = 0.83$).

To be comparable with the cefotaxime data, a Dunn's test was also performed. Using this test, concentrations at 62.5 µg/L and above were significantly different to the control ($p < 0.05$).

Table 7. Summary of the statistical tests performed for each antimicrobial and the endpoints determined. For Spearman's Rank column, values in bold are the $r_s$ value, italics is the corresponding p value. 'KW' = Kruskal Wallis test, * = with ANOVA.

| Antimicrobial | Spearman's Rank | Day 0 prevalence KW | Statistical LOEC (µg/L) | MSC (µg/L) |
|---|---|---|---|---|
| TAX | **0.681258** | *0.4716* | 125 | 30 |

| | | | | |
|---|---|---|---|---|
| | *2.594e-07* | | | |
| CLA | **0.02906765** *0.8606* | *0.8** | 750 | 65 |
| CIP | **0.7005894** *4.622e-10* | *0.2496* | 15.625 | 10.5 |
| TRMP | **0.9139325** *< 2.2e-16* | *0.4276* | 62.5 | 24 |

Table 8. The estimated MSCs and experimentally derived LOECs in µg/L, derived in this study. TAX = cefotaxime, CLA = clarithromycin, CIP = ciprofloxacin, TRMP = trimethoprim. Also shown is the fold difference between the two values (LOEC/MSC, rounded to 1 decimal place). Experimentally derived MSCs marked with a * were determined by Dunn's test; all others were fit to GLMs. Significance = $p$ <0.05.

| Antimicrobial | MSC (µg/L) | Experimentally derived LOEC(µg/L) | Growth based LOEC (µg/L) | Fold difference (LOEC/ MSC) | Fold difference (growth LOEC/ MSC) |
|---|---|---|---|---|---|
| TAX | 0.4 | 125* | 31.25 | 312.5 | 78.13 |
| CLA | 65 | 750* | 5000 | 11.54 | 76.92 |
| CIP | 10.5 | 15.63 | 1.95 | 1.49 | 0.19 |
| TRMP | 24 | 62.5 | 31.25 | 2.60 | 1.30 |

### 3.3.3. Statistical comparison of methods

Bland-Altman plots were generated to compare the endpoints determined using the three different approaches – long term selection coefficient MSCs, long term LOECs, and growth based LOECs. This demonstrates whether the variation between two sets of measurements are constant, as opposed to whether a significant relationship exists (or not) between two measurements from two different methods (which would be determined with a correlation). Plots where the line of equality of differences (0 on the y axis) resides within the 95 % confidence intervals (shown here in brown) around the mean of the differences (shown here in red), indicate good agreement between the two methods of measurement (i.e. acceptable bias (Giavarina, 2015)). The light blue and dark blue lines represent the upper and lower 95% agreement limits; and upper and lower 95% confidence limits for these agreement limits, respectively.

Figures 46 – 48 are Bland-Altman plots comparing: MSCs with long term statistically derived LOECs, MSCs with growth based LOECs, and long term statistically derived LOECs with growth based LOECs (respectively). All three

methods are in good agreement with each other, but the smallest critical difference was between the long term and growth based LOECs, indicating these provide the most similar endpoints (Figure 48).

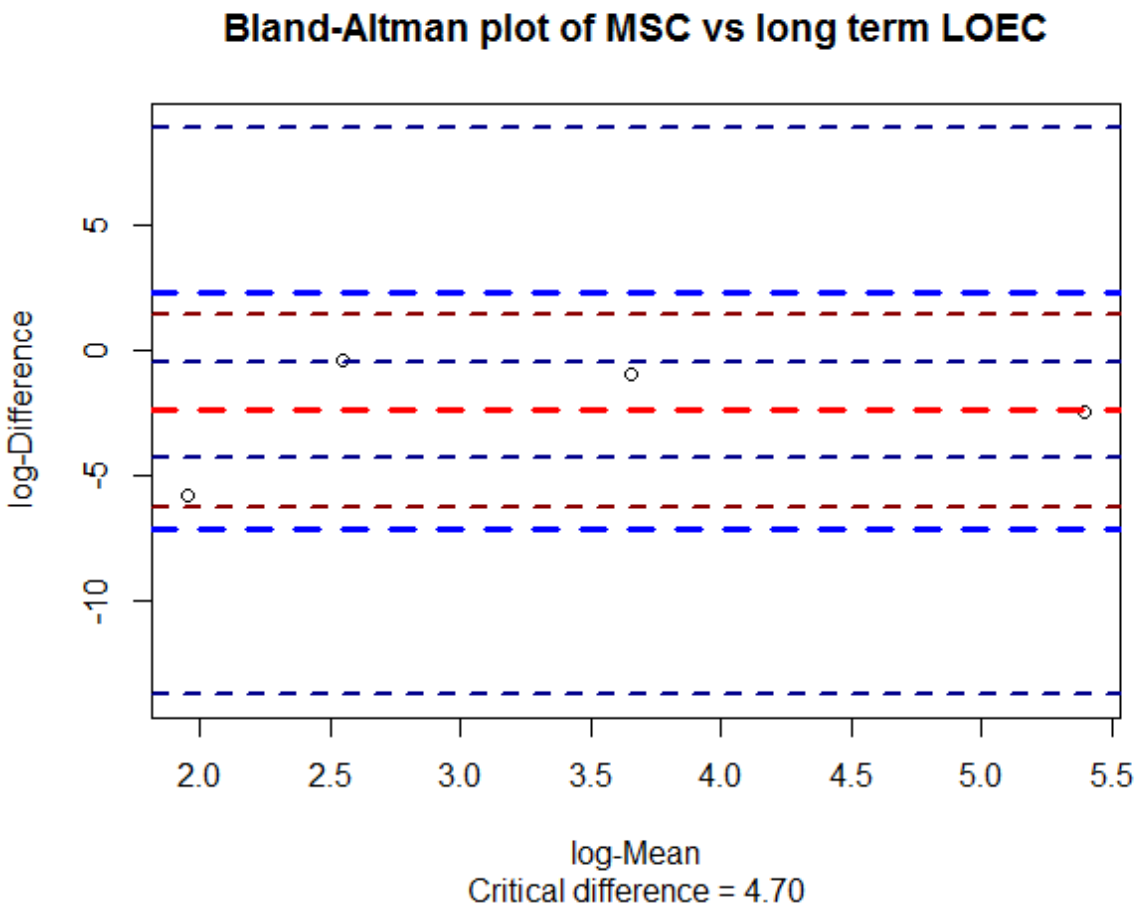


Figure 46. Bland-Altman plot with 95 % confidence intervals (shown in brown and blue), using the log transformed MSC and statistical LOEC for the long term data. The 95 % confidence intervals (brown, dashed lines) around the mean differences (red dashed line) must encompass 0 on the y axis (i.e. complete equality between measurements) in order for the bias between measurements to be acceptable.

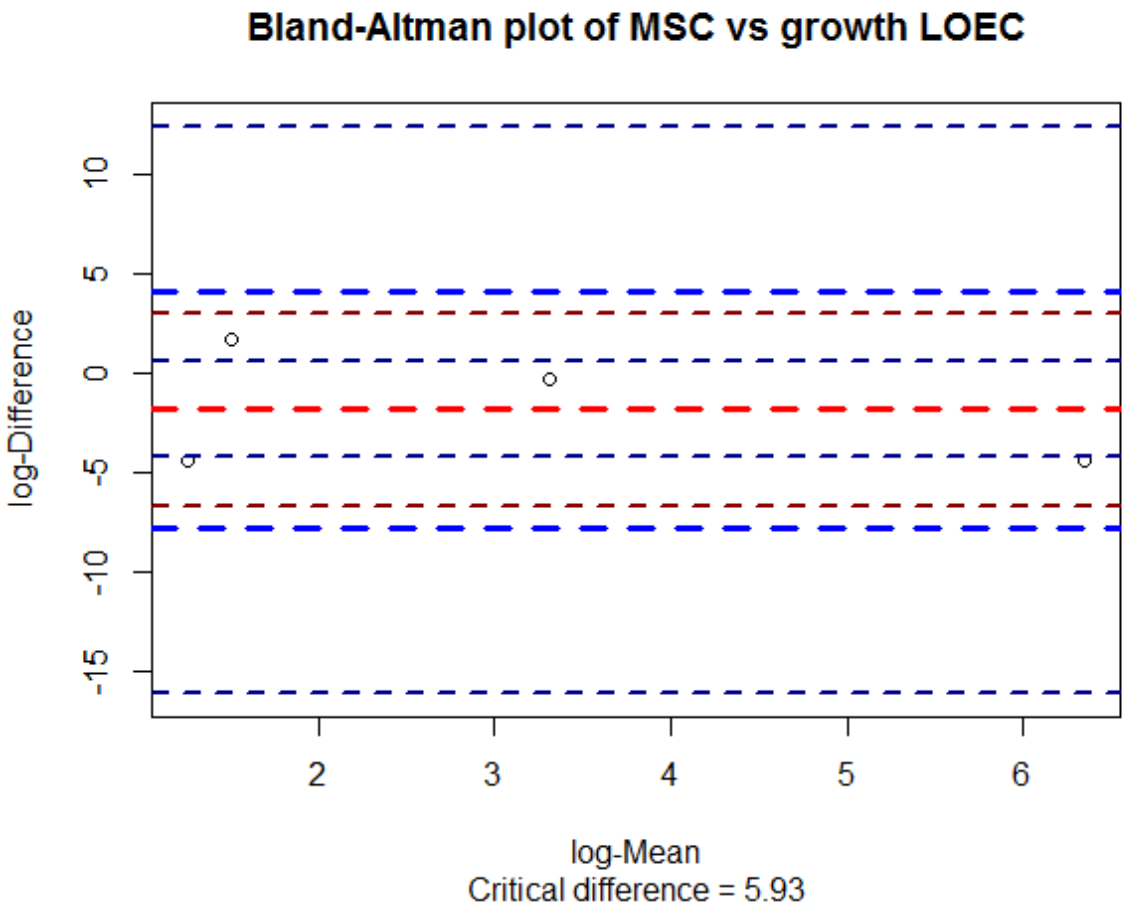


Figure 47. Bland-Altman plot with 95 % confidence intervals (shown in brown and blue), using the log transformed MSC and the growth LOEC data. The 95 % confidence intervals (brown, dashed lines) around the mean differences (red dashed line) must encompass 0 on the y axis (i.e. complete equality between measurements) in order for the bias between measurements to be acceptable.

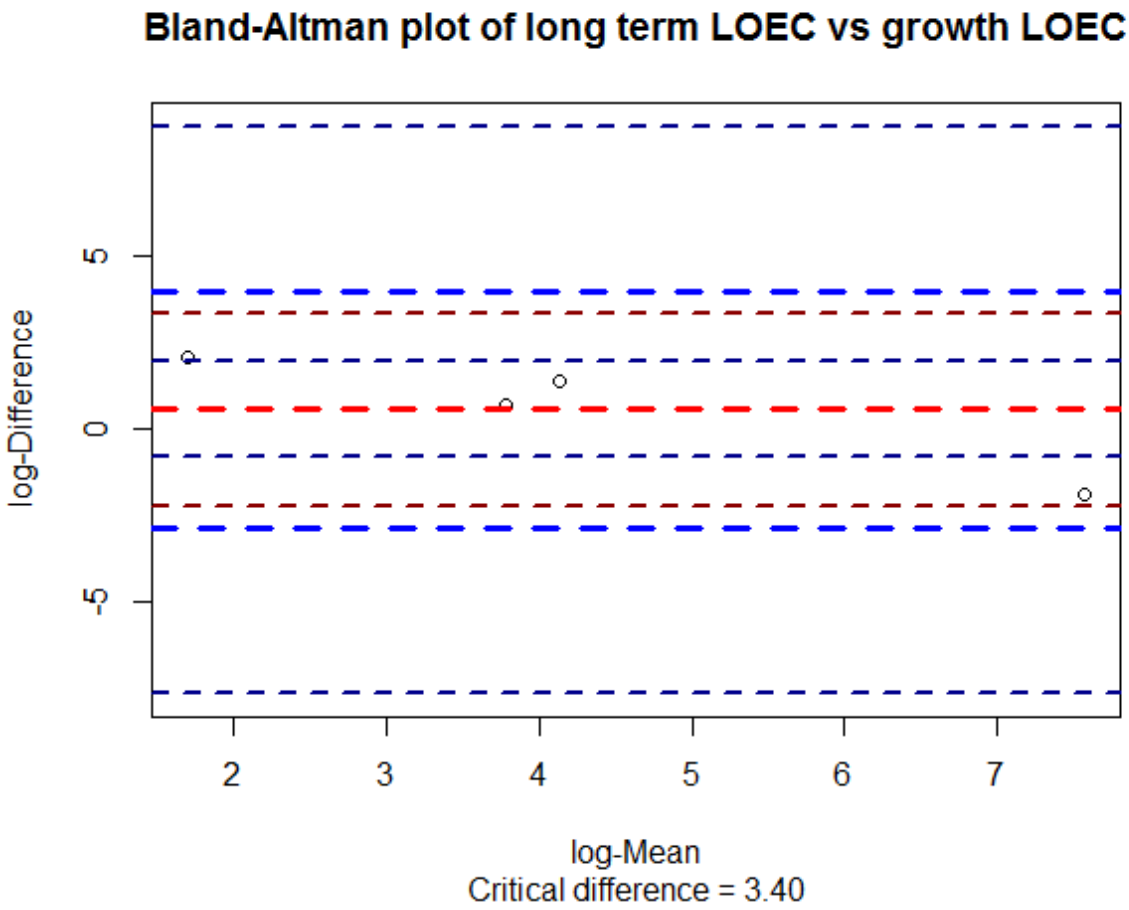


Figure 48. Bland-Altman plot with 95 % confidence intervals (shown in brown and blue), using the log transformed LOECs from the long term and growth experiments. The 95 % confidence intervals (brown, dashed lines) around the mean differences (red dashed line) must encompass 0 on the y axis (i.e. complete equality between measurements) in order for the bias between measurements to be acceptable.

### 3.3.4. Utility of the growth based assay with different bacterial communities

As a brief pilot experiment, the growth based assay was repeated with TAX using untreated waste water or treated waste water (effluent). The growth of these two complex communities is shown in Figure 49 and 50. The initial starting bacterial density was much lower in the effluent (Wilcoxon rank sum test, $p < 0.001$), leading to a longer lag phase. As with the influent experiments for multiple compounds, selected time points (namely 6, 9, 12 and 24 hours) were assessed for the strength of a dose-response relationship using a Spearman's rank test for

correlation. The $r_s$ and $p$ values for the relationships for both the influent and effluent communities are summarised in Table 9. The dose response relationship was apparent at 6 hours for the influent and 9 hours for the effluent; however, the dose-response relationship was apparent in effluent up until the 24 hour time point. Interestingly, at 12 hours, there was a significant positive correlation between growth and antibiotic concentration (Spearman's rank, $r_s = 0.54$, $p = 0.002$). This means there was higher growth with antibiotic than without – which is also apparent until 24 hours (though the correlation is no longer significant by this point) for the majority of concentrations (Figure 49). This is the case for all concentrations exceeding 39.06 µg/L.

Dunn's tests were used to calculate the growth based LOEC at the time point with the most significant negative correlation between growth and TAX concentration, for both influent and effluent. LOECs in both complex communities were 39.06 µg/L using this approach (Dunn's test, $p < 0.005$). For the influent community, Dunn's tests performed at the other time points yielded different or no significant differences; whereas in the effluent community, the LOEC remained the same no matter the time point chosen.

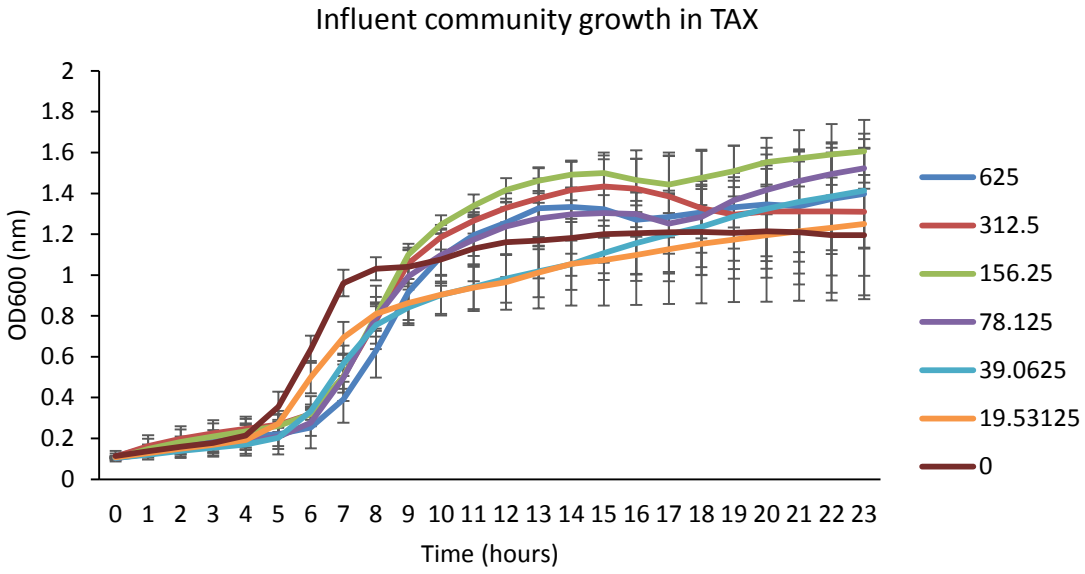


Figure 49. Average (n=6) growth (optical density measured at 600 nm) of the influent community in the presence of different concentrations (µg/L) of cefotaxime, over 24 hours. Shown with standard deviation bars.

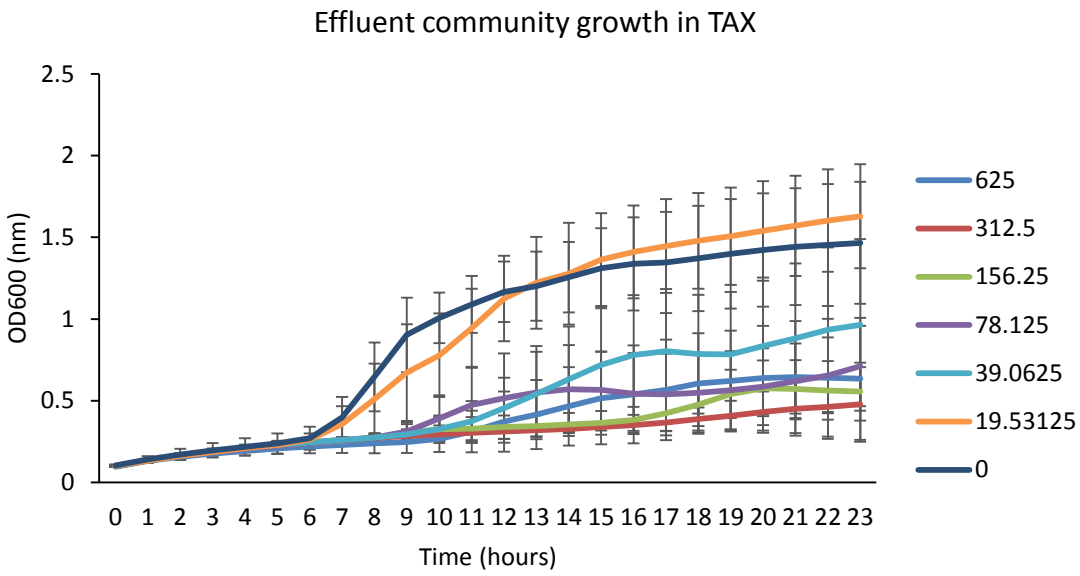


Figure 50. Average (n=6) growth (optical density measured at 600 nm) of the effluent community in the presence of different concentrations (µg/L) of TAX, over 24 hours. Shown with standard deviation bars.

Table 9. The $r_s$ (bold) and *p* (italicised) values for Spearman's rank correlations performed at different time points during growth rate assays of influent and effluent complex communities under TAX exposure.

| Community | 6 hours | 9 hours | 12 hours | 24 hours |
|---|---|---|---|---|
| Influent | **-0.6522644**<br>*2.866e-06* | **0.1271957**<br>*0.4221* | **0.5382491**<br>*0.0002362* | **0.1158957**<br>*0.4648* |
| Effluent | **-0.5173414**<br>*0.0004508* | **-0.7652939**<br>*3.582e-09* | **-0.7141509**<br>*1.093e-07* | **-0.6443013**<br>*4.138e-06* |

### 3.3.5. Pilot test at lower temperature and lower nutrient levels

A brief pilot experiment was conducted to assess the growth of the influent and effluent complex communities at lower temperature (20 °C), in broth and also M9 buffer (lower nutrient). As this was a simple pilot study, data in Figure 51 are based on a single replicate only. At lower temperatures but still with high nutrient levels, both effluent and influent communities reach exponential phase within a day. However, in M9 buffer, exponential growth phase begins around the same time (a day), but growth thereafter is much slower. The effluent community grew

better than the influent community in M9, with a higher final OD and longer exponential growth phase.

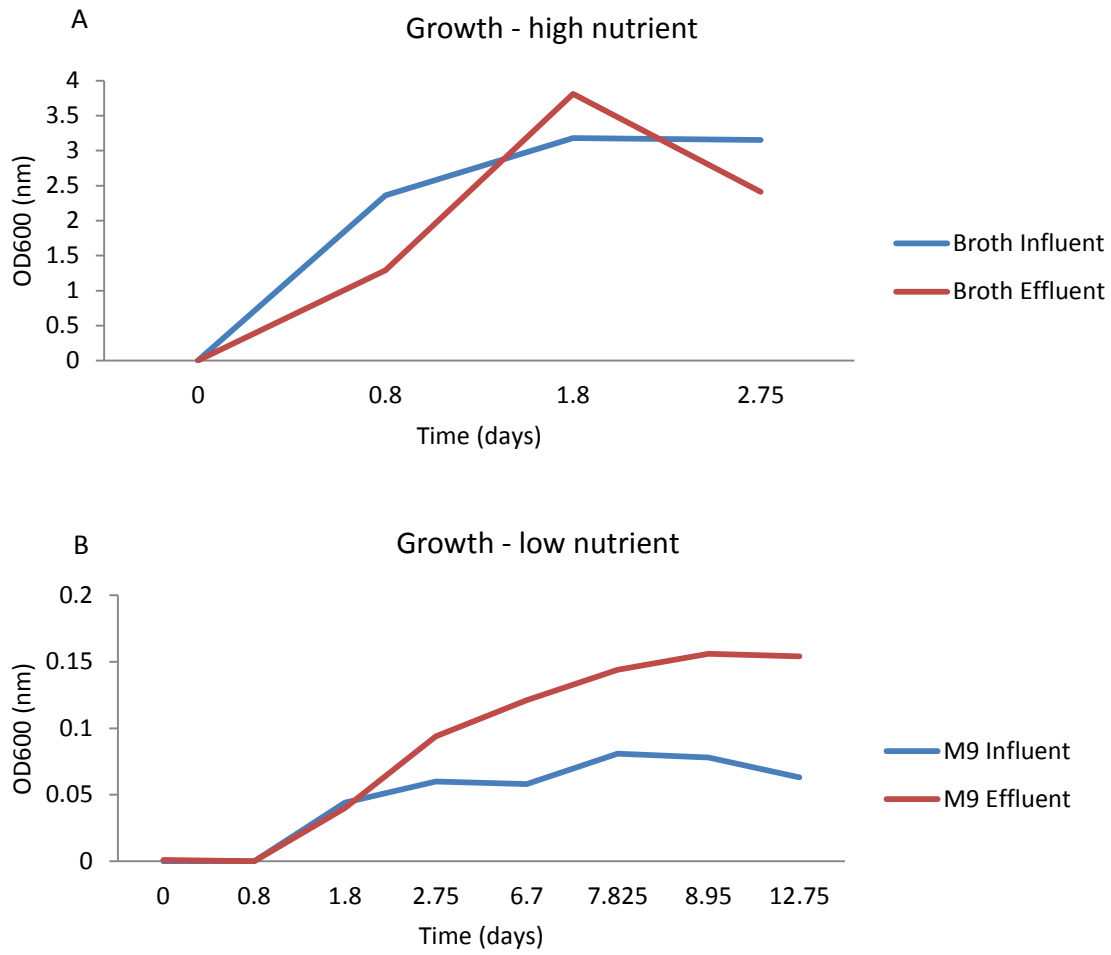


Figure 51. Growth of influent and effluent complex communities at low temperature (20°C) in broth (A) and M9 buffer (B), over time. Single replicate only.

## 3.4. Discussion

### 3.4.1. Targeted genes

The *qnrS* gene proved to be a poor gene target for determining an MSC for CIP, with prevalence frequently decreasing over time, and high variability between replicates within treatments. The cause of which is unknown, but could be due to

the fact *qnr* copy number can greatly influence fluoroquinolone MIC (Redgrave et al., 2014), and perhaps the *qnr* gene family should have been targeted instead of a single variant. In addition, the reduction on MIC by *qnrS* is small, and can result in increased levels of resistance by facilitating mutation-based resistance to arise (e.g. *gyrA* (Kehrenberg et al., 2007)). It may also be a result of the variety of resistance genes present in the complex community – where other resistance mechanisms, perhaps with lower fitness costs or that conferred increased resistance, were preferentially selected for over *qnrS*.

However, the *intI1* gene proved to be a good target for both CIP and TRMP, exhibiting a dose response relationship with antibiotic concentration. Selection for this gene aIso indicates co-selective potential of a compound, as gene arrays within class 1 integrons often harbour multiple different resistance genes conferring resistance to multiple antibiotic classes (Partridge et al., 2009). Use of *intI1* compared to other gene targets should be assessed, as it may be a suitable proxy for all resistance genes. This concurs with previous literature which propose *intI1* prevalence as a marker of anthropogenic impact and resistance gene prevalence (Berglund et al., 2014, Kotlarska et al., 2014, Gillings et al., 2009, Gaze WH, 2011, Gaze et al., 2005, Abella et al., 2015b, Gillings MR, 2008, Gillings et al., 2015, Amos et al., 2015, Jechalke et al., 2013).

### 3.4.2. MSCs and PNEC$^{R}$s

The MSCs and LOECs determined in this chapter using long term data were compared to previously available MSCs, PNECs and MECs. The MSCs would be more protective against resistance selection in the environment than statistically derived LOECs based on gene prevalence, as they are less effected by large, inherent variations in complex bacterial community treatment replicates. They are also based on qPCR data, shown previously to be the most sensitive methods used to determine effect concentrations (Lundstrom et al., 2016).

Conversely statistically derived LOECs are empirical (not estimated). As they are based on direct, quantitative measures, they could be considered as an acute effect concentration, and an assessment factor of 1000 (Straub, 2013) could be used to calculate an acute PNEC$^{R}$. Table 10 shows the MSCs and PNEC$^{R}$s (long term LOEC/1000) determined in this chapter, alongside the previous predicted PNEC$^{R}$ (Bengtsson-Palme and Larsson, 2016).

The CLA MSC was 65 µg/L and the LOEC was 750 µg/L. PNECs reported here are over 100 and 1000x lower than these two values, indicating the ecotoxicological PNECs may be protective. Even with the LOEC converted to a $PNEC^R$ (0.75 µg/L), the ecotoxicological PNECs remain protective.

The CIP MSC was 10.5 µg/L. This in within the range of MSCs determined previously for chromosomal resistance mutations (Gullberg et al., 2011); however it exceeds the reported PNECs by around 2 orders of magnitude. The long term LOEC was higher, at 15.63 µg/L. In which case, both the MSC and statistical LEOC as a $PNEC^R$ would indicate current ecotoxicological tests are protective against resistance gene selection.

For TRMP, the MSC was 24 µg/L. The previously derived MSC using a resistance plasmid harbouring a *dhfr* gene was 33 µg/L (Gullberg et al., 2014). Although these values are very similar, community effects may reduce MSCs; this was observed when comparing the single species and community data in chapter two; though this large disparity was probably due to indirect selection due to the sociality of the resistance mechanism. Compared to the previously reported PNECs presented at the beginning of this chapter, the acute toxicity PNEC is protective against selection (using the MSC endpoint); however, the chronic PNEC is not. The calculated $PNEC^R$ based on the above data (i.e. the LOEC with safety factor applied), suggests the reported ecotoxicological PNEC is not protective of resistance selection for neither acute nor chronic exposure.

When comparing $PNEC^R$s to previously estimated PNECs for resistance (Table 10), remarkably, the cefotaxime $PNEC^R$ and estimated PNEC for resistance were identical (both 0.125 µg/L). Unfortunately when considering all compounds tested in this study, neither the $PNEC^R$ (this study) nor predicted $PNEC^R$ (Bengtsson-Palme & Larsson, 2016) were consistently the most protective indicating that though the estimates appear useful in guiding experiments to determine MSCs or LOECs, actual experimental data is still required in order to be fully protective of the environment.

### 3.4.3. Assessing the risk of resistance selection *in situ*

The risk quotient (RQ) is determined as the either the Predicted Environmental Concentration (PEC) or MEC/PNEC. If >1, there is an estimated, or real risk to the environment, respectively. Table 10 summarises the lowest MSCs and

PNEC$^R$s (LOEC/safety factor of 1000) determined in this study; previously estimated PNEC$^R$s (Bengtsson-Palme and Larsson, 2016), MECs and calculated 'real' RQs. This captures the different possibilities to analyse effect concentrations, in terms of real world implications.

CLA poses the most significant risk to the environment in terms of resistance selection, with extremely high RQs for both the MECs reported here (the highest MECs for the respective environments, according to the UmweltBundesamt database (2016)), irrespective of effect concentration used (i.e. MSC or PNEC$^R$). CIP also poses a significant risk to the environment as it has extremely high RQs, regardless of environmental compartment or whether the MSC or PNEC$^R$ is used. Conversely, the selective potential of TRMP is only hazardous when using PNEC$^R$ data. The risk of TAX selecting for resistance in hospital effluent is incredibly high when using PNEC$^R$ data, indicating this could be a hotspot for resistance selection. However, there is a possible risk even in waste water effluent when using PNEC$^R$ to calculate the RQ.

Table 10. Table showing the MSCs and PNEC$^R$s determined in this study alongside previously estimated PNEC$^R$s. MECs shown are the highest reported MECs for a particular environmental compartment in the Umweltbundesamt database (2016); or by Gomez et al. (2007) (indicated by '*'). RQs are derived by MEC/MSC or PNEC$^R$: red, yellow and green RQ's represent unacceptably high, medium and low risk respectively.

| Antibiotic | MSC (µg/L) | PNEC$^R$ (µg/L) This study | PNEC$^R$ (µg/L) (Bengtsson-Palme and Larsson, 2016) | MEC in µg/L (environmental compartment) | RQ (MSC) | RQ (PNEC$^R$, this study) | RQ (PNEC$^R$, Bengtsson-Palme and Larsson, 2016) |
|---|---|---|---|---|---|---|---|
| TAX | 0.4 | 0.125 | 0.125 | 150 (hospital effluent)*<br>0.09 (waste water) | 375<br>0.225 | 1200<br>0.72 | 1200<br>0.72 |
| CLA | 65 | 0.75 | 0.25 | 14,600 (WWTP effluent)<br>100 (surface water) | 224.61<br>1.54 | 19466.67<br>133.33 | 58400<br>400 |
| CIP | 10.5 | 0.156 | 0.064 | 54.05 (hospital effluent) | 5.15 | 346.47 | 844.5 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | 3.7 (waste water) | 0.35 | 23.72 | 57.81 |
| TRMP | 24 | 0.063 | 0.5 | 1.5 (hospital effluent) | 0.06 | 23.81 | 3 |
| | | | | 1.81 (waste water) | 0.08 | 18.73 | 3.62 |

### 3.4.4. Growth based assays for rapid data generation

The MSCs estimated for the long term assay data using selection coefficients were consistently lower than using statistical based LOEC methods. This is likely due to the extremely high variation between replicates meaning that traditional statistical techniques underestimate the threshold at which biological effects occur. Though this is expected in a natural, complex community, this no doubt affects the power of the statistical test. This could be overcome by increasing replication; however this would also greatly increase sample processing time (which may compromise the data quality, i.e. if day 0 samples were incubated with antibiotic for a prolonged time period before being processed), as well as data generation costs (increase number of samples requiring qPCR analysis).

This problem does not apply to the growth rate assay as during the exponential growth phase, samples within each treatment exhibit, in relative terms, extremely low variability (Figures 31 - 37, which show standard deviation). Furthermore, if additional replicates were required, this could be easily be achieved with the growth based assays, which are conducted in 96 well plates. In this chapter, six replicates were sufficient to generate LOECs that were highly comparable to MSCs derived from long term assays. Using the percentage of average differences as opposed to actual differences for generating Bland-altman plots would have been preferable as the measurements spanned a large concentration range (Giavarina, 2015); however, percentage differences were not normally distributed and so were unusable. Log transforming the original measurement data in this case was preferable, as the differences were then normally distributed, and the large disparity between concentration measurements was adjusted.

Growth based assays are simple, quick and cheap. They lend themselves to validation according to OECD guidelines (OECD, 2005) to be used in ERA, as the lack of time, money and specialist equipment required facilitates inter-

laboratory testing, and high reproducibility. Growth data can be generated and analysed for a minimum of 2 compounds per day, meaning MSC data could be rapidly produced for ERA. In time, the assay could be further developed to test different bacterial communities, though these would need to be individually optimised. This was shown with the pilot effluent experiment, which likely due to the reduced bacterial density at the start of the experiment required longer to exhibit the strongest dose response relationship (at 9 hours instead of at 6). Critically the effect concentrations derived using these two different time points were the same, suggesting the assay is likely to be reproducible with a range of complex community inocula. Further experiments are required to confirm that this approach will always yield the same LOEC. In addition, the dose response relationship in the effluent extended over the majority of the duration of the assay, indicating effluent could be a better inoculum were this assay standardised to a single complex community. However, without further experiments, it is unknown whether this is a cell-density or community effect. Also noted was that, in some cases, growth of the complex community was higher towards the end of the experiment in the presence of the antibiotic than without (for CLA, ERY and TAX influent). This suggests adaptive shifts in the community, and or utilisation of degraded antibiotics as a carbon source.

To overcome the issue of the growth phases emerging at different time points, the starting density of the assay could be standardised; or pilot assays could be used to pinpoint the exponential growth phase and then the most significant dose-response relationship determined, to be used as the assay 'endpoint'. The latter is recommended, particularly if the assay were to be developed to be more environmentally relevant (e.g. through reducing incubation temperature, or nutrient content); as slower growing communities would likely be favoured by these conditions so the 6 hour time point is unlikely to be transferable. Indeed, the simple pilot experiment comparing the growth of influent and effluent showed that growth of the effluent based community was better in lower temperature and lower nutrient than the influent which may be due to adaptation to lower environmental temperatures.

Finally, another strength of the growth based assay compared to the long term experiments is that it does not limit the MSC estimation to a particular gene, or gene class. As it examines phenotypic differences in growth rate, this will capture any competition and selection occurring for all the available resistance

genes and mutations present in that community. This is in keeping with the traditional definition of a MSC – defined as the point at which there is reduction in growth rate of susceptible bacteria (Figure 3 chapter two, taken from Gullberg et al. (2011)).

The growth based LOECs could also be used to inform the concentration ranges for future, long term experiments. The MSCs for AZ and ERY are predicted to be between 42 and 1000 µg/L and 1053 and 25,000 µg/L, respectively – when using *ermF* as the gene target. The upper boundary is the growth based LOEC, and the lower boundary is this value divided by the average fold difference (23.75) between the growth LOEC and MSC for all compounds in this study (Table 8). Long term experiments should be performed to elucidate the accuracy of these estimations.

### 3.5. Conclusions

For the first time, different methods for determining antimicrobial MSCs and LOECs in the same experimental system have been directly compared. Data was generated for a range of antimicrobials spanning different drug classes; through assessing both genotypic effects (target gene prevalence) and phenotypic effects (growth based assay). Genotypic data was used to derive MSCs and LOECs, and phenotypic (growth based) data was also used to derive LOECs. All methods were compared using Bland-Altman plots to assess the level of agreement between different methods, which was high.

This study indicates a real risk of *in situ* selection occurring in the environment, (based on RQs calculated from MSCs or $PNEC^{R}$s, and MECs) for certain compounds, or in certain environmental hotspots, such as hospital effluent. Further work should continue to determine MSCs and RQs for selection by different compounds, and the environmental applicability of the tests should be assessed.

Repeatedly, it has been shown there are no rules which can be applied to all antimicrobial compounds, which can be used to accurately estimate MSCs - they will need to be determined on a case by case basis. Growth based assays are shown here to be a rapid, simple and cheap method to reliably estimate MSC data, which are currently severely lacking.

# Chapter four: Using PCR and next-generation sequencing to study class 1 integron array diversity under selective pressure

## 4.1. Introduction

### 4.1.1. Integrons

Integrons are genetic backbones which often carry multiple antibiotic resistance gene cassettes in 'arrays' (Partridge et al., 2009). Most gene cassettes do not carry their own promoter, which affects expression of the genes in the array. Class 1, 2 and 3 integrons contain a Pc promoter in *intI*, which promotes expression of downstream gene cassettes with decreasing expression the more distal the gene (Partridge et al., 2009, Hall, 2012). Infrequently, a gene cassette can mobilise into a plasmid or other DNA without the *attI* site being present, but the gene will remain unexpressed unless its cassette also carries a promoter, or its new location is downstream of a promoter (Hall, 2012). Integrase expression can be induced by antibiotics (Baquero et al., 2013) sometimes under the control of the SOS response, which is also induced by low concentrations of antibiotics (Guerin et al., 2009, Guerin et al., 2010, Guerin et al., 2008). This means novel (to the host) resistance gene cassettes can be acquired or pre-existing resistance cassettes can be reshuffled nearer to the promoter, increasing their expression. Conversely, reshuffling of resistance cassettes away from the promoter allows genes to be silenced (i.e. to reduce fitness cost), which may be another mechanism whereby resistance may be maintained in the complete absence of, or in presence of only low concentrations, of antibiotics (Guerin et al., 2009).

Little is known about class 1 integron expression gene cassette array dynamics, particularly in a complex microbial community, or when exposed to sub-lethal, antimicrobial concentrations. These antimicrobial concentrations are becoming more relevant to the study of antimicrobial resistance, owing to an increasingly large body of work indicating that selection for antibiotic and antimicrobial resistance can occur at very low antimicrobial concentrations at the chromosomal and plasmid level (Negri et al., 2000, Negri et al., 2002, Gullberg et al., 2014, Gullberg et al., 2011). The lack of understanding of integron diversity and gene cassette array plasticity is in part due to a lack of suitable methods, discussed further below. The aim of this study was to design a novel method to

study gene cassette diversity and their arrangement in class 1 integrons harboured by bacteria within a complex bacterial community, and whether these are affected by different selective pressures.

### 4.1.2. Class 1 integrons

Class 1 integrons comprise of a 5' conserved sequence (CS) which includes the integrase (*intI1*) gene, *attI* site and the Pc promoter; and a 3'CS which often includes a *qacEΔ1* gene, *sul1* gene and *orf5* (an ORF with unknown function (Canal et al., 2016)). Integration of free gene cassettes is performed by *intI*1 between the *attI* site on the integron, and the *attC* site (sometimes known as the 59 base element (Mazel, 2006)) present on the gene cassette. The gene cassette closest to Pc is most strongly expressed; the gene cassette furthest downstream in the array is expressed the least (Partridge et al., 2009). 'Complex' class 1 integrons have also been described, which can harbour additional resistance gene cassettes between insertion sequences and partial copies of the 3' CS (Partridge et al., 2009).

Class 1 integrons are often described as being good indicators of anthropogenic impact, pollution and presence of antibiotic resistance genes; and can be found both in bacteria in the clinic and in natural environments (Berglund et al., 2014, Kotlarska et al., 2014, Gillings et al., 2009, Gaze WH, 2011, Gaze et al., 2005, Abella et al., 2015b, Gillings MR, 2008, Gillings et al., 2015, Amos et al., 2015, Jechalke et al., 2013). Class 1 integrons are usually more abundant than other integron classes (Stalder et al., 2013) and are therefore key vectors for the transmission of antimicrobial resistance, both within and between natural and man-made environments as they can integrate a number of genes conferring resistance to both antibiotics and biocides. The class 1 integrons have therefore been chosen for investigation in this study.

### 4.1.3. Previous methods studying integron gene cassette diversity

Understanding the impact of different selective pressures on integron prevalence, gene cassette diversity and gene cassette location (in relation to Pc), is critical for understanding the mobilisation of antimicrobial resistance genes from the

clinic to the environment, and *vice versa*. However, both gene cassette diversity and location are still relatively understudied due to several methodological issues.

Class 1 integrons are at relatively low abundance in the metagenome (so study by shotgun metagenomics often does not have enough depth). Primer walking (with Sanger sequencing) could be used to determine downstream primer sites, but this is slow, laborious and is not high-throughput enough to ensure the target is representative of the metagenome. Integrons cannot be fully amplified by conventional PCR without bias for two main reasons. Firstly, potentially very long gene cassette arrays could be present. These often contain many repeats (Partridge et al., 2009) which could compromise amplification. Second, using specific primers (based on *a priori* knowledge of downstream sequences) results in amplifying the same type of integron repeatedly introducing significant bias, meaning much diversity could remain uncaptured.

Despite these caveats, integrons and associated cassette diversity have generally been investigated with PCR. There have been several studies which have used the primers developed by Levesque et al. (1995), which target the 5' and 3' CS's in class 1 integrons (Canal et al., 2016, Stalder et al., 2013, Zhang et al., 2009a). However, the length of these amplicons was only approximately 1600 bp, and single colonies were used as the PCR template (Zhang et al., 2009a, Canal et al., 2016). Therefore only 'simple' class 1 integrons were studied, and within that group, only a few gene cassettes captured. Further bias arises from targeting gene cassettes from a small subsample of bacterial isolates, as opposed to the whole community. Indeed, the integron-bearing colony may be multidrug-resistant, but the gene cassettes found may only confer resistance to a few compounds (Canal et al., 2016). While this could be due to resistance genes being harboured elsewhere (e.g. chromosomally, on plasmids or within other integron classes), it could also indicate incomplete capture of gene cassettes.

Another primer pair was developed by Stokes et al. (2001) which targets the 59 base element (*attC* site) on gene cassettes. This allowed characterisation of gene cassettes within total environmental DNA, a significant improvement on PCR performed on individual isolates. However, this is still likely to bias for shorter gene cassette arrays due to competition during PCR amplification. This approach therefore still does not fully capture all gene cassette diversity (Stokes et al., 2001) and no information on integron class or location within the array is

generated. Since the original publication, amplicons generated using the 59 base element primers have been sequenced with Illumina (similar to a 16S amplicon sequencing approach). This newly developed, high-through put method has been used to characterise gene cassette function in different environments, and relate this to anthropogenic impact (Gatica et al., 2016).

Another method was developed recently which makes use of the next-generation sequencing (NGS) platform developed by Pacific Biosciences (PacBio) and PCR. Inverse PCR was used to amplify outward from selected antibiotic resistance genes, to determine their mobilisation potential by searching for mobile genetic elements such as integrons (Pärnänen et al., 2016). While this method overcomes the issues arising from lack of depth when using metagenomics, this is not a complete picture of gene cassette diversity as resistance genes are selected *a priori*. Additionally, the method is not integron specific.

## 4.2. This study

This study aimed to develop a novel, unbiased PCR method to amplify gene cassette arrays present in class 1 integrons. This method is theoretically unbiased due to the use of unspecific, downstream primer sites. Long-range PCR was used to generate long amplicons. Amplicons were sequenced using PacBio NGS technology, to sequence amplicons in a single read.

### 4.2.1. A novel PCR approach: FUN-PCR

FPNI (fusion primer and nested integrated) PCR is a method that was developed for rapid chromosome walking, as it can be used to walk from a region with known DNA sequence to a region of unknown DNA sequence. The basic premise is the use of a forward primer based on a known DNA sequence, and a mixture of random, degenerate reverse primers (which also contain a known sequence at the 5' end, the 'fusion' sequence). These are used in a PCR reaction with high and low stringency cycles to allow amplification of specific product from the forward primer; and a mixture of unspecific and specific product from the reverse primers. Subsequent rounds of nested PCR based on the known, specific regions amplified with the forward primer, and the conserved regions ('fusion' regions) in

the reverse primers eliminate non-specific products (Figure 52 (Wang et al., 2011)): “In the first PCR step, single stranded copies of the target template are generated in the high stringency cycles, and double stranded products are produced in the low stringency cycle (in total, involving 3-5 repeated PCR cycles); in this primary step, amplification of the target products is likely to be accompanied with other, nonspecific, products. In the secondary and tertiary PCR steps (nested PCR), the target DNA is exponentially amplified by the gene specific and adaptor specific primers, while non-target genes are not amplified because there is no corresponding gene specific primer (and/or amplification was suppressed by the stem-loop structure of the DNA).” In the current study, FPNI PCR was modified using reverse primers based on Type II DNA restriction sites. This method has been termed FUN (Fusion Unspecific Nested) -PCR.

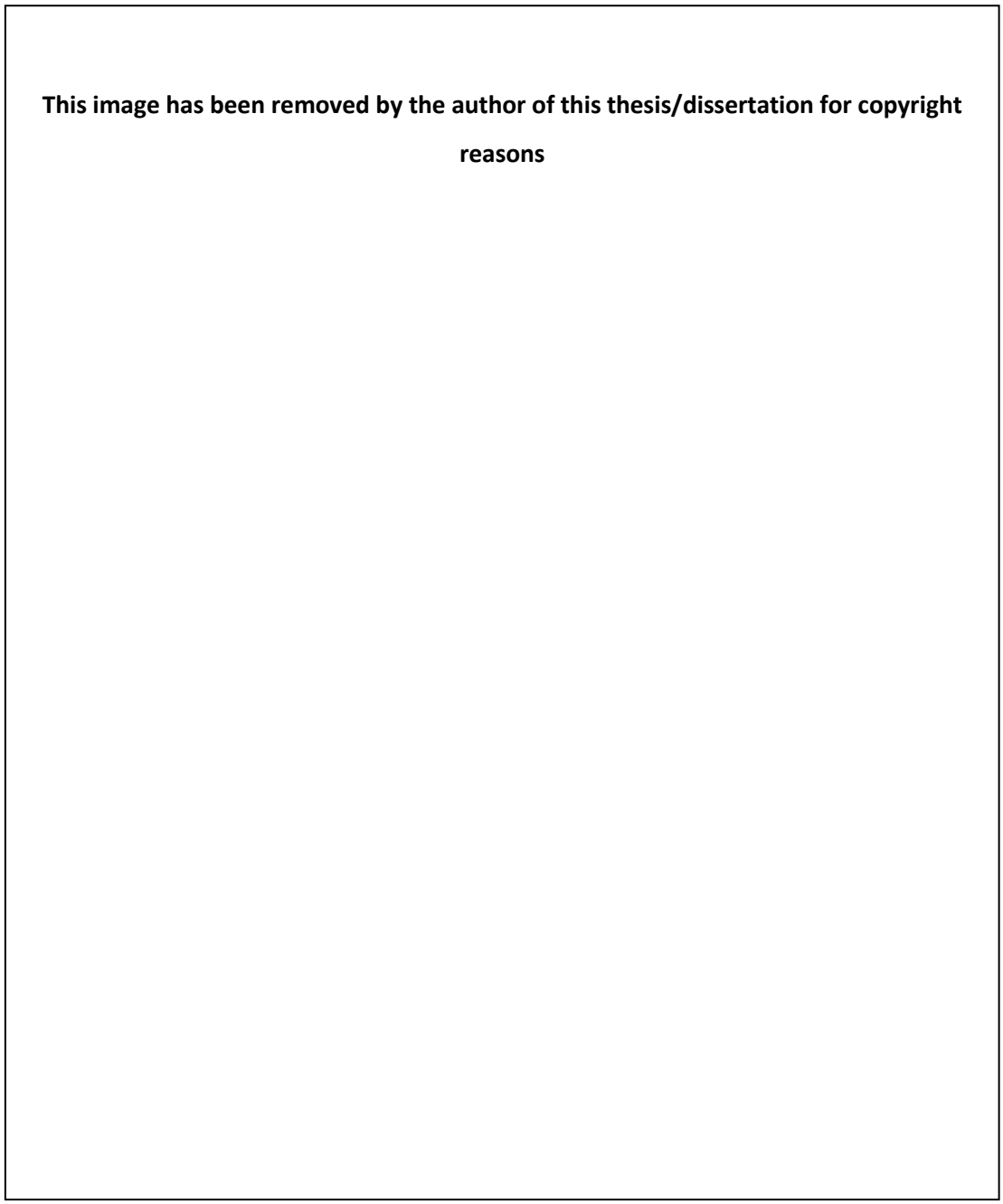


Figure 52. Figure 2 taken from Wang et al. (2011). “A general (theoretical) scheme for FPNI-PCR (PCR based method for genomic walking or tagged flanking sequence cloning).”

Type II restriction sites have been used for recombinant DNA technology in molecular biology since the discovery of the first restriction enzyme HindII in 1970 (Loenen et al., 2014). Restriction sites are conserved DNA sequences ranging from 4 bp to 9 bp in length. The length of the restriction site is linked to how frequently the site will appear in any given DNA sequence; therefore the longer the restriction site, the less likely and therefore less frequently it will

appear. Here, a range of different reverse primers containing different length restriction sites (and a conserved 'fusion' sequence for subsequent rounds of nested PCR) were tested, and compared to the use of a primer used previously targeting the *qacEΔ1* gene (Gaze et al., 2011). Following subsequent rounds of nested PCR, amplicons were sequenced using SMRT (Single Molecule Real-Time) sequencing developed by PacBio.

### 4.2.2. Use of PacBio NGS

PacBio sequencing is a NGS method that offers an average read length of > 10,000 bp, an N50 of over 20,000 bp and a maximum read length of in excess of 60,000 bp (Rhoads and Au, 2015). This is useful for investigating integron diversity, as longer read length will be unaffected by long stretches of repeats, which are often found in integrons (Partridge, 2009). Additionally, larger gene cassette arrays maybe be sequenced in a single read. Though PacBio sequencing has lower accuracy than other NGS methods (the error rate is 11 – 15 %), this can be mitigated through increased coverage, by repeatedly sequencing smaller length reads (Rhoads and Au, 2015).

### 4.2.3. Studying the effects of different selective pressures

In this study, amplicons around 1 – 5 kb in length generated by FUN-PCR underwent PacBio sequencing. The DNA template was from an experimental evolution experiment where a natural, complex, bacterial community (from untreated waste water) was exposed to sub-lethal concentrations of different antimicrobials (two antibiotics and one biocide) in daily passage experiments for 7 days. Selective compounds were the quaternary ammonium compound (QAC) benzalkonium chloride (BKC), and two antibiotics trimethroprim (TRMP) and ciprofloxacin (CIP) which have been classed as 'essential' antibiotics by the WHO (WHO, 2017). Further rationale for selecting these compounds in terms of environmental and clinical relevance can be found in chapter three. This experimental system is based on the previous studies designed for determination of minimal selective concentrations (MSCs) for several different antibiotics (see chapters two and three).

### 4.2.4. Author contributions

I applied successfully for the MRC Clinical Infrastructure pilot project, grant number: MR/M008924/1 which covered costs for PacBio SMRT cells and staff time at Exeter Sequencing Service (ESS) to prepare libraries, sequence and perform initial quality analysis of sequence data (equating to ~£2000).

I designed and performed the evolution experiment and subsequent sample preparation. Lihong Zhang selected the restriction enzyme sites and advised on primer design. I designed all primers and performed all PCR optimisation steps and final PCRs; as well as DNA preparation and quality control. Library prep, size fractionation and sequencing was overseen and undertaken by Karen Moore and Jeremie Poschman of ESS.

Demultiplexing of sequences was performed by Paul O'Neill of ESS. I designed the sequence analysis pipeline, performed all sequence analysis and data analysis.

## 4.3. Materials and Methods

### 4.3.1. Evolution experiment

Untreated waste water collected from Falmouth sewage treatment plant (serves population of 43,000), Cornwall, UK in October 2015 was used as the natural, complex bacterial community sewage inoculum. This was washed by spinning down and resuspension in equal volume 0.85 % sterile saline twice before use. Iso-sensitest broth (Oxoid) was inoculated with a 100x dilution of this suspension and split into 3 replicates for each compound, and for the no treatment control.

Selective compounds used were BKC, CIP and TRMP at half the clinical breakpoint concentrations (EUCAST, 2014) or MIC (see chapter five for BKC MIC) for *Enterobacteriaceae*, equating to 8 mg/L, 0.5 mg/L and 2 mg/L respectively. The microcosms were then incubated overnight at 37 °C, shaking at 180 rpm.

Each day a 100x dilution of the overnight culture was inoculated into fresh media containing the same selective compound. This was repeated for a total of 6 days. On the 7$^{th}$ day, 500 µl overnight culture was spun down at full speed for

2 minutes and resuspended in equal volume of 20 % glycerol, and stored at -80 °C.

### 4.3.2. DNA extraction

Total DNA was extracted from each replicate and 1 ml of the untreated waste water inoculum using the MoBio ultraclean kit, according to instructions but with the initial spin extended to 3 minutes. All DNA was stored at -20 °C until use.

### 4.3.3. Primer design and PCR conditions

All primers were synthesised by IDTDNA. Forward primers were based on the *intI1* sequence (NCBI reference sequence NG_039604.1), and reverse primers comprised of a restriction site plus fusion primer sequence (for round 1 PCR); or two fusion sequences for nested PCR (rounds 2 and 3). Primers for barcoding comprised of the last nested primer and a unique barcode for PacBio sequencing, available online (https://github.com/PacificBiosciences/Bioinformatics-Training/blob/master/barcoding/pacbio_384_barcodes.fasta).

Table 11 contains a full list of primer sequences. All PCR reactions were performed with LA Taq enzyme (ClonTech) and using the cool start method (on ice), with optimal conditions based on gradient PCR. The reaction for the first PCR was as follows: 0.5 µl LA Taq, 8 µl dNTPs, 5 µl Buffer ($MgCl_2$ plus), 2.5 µl IntI14 primer (10 µM), 2.5 µl reverse primer (10 µM), 5 µl template and sterilised water to a final volume of 50 µl. The programme was: 94 °C 1 min, two cycles of 98 °C 10 sec, 62 °C 30 sec, 72 °C 15 min followed by 1 cycle of 98 °C 10 sec, 25 °C 2 min, 72 °C 15 min for a total of 6 cycles; followed by 98 °C 10 sec, 62 °C 30 sec, 72 °C 15 min for two cycles and then a final extension at 72 °C 15 min.

The second PCR reaction was as above, but in a 20 µl reaction with IntI13 as forward primer (10 µM) and FSP1 as reverse primer (10 µM). 1 µl of PCR product from PCR 1 was used as template. The programme was as follows: 94 °C 1 min, 30x (98 °C 10 sec, 52 °C 30 sec, 72 °C 15min), 72 °C 15 min.

The final nested PCR reaction used 1 µl of gel purified product sized 1.5 – 10 kb (NucleoSpin) from PCR 2 diluted 100x, in a 20 µl reaction as above with

IntI5 as forward primer (10 µM) and FSP2 as reverse primer (10 µM). The programme was as above for round 2, except the annealing temperature was increased to 55 °C.

The final PCR for barcoding ready for library preparation and to allow pooling of samples for sequencing was either a 20 or 50 µl reaction as above, but with forward and reverse primer volume doubled to 2 µl or 5 µl each, respectively. 1 µl or 2.5 µl of gel purified product (1.5 – 10 kb in size) from PCR 3 diluted 10x or 100x to a final reaction concentration of 1 – 5 ng per 20 µl reaction was used as template.

Following gel electrophoresis verification on a gel stained with ethidium bromide, the final PCR product was cleaned up with Zymo Clean and Concentrate kit into a final volume of 10 µl, according to instructions but with the following amendments: all spins were reduced to 13,000 x *g* (to reduce shearing), an additional wash step and additional drying step of 1 min were introduced (to reduce carry over of contaminants, salts etc); and the filter was incubated for 2 minutes after the addition of elution buffer before the final spin (to increase recovery). This then underwent a second clean and concentrate step by repeating the previous procedure. All products immediately underwent QC analysis before being pooled according to DNA concentration and frozen at -20 °C. Samples remained frozen until sequencing.

Table 11. Full list of primers used in this study, their sequence, gene/primer target, approximate product size in kb, and reference.

| Primer | Sequence | Target | Approx. product size (kb) | Reference |
|---|---|---|---|---|
| IntI4 | CATCACGAAGCCCGCCACA | *intI1* gene | - | This study |
| FP1-BbvC1-1 | GTAATACGACTCACTATAGGG CACGCGTGGTNNNNNNNNCC TCAGC | BbvC1 site | | This study, (Wang et al., 2011) |
| FP2-Bsu361-1 | GTAATACGACTCACTATAGGG CACGCGTGGT NNNNNNNN CCTNAGG | Bsu361 site | | This study, (Wang et al., 2011) |
| FP3-BamH1-1 | GTAATACGACTCACTATAGGG CACGCGTGGTNNNNNNNNGG ATCC | BamHI site | | This study, (Wang et al., 2011) |
| FP5-Taq1-1 | GTAATACGACTCACTATAGGG CACGCGTGGTNNNNNNNNNN TCGA | TaqI site | | This study, (Wang et al., 2011) |
| QacEcom1r | CCGACCAGACTGCATAAGCA | *qacE* gene | | (Gaze, et al., 2011) |

| | | | | |
|---|---|---|---|---|
| IntI3 | TTCGCGACGGCCTTGC | *intI1* gene, nested | - | This study |
| FSP1 | GTAATACGACTCACTATAGGGC | Primer, nested | Variable | (Wang et al., 2011) |
| IntI5 | CAGCGGTTACGACATTCGAA | *intI1* gene, nested | - | This study |
| FSP2 | ACTATAGGGCACGCGTGGT | Primer, nested | Variable | (Wang et al., 2011) |
| Barcode Forward | e.g. TCAGACGATGCGTCATCAGC GGTTACGACATTCGAA | Primer, nested | ~5 - 10 | This study |
| Barcode Reverse | e.g. GCAGAGTCATGTATAGACTAT AGGGCACGCGTGGT | Primer, nested | ~5 - 10 | This study |

### 4.3.4. Quality control and PacBio sequencing

All final PCR products were quantified using the QuBit fluorometer (2.0, Thermo Scientific) and 260/280 and 260/230 ratios determined with Nanodrop (Thermo Scientific). Concentrations ranged from 650 ng/µl to 1,630 ng/µl. All 260/280 ratios were between 1.88 and 1.90 and all 260/230 ratios were between 1.66 and 2.33. All replicates were pooled into a single sample based on DNA concentration to a total weight of 4.55 µg DNA per sample.

Library preparation and sequencing was carried out by ESS. Sample was first size fractionated by SAGE-ELF into a smaller (1 – 5 kb) and larger (5 kb plus) fraction. The smaller fraction was sequenced successfully, but the larger fraction could not be recovered and therefore there is no data for the larger amplicons.

### 4.4.4. Sequence analysis pipeline

All sequences were demultiplexed by Paul O'Neill at ESS. All subsequent sequencing analysis was performed in a unix environment (Ubuntu).

First, the average length of reads for each fastq file was determined:

```
$ awk 'NR%4==2{sum+=length($0)}END{print sum/(NR/4)}'input.fastq
```

Then fastq files were converted to fasta files, and the number of reads counted:

```
$ sed '/^@/!d;s//>/;N' sample1.fastq > sample1.fasta

$ grep –c '>' sample1.fasta
```

ABRicate (Seeman, 2017) (version 0.4) was used to identify reads containing *intI1* which could then be binned into a new fasta file. ABRicate uses BLAST (version 2.2.30) software to BLAST reads against all the main antibiotic resistance gene databases individually – Resfinder, ARG-ANNOT, CARD and NCBI (as well as PlasmidFinder and VFDB (Virulence Factor Database)). The output is a tab delimited file containing the name of the read with the hit, the start and end of the hit within that read, the name of the resistance gene, % coverage, % identity, and the database the hit is matched to. Personal databases can also be created and used with ABRicate. To determine the number of reads within each file which contained *intI1*, I created a database 'class1' which contained a single fasta sequence of a class 1 integrase, truncated to the be the same size as the expected amplicon sequence. The original fasta sequence used for the design of the PCR primers was removed from the NCBI site (05/07/17), so another sequence was used in its place (M569736.1). This was confirmed with MEGA6 (Tamura et al., 2013) to contain the primer sites and have 100 % identity to the previous sequence for the amplicon derived following all rounds of nested PCR. The fasta file was in the format: ">database~~~~geneID~~~~accession gene details" followed by the nucleotide sequence, as specified on the github website for ABRicate. The commands for creating a database were as follows:

```
$ cd /path/to/databases

$ mkdir class1

$ cd class1

$ cp /path/to/fasta/file/class1.fasta sequences

$ makeblastdb –in sequences -title tinyamr -dbtype nucl -
parse_seqids -hash_index
```

Reads with one or more integrase hits were outputted into a tab delimited file:

```
$ abricate --db class1 example.fasta > example.intI1hits.tab
```

This tab delimited file was sorted in excel by the start position of the integrase gene hit. The read names containing *intI1* hits which started within the first 75 bp of the sequence were saved into a new csv file. The 75 bp was selected as it is half of the maximum expected, known amplicon sequence (150 bp); and 25 amino acids (i.e. 75 nucleotides) has been cited as being the minimum coverage

required for a hit with > 90 % identity to a known resistance gene (Yang et al., 2013). Cut offs of > 80 % coverage and > 80 % identity were also applied. The lower identity was used to account for the higher error rate of PacBio of 11 – 15 % (Rhoads and Au, 2015).

The reads names were then extracted from the csv file into a text file. This was used to bin only those reads containing *intI1* from the original fasta file into a new fasta file:

```
$ grep $'beginning_of_reads_names*' sortedhits.csv >
readnames.txt

$ perl -ne 'if(/^>(\S+)/){$c=$i{$1}}$c?print:chomp;$i{$_}=1 if
@ARGV' readnames.txt original.fasta > intI1filtered.fasta
```

For identifying resistance genes, *intI1* filtered fasta files underwent BLAST against the ARG-ANNOT and CARD databases using ABRicate. CARD was chosen as the largest database which included resistance mutations (McArthur et al., 2013), and ARG-ANNOT on the basis it has been shown to generate a greater numbers of hits, particularly for unknown (previously identified) resistance determinants (Gupta et al., 2014).

```
$ abricate --db argannot intI1filtered.fasta > arghits.tab

$ abricate --db card intI1filtered.fasta > cardhits.tab
```

A secondary database was created (see above) to BLAST for the *qac* resistance genes. Nine *qac* genes in total were included in the database, including the genes listed in the review on integron cassettes by Partridge et al. (2009), and two more recent reviews on *qac* genes (Jaglic and Cervinkova, 2012, Wassenaar et al., 2015). Only genes with full cds were used. The genes and accession numbers were as follows: *qacA, qacB, qacC, qacE, qacE2* (also known as *qacG* or *qacG2* (Partridge et al., 2009)), *qacEdelta1, qacF, qacH* (also known as *qacI* (Partridge et al., 2009)), and *qacJ* - corresponding to the accession numbers NG_048037.1, AF053772.1:1144-2688, U15783.1:1931-2254, U67194.4:33897-34229, KF856624.1:29665-30012, AF034958.3:2039-2371, AF205943.1:1374-1706 and NG_048046.1:101-424, respectively. Filtered files were searched for *qac* genes using the ABRicate command above, with the database specified as ‘qac’.

The tab files were then sorted in excel. Hits were filtered based on length (nucleotide sequences ≥ 75, based on the previously published cut off of ≥ 25

amino acids (Yang et al., 2013b)) and % identity (≥ 80 % nucleic acid identity, reduced slightly from the 90 % amino acid identity cut off published previously (Yang et al., 2013b)). This was again lowered to account for the higher error rate of PacBio sequencing, of around 11 – 15 % (Rhoads and Au, 2015).

### 4.4.5. Sequence data analyses

The percentage of *intI1* filtered reads containing resistance genes for each database was calculated for each file, and averaged across antimicrobial treatments. Percentage of *intI1* filtered reads containing each type of resistance gene (for each resistance gene database) were also calculated for each file, and averaged across antimicrobial treatments.

Heatmaps were generated using the python packages pandas (McKinney, 2010), matplotlib (Hunter, 2007) and seaborn (Waskom, 2016). Statistical analyses were performed in R studio (RStudio, 2015).

## 4.5. Results

### 4.5.1. PCR optimisation and final primer selection

Initially, all reverse primers (FP1-BbvC1-1, FP2-Bsu361-1, FP3-BamH1, FP5-Taq1-1 and QacEcom1r) were tested, with the two 7 bp restriction site primers (FP1-BbvC1-1 and FP2-Bsu361-1) mixed 1:1, using the untreated waste water DNA as template. The low stringency cycle's annealing temperature was set to a gradient between 25 °C and 50 °C, in increments of 5 °C. Products from these reactions then underwent PCR reaction 2, where the annealing temperature was tested at 52 °C and 57 °C. Gel electrophoresis showed successful amplification for the FP1-BbvC1-1 and FP2-Bsu361-1 combination at 30 °C then 52 °C; and FP5-Taq1-1 and QacEcom1r primers at 30 °C and then 52 °C for the first and second PCRs, respectively. FP5-Taq1-1 also yielded a product at 30 °C and then 57 °C for the first and second PCRs, respectively.

Product from PCR 2 around 7 kb in size (+/- 3 kb) was gel purified then diluted 100x and used as template in PCR 3. Here, the brightest band was for the FP1-BbvC1-1 and FP2-Bsu361-1 combination so this was selected to be used

for amplification of all the evolution experiment replicates (Figures 53 and 54 for the round 2 and round 3 PCR products).

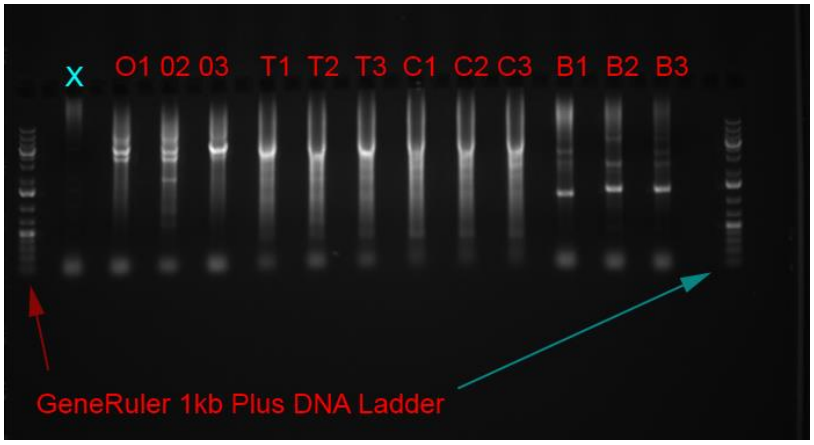


Figure 53. PCR 2 amplification products using 7 bp cutter primers FP1-BbvC1-1 and FP2-Bsu361-1 in combination for the evolution experiment replicates (0 = no treatment, T = trimethoprim, C = Ciprofloxacin, B = BKC). A 1 % agarose gel stained with ethidium bromide run at 120 V for 40 minutes, with GeneRuler 1 kb Plus DNA Ladder (ThermoScientific). 5 µl sample with 1 µl DNA loading dye (ThermoScientific).

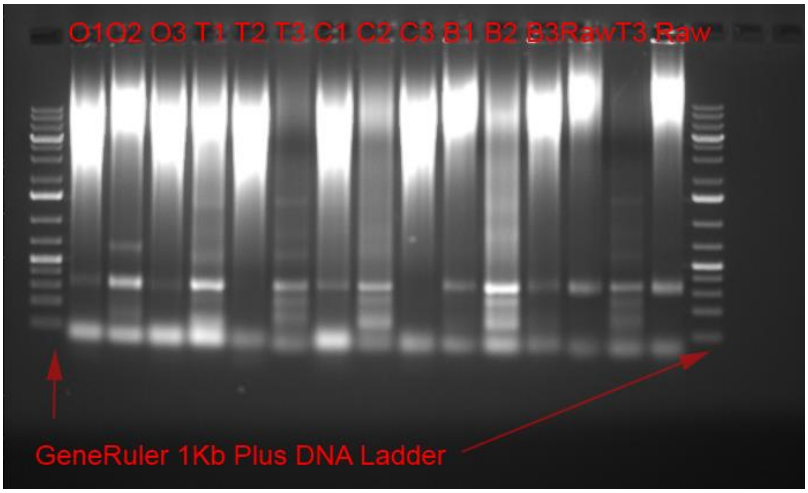


Figure 54. Barcode PCR amplification products using the 7 bp restriction site primers FP1-BbvC1-1 and FP2-Bsu361-1 in combination, for the evolution experiment replicates (O = no treatment, T = trimethoprim, C = Ciprofloxacin, B = BKC). A 1 % agarose gel stained with ethidium bromide run at 120 V for 40 minutes, with GeneRuler 1 kb Plus DNA Ladder (ThermoScientific). 5 µl sample with 1 µl DNA loading dye (ThermoScientific).

### 4.5.2. PacBio sequencing QC

The first two SMRT Cells bearing the smaller size (~1 – 5 kb) samples were successfully sequenced. The majority of reads of insert were around 1 kb in length, with a peak of around 7,000 and 15,000 reads at 1 kb for SMRT cells 1 and 2 respectively. The majority of reads of insert were also of high quality (Figure 55).

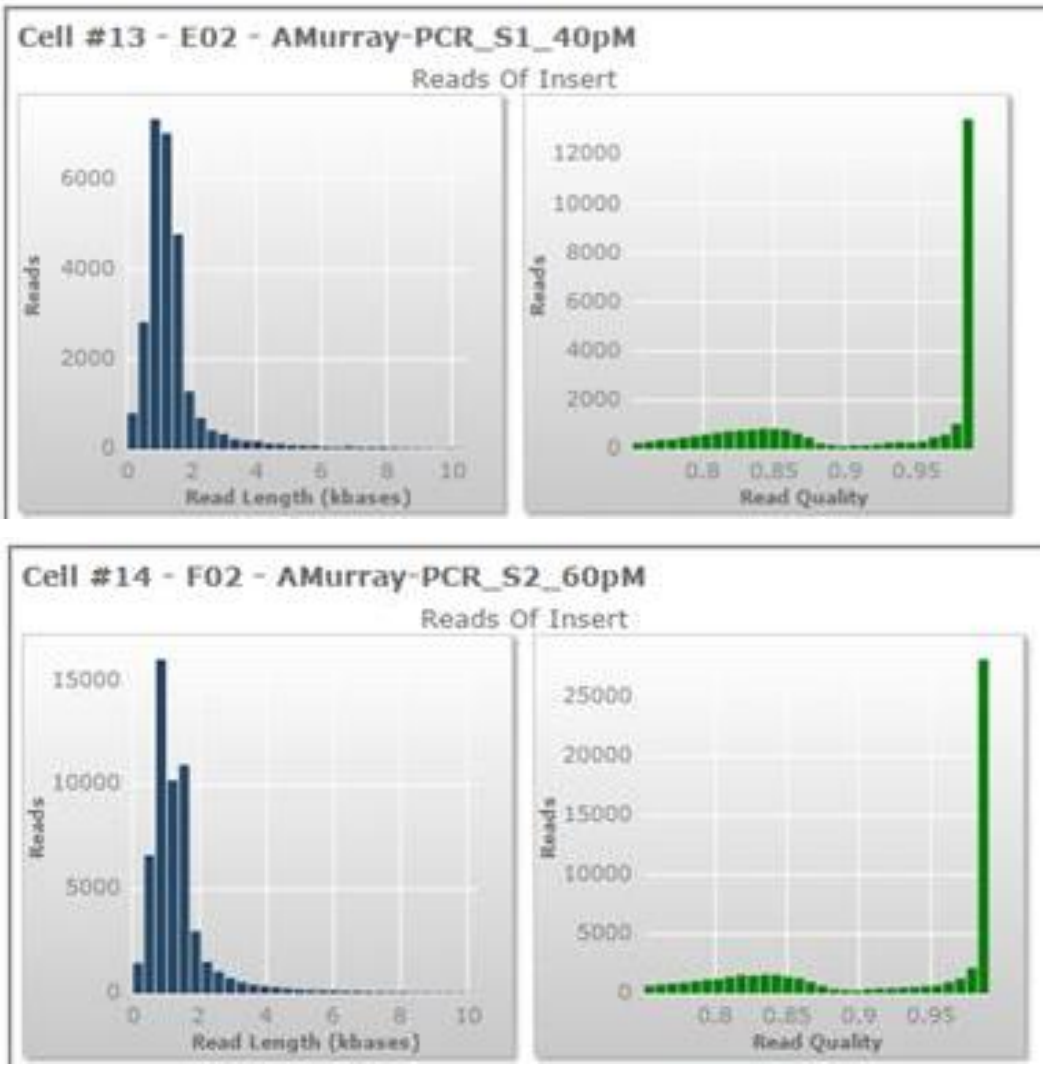


Figure 55. The average read length (left charts) for numbers of reads of insert and read quality (right charts) for SMRT cells 1 (top) and 2 (bottom).

### 4.5.3. *IntI1* detection

The average length of the reads for all treatments was 1,189 bp, with a maximum length of 2,449 bp and a minimum length of 940 bp. The breakdown of the numbers of reads at each stage of filtering can be seen in Table 12.

Table 12. Table showing the numbers of reads at each stage of intI1 filtering.

| **INT HITS** | | | | | | |
|---|---|---|---|---|---|---|
| Treatment | Replicate | No. reads | Int hits | Start < 75 | > 80 % coverage | > 80 % ID |
| BKC | 1 | 25025 | 21598 | 9101 | 8828 | 7992 |
| | 2 | 480301 | 361978 | 162515 | 144808 | 140775 |
| | 3 | 18000 | 13980 | 5871 | 5075 | 4930 |
| TRMP | 1 | 276583 | 238979 | 108309 | 100714 | 97879 |
| | 2 | 25882 | 22548 | 10120 | 9400 | 9127 |
| | 3 | 213261 | 192223 | 93150 | 88066 | 85755 |
| CIP | 1 | 58554 | 48305 | 22128 | 20411 | 19874 |
| | 2 | 288586 | 210441 | 93044 | 81653 | 79182 |
| | 3 | 15599 | 11981 | 5767 | 5293 | 5150 |
| Control | 1 | 5348 | 2230 | 746 | 626 | 605 |
| | 2 | 188369 | 122288 | 57068 | 50716 | 49631 |
| | 3 | 12982 | 12179 | 3077 | 2826 | 2738 |
| Raw | 1 | 17402 | 14615 | 5713 | 5308 | 5173 |

The average (n=3, except for the ‘Raw’ treatment, which was a single replicate only) number of reads containing an *intI1* hit within the first 75 bp of the start of the sequence, with greater than 80 % coverage and over 80 % identity to the expected amplicon sequence are shown in Figure 56. The CIP and TRMP treatment resulted in a significantly greater percent of reads containing *intI1* (Figure 56) compared to the no treatment control ($p$ = 0.007 and $p$ = 0.052, respectively, ANOVA with Tukey test).

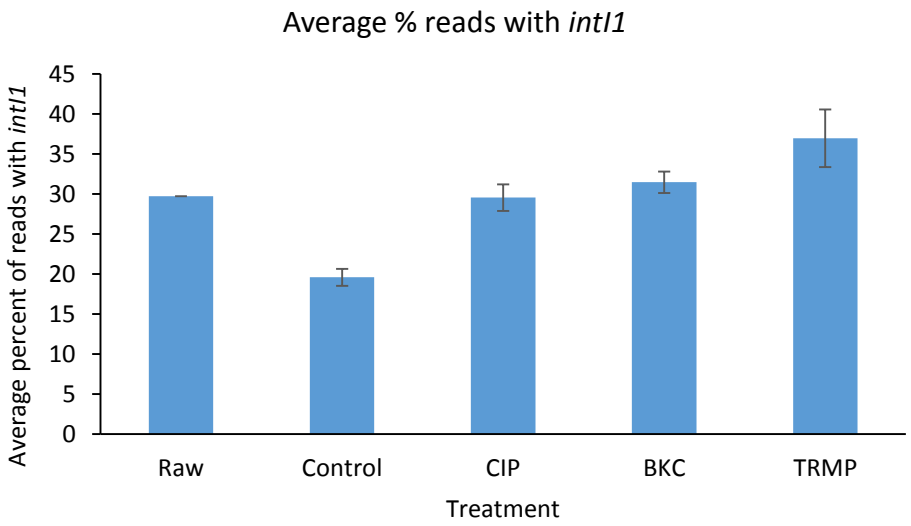


Figure 56. Average (n=3, except 'Raw' n=1) percent of total reads bearing hits for intI1 within the first 75 bp of the read, with a minimum of 80 % coverage and identity. Shown with standard error bars.

### 4.5.4. Antibiotic resistance gene detection with ARG-ANNOT and CARD

Resistance genes in the *intI1* filtered reads were identified by using ABRicate to BLAST against the ARG-ANNOT and CARD databases. Percentage of *intI1* filtered reads containing each gene hit were averaged within treatments. If the same gene was detected in both databases, these percentages were also averaged within treatments. Genes present in only one database are also discussed below.

The resistance genes detected by searching both ARG-ANNOT and CARD were *bla*$_{TEM}$, *mphA,* and *aad* (Figures 57 and 58). The β-lactamase gene *bla*$_{TEM}$ (Canton and Coque, 2006) had a relatively high prevalence compared to other detected resistance genes (though there were a greater number of hits with ARG-ANNOT than with CARD). The TRMP treatment had the highest prevalence, followed by the CIP and BKC treatments. All antimicrobial treatments had a significantly increased average percentage of *bla*$_{TEM}$ hits compared to the no treatment control ($p < 0.005$ for both CIP and TRMP, $p = 0.055$ for BKC, ANOVA with posthoc Tukey test). For *mphA,* exposure to BKC or CIP decreased *mphA* hits, whereas TRMP increased *mphA* hits (compared to the no treatment control – Figures 57 and 58). However, none of these differences were significant

(Wilcoxon Rank Sum tests). The aminoglycoside resistance gene *aad* (Partridge et al., 2009) was only detected following CIP exposure, and the percentage of *intI1* hits bearing *aad* was significantly different compared to the control ($p$ = 0.028, Wilcoxon Rank Sum test).

The most common cassette gene in *intI1* filtered reads was *catB* (found in the ARG-ANNOT search only), which encodes chloramphenicol resistance (Partridge et al., 2009). The no treatment control had the highest average percentage of *catB* hits, followed by the TRMP, CIP and BKC treatments (Figure 57). The differences between treatments were not significant (Kruskal Wallis).

The *strB* gene (detected only with the ARG-ANNOT search) encodes streptomycin resistance (Partridge et al., 2009). The number of *strB* hits decreased following culturing but increased persistence was observed following CIP exposure (Figure 58). Differences in the percentage of *intI1* hits bearing *strB* compared to the control were not significant for any of antimicrobial treatments (Kruskal Wallis test).

The *aph* genes encode aminoglycoside resistance (Partridge et al., 2009) and were only detected with the CARD search. *Aph* hits were greatest in the original inoculum but were almost completely lost following culturing (Figure 58). However, *aph* was enriched by antimicrobial exposure compared to the control, with the CIP exposure resulting in the highest percentage of *aph* hits (Figure 58). However, none of these differences were significant (ANOVA or Kruskal Wallis, as appropriate).

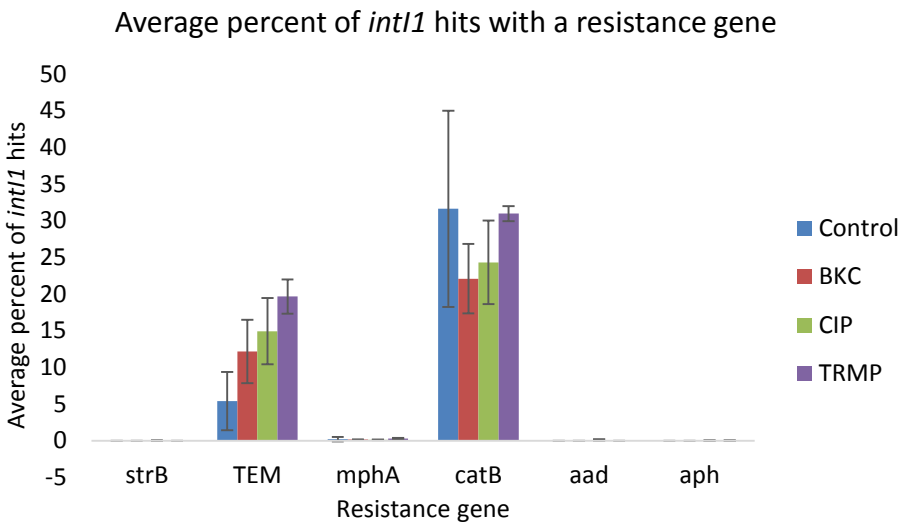


Figure 57. Average (n=6 for *bla*$_{TEM}$, *mphA* and *aad*, n=3 for *strB, aph* and *catB*) percent of *intI1* filtered reads containing different resistance genes hits of a minimum of 75 bp and 80 % identity, using both the ARG-ANNOT and CARD databases. Shown with standard deviation bars.

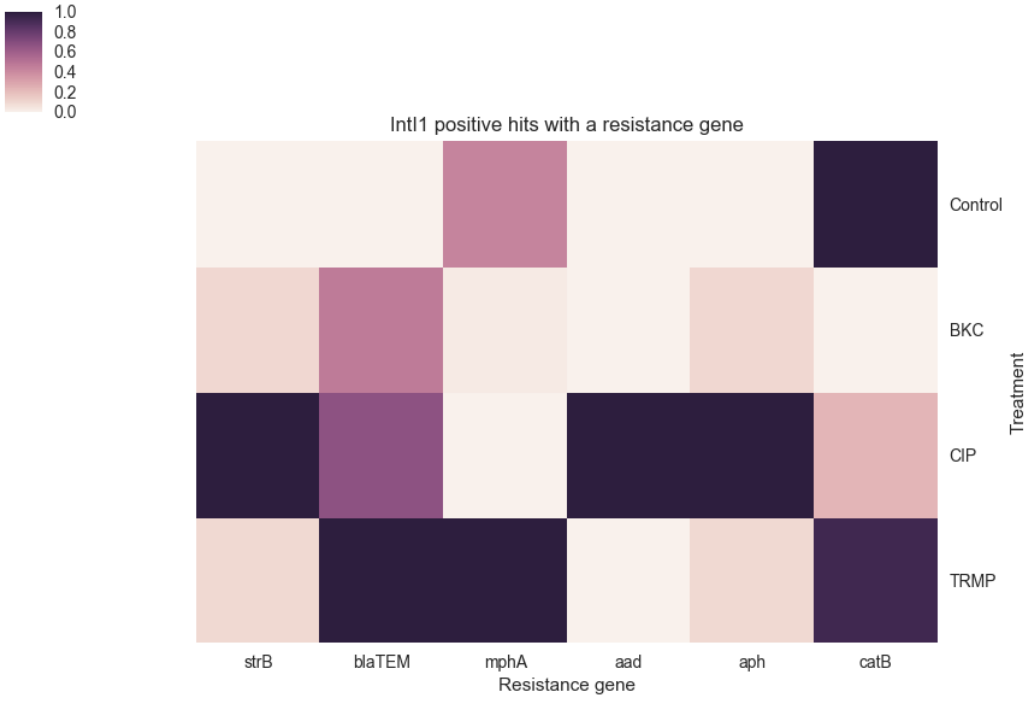


Figure 58. Average (n=6 for *bla*$_{TEM}$, *mphA* and *aad*, n=3 for *strB, aph* and *catB*) percent of *intI1* filtered reads containing different resistance genes hits of a

minimum of 75 bp and 80 % identity, using both the ARG-ANNOT and CARD databases. Average percentages are normalised within each resistance gene type (for each column, the minimum is subtracted from each value and then divided by maximum value).

Table 13. Average percentage (n=6 for *bla*$_{TEM}$, *mphA* and *aad*, n=3 for *catB*, *strB* and *aph*) of *intI1* filtered reads with different resistance gene hits.

| | Gene | | | | | |
|---|---|---|---|---|---|---|
| Treatment | *bla*$_{TEM}$ | *mphA* | *aad* | *catB* | *strB* | *aph* |
| Raw | 1.797796 | 0 | 0 | 16.60545 | 0.019331 | 0.019331 |
| Control | 5.412487 | 0.207635 | 0 | 31.62422 | 0 | 0 |

### 4.5.5. *Qac* gene detection

Surprisingly, there were no hits for any *qac* genes, for any of the replicates, for any treatment.

## 4.6. Discussion

### 4.6.1. FUN-PCR – fit for purpose?

The percentage of total reads with an *intI1* hit passing the acceptance criteria was, on average, around 30 %. As the coverage and identity cut offs were lowered to account for the higher error rate of PacBio sequencing (Rhoads and Au, 2015), the location of hit (i.e. within the first 75 bp of the read) resulted in the greatest reduction of hits. This approach was adopted due to the uncertainty and novelty of the FUN-PCR method, and to highlight resistance genes which would be closest to the *intI1* gene (and therefore Pc). However, this approach would have excluded the multiple integron hits which were detected within the same read. These could be PCR artefacts, but could also have been amplified from integrons within plasmids, where more than 1 integron may be present (Tosini et al., 1998). Further analysis could investigate associated resistance genes (if any) with other integrase hits further downstream.

The low diversity of resistance genes identified suggests FUN-PCR may be subject to some bias. Only five different resistance genes were detected with ARG-ANNOT, and four with CARD. While *catB, aph* and *aad* genes were amongst the genes identified which are also well known resistance gene cassettes (Partridge et al., 2009); compared to all known integron-associated resistance genes, very few were detected. It may be that the same integrons were repeatedly amplified during PCR, or that the original inoculum had a very low integron carriage and / or diversity. Based on the *intI1* qPCRs for the CIP and TRMP experiments in chapter three, there were on average around 250 *intI1* copies at day 0. As the same inoculum was used in this study, it could be the lack of resistance gene diversity is a reflection of the low class 1 integron number in the original inoculum. Though free gene cassettes (i.e. those not integron-associated) may have also existed in the original inoculum and could have been captured, the diversity and number of non-integron associated resistance gene cassettes in natural communities has not been studied; and so the likelihood of their integration into integrons cannot be predicted. Alternatively, greater gene cassette diversity may also have been carried within the larger size fraction of amplicons, which was unfortunately lost before sequencing.

The *bla*$_{\text{TEM}}$ and *mphA* genes are not listed in the 2009 review by Patridge et al., (2009) as common resistance cassettes, but these have been identified previously in a range of different integron backbones in different hosts (INTEGRALL (Moura et al., 2009) search, 12$^{th}$ July 2017). However in the INTEGRALL database, *mphA* is only reported twice, once in an *Aeromonas* species and once in a *Shigella* species. *Bla*$_{\text{TEM}}$ has five reports in the INTEGRALL database, with all of these hits also from *Enterobacteriaceae* hosts. The relatively high numbers of hits for these genes is explained by the inoculum used in this study – untreated waste water, which would include abundant *Enterobacteriaceae* spp. This could be confirmed with 16S sequencing of the communities.

Class 1 integrons contain a *qacEΔ1* and *sul1* gene in the 3' CS. As there were no hits for either of these genes, particularly under BKC exposure, it suggests the sequences were too short to include the 3' CS. Indeed, the average length of all the reads across all treatments was just over 1 kb. This could also explain the lack of observed resistance genes diversity, which again may have been more apparent in the lost, larger sized fraction of amplicons.

### 4.6.2. Sequence analysis pipeline

Integron_Finder (Cury et al., 2015) was used initially to search for integrase genes on each read within each multifasta library, using the options for linear sequences and 'local-max' searches (to increase the chances of a hit for an integrase gene). However, very few integrases were defined due to the amplified gene being truncated (as a result of the nested PCR) – so truncated, it would not be detected (*pers. comm.* Jean Cury).

The ABRicate tool successfully identified *intI1* hits and resistance gene hits. Were this method to be used again, the pipeline would benefit from development of additional commands to manipulate and filter hits within the tab file. Additionally, it would be advantageous to group multiple *intI1* hits within the same read, to determine the distance between those hits, and the distance to associated resistance genes. This could be useful for determining if resistance gene cassettes 'reshuffled' under selective pressure (antimicrobial exposure) to be closer to the promoter, where the genes would be more strongly expressed (Partridge et al., 2009). It appears the method did capture some gene cassette rearrangements, as the *aad* genes were undetected in both the raw inoculum and control treatment; but were detected following CIP exposure. The additional steps in the analysis pipeline would be most crucial for larger amplicons. However, as the average length of the reads was just over 1 kb, it can be assumed that on average, any resistance genes detected in this study are close to the promoter, and are therefore being highly expressed.

### 4.6.3. Selective effects of antimicrobial treatment

Both TRMP and CIP exposure resulted in significant increases in one or more resistance genes. TRMP and CIP significantly enriched for β-lactam resistance, while CIP also significantly enriched for aminoglycoside resistance (via *aad* genes, and a non-significant increase in *aph* was observed). Interestingly, neither TRMP nor CIP resistance gene cassettes were detected following TRMP or CIP exposure. These findings could suggest, as with the multiple *intI1* hits, that these integrons were located on multidrug resistance plasmids which may have also harboured genes conferring resistance to the exposure antibiotic; or that

resistance genes to the selecting antibiotics were positioned further downstream in the integron array (though these would still be less highly expressed than the co-selected resistance genes). Full metagenomic sequencing of the samples should be carried out to determine if any other resistance determinants were co-selected by the antimicrobial treatments; and if TRMP, CIP or BKC resistance determinants were directly selected for. If TRMP, CIP or BKC resistance genes were detected, it may be that integrons are not the key mobile genetic elements carrying these resistance determinants in this community. Unfortunately, this could not be completely confirmed without the larger sized amplicon fraction.

## 4.7. Conclusions

FUN-PCR is a novel method which can be used to amplify integrons and associated resistance gene cassettes in a complex bacterial community. Coupled with PacBio sequencing, it has the potential to identify resistance genes previously unrecognised as class 1 integron-associated gene cassettes, and to track gene cassette integration following exposure to different selective pressures.

This novel approach has shown TRMP and CIP are important co-selectors for antibiotic resistance, and may induce gene cassette 'reshuffling' (most likely via excision). Combined with the findings in chapters two and three, these results indicate the co-selective potential of a compound should always be considered in complex communities, and therefore when risk assessing antibiotics and identifying environmental protection goals in terms of antimicrobial resistance selection potential.

Though the concentrations used here were much greater than those found in the environment, findings from chapter one indicate that the strength of selection does not necessarily increase proportionally with antibiotic concentration. Therefore, there may be little difference in terms of *intI1* and associated, antimicrobial resistance gene cassettes selection at lower antimicrobial concentrations.

# Chapter five: Investigating co-selection for antibiotic and quaternary ammonium compound resistance in the environment – a functional metagenomics study

## 5.1. Introduction

Co-selection is the term used to describe indirect selection for one or more resistance mechanisms. There are two main mechanisms by which this can occur: co-resistance, and cross-resistance. Co-resistance is when two or more resistance genes are genetically linked, for example on a plasmid; and therefore only one compound needs to be present in order to select for the plasmid (and consequently both resistance mechanisms). Cross-resistance is when a single resistance gene can confer resistance to multiple compounds, for example a multi-drug efflux pump; and therefore a single selective compound can select for the gene (Baker-Austin et al., 2006, Larsson, 2014).

The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) confirmed that that co-selection for antibiotic resistance is likely to occur in the environment (SCENIHR, 2009). The most well studied compounds that co-select for antibiotic resistance are biocides and metals, and to a lesser extent, other antibiotics. Co-selection via co-resistance has been shown to occur under laboratory conditions (Tandukar et al., 2013, Gullberg et al., 2014) and there is evidence for co-selection of antibiotic resistance genes in the environment (Gaze et al., 2005, Gaze et al., 2011, Drudge et al., 2012, Berg et al., 2010, Berg et al., 2005). More recently, all genomes in the NCBI database were analysed for co-occurrence of antimicrobial resistance genes. 86 % of all genomes which harboured a metal or biocide resistance gene also contained an antibiotic resistance gene. Similarly, plasmids bearing metal or biocide resistance determinants were also significantly more likely to harbour antibiotic resistance genes, showing many of these are excellent candidates for co-selection to act upon (via co-resistance) (Pal et al., 2015). However, still more work is required to understand the role of co-selection in the maintenance and spread of mobile antibiotic resistance determinants (Andersson and Hughes, 2014).

So far in this thesis, research has focused on direct selection for antibiotic resistance *in vitro* in natural bacterial communities rather than in single “model” organisms. However, a holistic approach to tackle the global issue of antibiotic

resistance requires improved understanding of co-selection for antibiotic resistance by other micropollutants such as biocides as well as direct selection for antibiotic resistance as a result of exposure to antibiotics. Additionally, it is important to understand how selection for AMR occurs in the environment, and to study natural communities in an unbiased way. Functional metagenomics removes some causes of sampling or sequence analysis biases, as it allows functional screening to determine both novel and previously characterised genes alike; without subjecting the community to culture bias, and without requiring *a priori* knowledge of resistance genes (dos Santos et al., 2017). This chapter describes an un-replicated, functional metagenomics field study which investigated co-selection for antibiotic resistance in quaternary ammonium compound (QAC) impacted environments.

### 5.1.2. Quaternary ammonium compounds (QACs) and resistance genes

There is a vast range and chemical diversity of QACs which will not be discussed here. The QACs discussed further and which formed part of this study are the first generation QACs, benzalkonium chlorides ('BKC', such as BAC-12, BAC-14 etc.), and cetyltrimethylammonium bromide (CTAB), a third generation QAC (Gerba, 2015).

QACs are biocides, antimicrobial compounds used not as therapeutic agents, but as surfactants, detergents and disinfectants (Oh et al., 2013) and as preservatives in personal care products (Buffet-Bataillon et al., 2012). Their use precedes that of antibiotics (Gillings et al., 2009) and they are also estimated to be produced in quantities several orders of magnitude greater than antibiotics (Calero-Caceres et al., 2014), with annual estimates for surfactant production at greater than 18 million tons (Cirelli et al., 2010).

QACs' mode of action is to first interact with, then penetrate, the bacterial cell wall; then to disrupt the cytoplasmic membrane which causes leakage of intracellular constituents, including autolytic enzymes. These enzymes then degrade the cell wall, resulting in cell lysis (Zhang et al., 2015, Gerba, 2015).

Biocides are used at concentrations up to 1000x greater than MIC (in order to reduce the likelihood of resistance emerging at sub-inhibitory concentrations), due to the fact toxicity in humans is not a problem as with antibiotic chemotherapy (Chapman, 2003). However, though QACs are bactericidal at around 10 mg/L

and bacteriostatic at around 0.5 mg/L (Gerba, 2015), toxicity to the common ecoxtoxicological model species is quite high, with EC50's of benzalkonium for fish and invertebrates around 280 µg/L and 5 µg/L respectively. The toxicity to model bacterial species is an EC50 of benzalkonium for *Vibrio fischeri* determined as 0.5 mg/L, and for *Pseudomonas putida* as 6 mg/L (Zhang et al., 2015). These high effect concentrations for the target organism are again alarming (as discussed in chapters one and three), as it suggests the current ecotoxicological tests may be unsuitable for determining the selective potential of a compound (as opposed to toxic effect). However, for QACs it appears that non-bacterial ecotoxicological model species are more protective of the environment and natural ecosystems, which not the case for antibiotics (see chapter one and chapter three).

Measured Environmental Concentrations (MECs) of QACs tend to be very high. For example in Sweden, average concentrations across several different WWTPs of a BKC homologue was 89 µg/g in activated sludge (dry weight), 30 µg/L in WWTP influent and 0.3 µg/L in treated effluent. Another QAC, CTAB, was measured at 370 µg/L in sludge, 54 µg/L in influent and 0.3 µg/L in effluent (Östman et al.). In previous studies, a BKC (BAC-12) was measured at 170 µg/L in influent (Martinez-Carballo et al., 2007). Finally, QAC concentrations in surface water tend to be in the µg/L range (Zhang et al., 2015), though have also been detected up to 5 mg/L in hospital effluent (Kummerer, 2009b).

The majority of MECs should not be toxic according to the effect concentrations, but this is not due to WWTP degradation. Rather, due to the cationic charge of QACs, the majority bind to sludge, (Zhang et al., 2015), which explains the very high sludge MECs. However, this sludge would then be applied to agricultural land as fertiliser, and though there would be some sorption to soil, it is likely there would still be an effect on the soil biota (Cirelli et al., 2010).

Due to their wide use at very high concentrations and poor biodegradability (Buffet-Bataillon et al., 2012, Oh et al., 2013, Chapman, 2003, Zhang et al., 2015), QACs could confer a stronger selective pressure than low, environmental concentrations of antibiotics. For example, QAC concentrations in surface water tend to be in the µg/L range (Zhang et al., 2015), whereas antibiotics are typically in the low ng/L range (Homem and Santos, 2011). Though some argue novel resistance to QACs is unlikely to develop due to the nature of its antimicrobial

activity (Gerba, 2015), this does not mean pre-existing QAC or antibiotic resistance mechanisms would not be co-selected for by QACs.

In fact, the nature of and genetic context of QAC and antibiotic resistance genes lend themselves to be ideal co-selection candidates. For example, the co-selective potential of QAC's was shown to be the highest of all biocides and metals studied (alongside mercury (Pal et al., 2015)). The *qac* efflux genes (e.g. *qacE, qacEΔ1 and qacH*) are excellent examples of genes which facilitate co-selection through both cross-resistance (as it encodes a multi-drug efflux pump) and co-resistance (as it is often located on integrons, which in turn carry a vast diversity of antibiotic resistance genes). These *qac* efflux genes have been shown to have a relatively high prevalence in polluted environments (Gaze et al., 2005, Gaze et al., 2011, Gillings et al., 2009). In fact, most resistance to QACs is via efflux pumps such as the *qac* genes, which tend to belong to either the small multi-drug proteins, or the major facilitator family. These can all be found on a range of multi-drug resistant plasmids (Chapman, 2003), which again make co-selection likely.

### 5.1.3. This study

This study aimed to investigate co-selection of antibiotic resistance in QAC-exposed environments. Functional metagenomic libraries generated previously by Lihong Zhang from: reed bed (RB) soil used to remediate the effluent of a textile factory; sewage cake (SC); and 'pristine' grassland (GL) from the Rothamsted site, England (protected from anthropogenic impact); were screened for BKC and CTAB resistance. BKC and CTAB are both common QACs with applications as preservatives in pharmaceuticals, personal care products and in household cleaning products, detergents and fabric softeners (Buffet-Bataillon et al., 2012, Östman et al., 2017), and so both the RB and SC environments were expected to have much higher QAC exposure compared to the GL environment.

Initially, all libraries were screened on CTAB and BKC to determine levels of QAC resistance. Then occurrence of co-selection was investigated, by determining the proportion of CTAB resistant clones from both exposed libraries that were also resistant to two antibiotics: ampicillin (AMP) and trimethoprim (TRMP). The MICs for unique BKC resistant clones were determined and these clones then underwent full sequencing to search for co-resistance and cross-

resistance candidates; and transposon mutagenesis to functionally identify the genes conferring BKC resistance. A subset of these ORFs which were common to virtually all knock outs was sub-cloned to screen for cross-resistance to a range of antibiotics.

#### 5.1.3.1. Choice of antibiotics for investigating co-selection

TRMP was chosen as its target is dihydrofolate reductase, and resistance arises due to acquisition of alternative enzymes encoded by the *dfr* genes. 30 *dfr* genes have been recorded, and they are frequently associated with class 1 and class 2 integrons, which have been found extensively in environmental bacteria (Abella et al., 2015a, Abella et al., 2015b, Berglund et al., 2014, Gatica et al., 2016, Gaze et al., 2005, Gaze et al., 2011, Gillings et al., 2015), and which often harbour QAC resistance genes such as *qacE* (Gillings et al., 2009, Partridge et al., 2009). Due to the increased in resistance in recent years TRMP use has reduced. Despite this, *dfr* gene prevalence has not decreased suggesting the genes confer a low fitness cost (Brolund et al., 2010). Low fitness cost and well-defined integron association makes TRMP co-selection likely.

Additionally, TRMP is commonly used to treat urinary tract infections (UTIs), but is not metabolised fully within the body (Brolund et al., 2010), nor is it biodegradable (Sirtori et al., 2010). Concentrations of TRMP in WWTP effluent range from 20 – 1340 ng/L (Le-Minh et al., 2010), which though greatly below the MIC of the host with an empty vector, may still be high enough to select for resistance (Gullberg et al., 2011).

AMP is a β-lactam antibiotic and prevents cell wall synthesis. Resistance is commonly mediated by β-lactamases which cleave the β-lactam ring such as a*mpC*, *bla*$_{TEM}$*, bla*$_{SHV}$ and others, which have been isolated in natural environments and shown in some cases to be integron associated (Henriques et al., 2006). As well as resistance via target modification and reduced permeability, β-lactam resistance can also be conferred by efflux pumps (Reygaert, 2011), and *qacEΔ1* has been shown to confer ampicillin resistance (Kücken et al., 2000). For this reason, AMP was chosen alongside TRMP as it is a likely co-selection candidate based on environmental detection of resistance determinants and integron association.

For the disc diffusion assay, a range of antibiotics were chosen based on resistance profiling conducted in previous work for commonly identified resistance genes (Kazimierczak et al., 2009, Nakao et al., 2006). These were a range of tetracyclines, and imipenem. Cefotaxime (TAX) and sulfamethoxazole (SMX) resistance was also evaluated due to association of ESBLs and SMX resistance genes such as *sul1* with integrons (Henriques et al., 2006, Partridge et al., 2009), and due to the fact SMX is commonly used with TRMP to treat UTIs (Sirtori et al., 2010).

#### 5.1.3.2. Primary aims of the study

- Determine if the studied QAC-exposed environments have higher levels of QAC and antibiotic resistance compared to the 'pristine' study environment.
- Determine the genes responsible for these resistance(s).

#### 5.1.3.3. Secondary aim of the study

- Sub-clone the most common ORFs conferring QAC resistance and perform disc diffusion assays to generate antibiotic susceptibility profiles, to determine level of cross-resistance.

### 5.1.4. Author contributions

Lihong Zhang generated the metagenomic libraries. I received input from both Lihong Zhang and William Gaze on the initial experimental design for QAC and co-selection screening, and transposon mutagenesis. I selected compounds for subsequent experiments to screen for co-selection. I designed and performed all experiments, and analysed all the numerical and sequence data. I wrote this chapter and a manuscript for publication.

## 5.2. Materials and methods

### 5.2.1 Sampling sites and metagenomics library construction

SC (fully digested, dehydrated and limed sewage) was collected from a West Midlands WWTP. The RB soil was amended with effluent from a textile mill with high usage of quaternary ammonium compounds (QACs). The grass land soils were taken from the Rothamsted Park Grass experimental plots.

### 5.2.2. Culturing and identifying unique inserts

All culture media (liquid and in agar) contained tetracycline ('TET' 12.5 mg/L) for vector maintenance. Incubation was stationary or shaking (150 - 180 rpm) at 35 – 37 °C overnight.

Libraries were screened by plating on five LB agar plates containing MIC (of host with empty vector) concentrations of BKC and CTAB (16 mg/L and 32 mg/L respectively) with 100 µl of RB cells, GL cells or SC cells diluted to $10^{-1}$, which equates to at least one coverage per library across the five plates.

Plasmid DNA extraction was performed on a random selection of 24 BKC resistant clones from both the RB and SC library alongside the single BKC resistant clone isolated from GL with the GeneJet MiniPrep Kit (ThermoScientific), according to instructions for low plasmid copy number.

Restriction digest with EcoRI and BamHI FastDigest Green (Fermentas, now ThermoScientific) identified unique inserts. Total reaction volumes for each sample were 1 µl Buffer, 0.3 µl enzyme, 4.7 µl sterile $dH_20$ and 4 µl of plasmid DNA (due to low DNA concentration). Reactions were incubated in a heat block at 37 °C for 20 minutes. Reactions were loaded to a 0.8 % agarose gel stained with EtBr for gel electrophoresis at 110 Volts for 45 minutes to identify unique inserts.

### 5.2.3. Gene knock out and sequencing

Unique vector inserts underwent transposon mutagenesis using the EpiCentre Kan-25 EZ Kit, according to manufacturer's instructions. Unique vector inserts were combined in equal proportions and were also mutagenised individually (for

quick screening and complete recovery, respectively). Mutagenised vectors were electroporated into electrocompetent EC100 cells (Epicentre) by mixing 1 µl mutagenised DNA with 50 µl cells in 2 mm cuvettes and shocking at 2200 - 2500 V. Successfully shocked cells (pulse time > 4.0 ms) were recovered in 500 µl SOC media and incubated for 60 – 90 minutes. 100 µl of cells were diluted to $10^{-3}$ in LB broth and spread on plates containing TET only, to assess transformation success. The remainder of cells were spread in 100 µl aliquots onto LB agar plates containing kanamycin ('KAN') 50 mg/L (to select for clones with successful transposon insertion) and TET.

ClonTech chemically competent (Stellar™) cells were also used for transformation according to manufacturer's conditions. 1 µl plasmid DNA was combined with 40 µl chemically competent cells, and incubated on ice for 5 minutes. Cells were then shocked at 45 °C for 45 seconds, and recovered immediately in 500 µl SOC media. After 60 minutes shaking at 180 rpm at 37 °C, 100 µl cells were spread per selective plate.

Following incubation overnight, clones were randomly selected to be functionally screened for resistance gene knockouts. Mutants were spotted onto LB agar plates containing kanamycin (50 mg/L) and TET and also onto LB agar plates containing KAN (50 mg/L), TET and BKC at 16 mg/L, 24 mg/L or 32 mg/L (to assess the extent to which resistance was lost). Over 120 mutants were screened per mutagenesis reaction. Clones unable to grow on one or more of the BKC plates were picked from the plates without BKC, and grown overnight in 10 ml (due to low plasmid copy number) LB broth for plasmid extraction as described previously.

Restriction digest (as above) determined unique mutants for the transposon reactions with mixed vector, and these along with the single transposon knock outs were sent for sequencing (GATC) using the forward and reverse primers provided in the EpiCentre Kan-25-EZ Kit at a 1:9 ratio of primer to DNA (10 µl total), due to low DNA concentration (~30 ng/µl, nanodrop).

For sequence analysis, ab1 files were first examined for sequence quality, and the ends were trimmed of bases with low or indistinct peaks. For the mutants, the transposon sequence was first removed and then forward and reverse sequences were combined to give the knock out gene sequence using MEGA5.2 (Tamura et al., 2011). GenBank ORF finder was used to identify potential ORFs which then underwent BLASTp.

Entire inserts were also sequenced to search for co-resistance, by primer (IDTDNA) walking using plasmid DNA extracted as above (sequencing performed by Macrogen Europe). Read quality was assessed as above and alignment performed in MEGA 6.0 (Tamura et al., 2013).

### 5.2.4. Phylogenetic analysis

Phylogenetic analyses were performed in MEGA 6.0 (Tamura et al., 2013). UDP-like proteins identified in this study were aligned, trimmed and used to generate a maximum likelihood tree (with default settings) with 500 bootstrap replicates using a UDP-galactose-4-epimerase sequence from GenBank (Accession NC004663.1). Maximum likelihood was chosen as the most robust analysis available for the small selection of sequences, and sequence length. 500 bootstrap replicates were used as the uppermost value to yield reliable results (Pattengale et al., 2010).

### 5.2.5. Cloning UDP-galactose-4-epimerase -like ORFs and expression

Two ORFs (R11 and S78) and the positive control (included in the Champion pET101 expression kit) were successfully amplified using Q5 enzyme (New England Biolabs) in the following reaction: 10 µl buffer, 1 µl dNTPs, 5 µl primer pair, 0.5 µl Q5 and 43.5 µl sterile water. Cycling conditions were as follows: 98 °C for 30 seconds, followed by 30 cycles of 98 °C for 10 seconds, 62 °C for 30 seconds, 72 °C for 1minute and a final extension of 72 °C for 10 minutes. The other ORF (R161) was amplified using HotStart OneTaq (New England Biolabs) in a 20 µl reaction comprising of 10 µl HotStart OneTaq Master Mix, 2 µl forward and reverse primer combined (10 µM each), 7 µl sterile water and 1 µl template. Cycling conditions were as follows: 95 °C for 1 minute, followed by 30 cycles of 95 °C for 1 minute, 53 °C for 30 seconds and 68 °C for 1 minute, and a final extension of 5 minutes at 68 °C. Primer sequences for PCR reactions are shown in Table 16 (Appendix).

Products were run on a 0.8 % agarose gel to verify successful amplification of a single, specific band. All reactions were cleaned up using the NucleoSpin Clean and Concentrate kit (Mackery-Nagel), according to manufacturer's

instructions. PCR products were also sequenced before cloning (Macrogen Europe) using the same primers in ratios described previously to verify high fidelity amplification.

R11 and the positive control vector were successfully cloned into the pET101 vector using the Champion pET Directional Expression kit (InVitrogen) according to the manufacturer's instructions, and were electroporated as above. The other two ORFs (R161 and S78) could not be successfully cloned using the pET101 kit, so they were cloned in a Hot-Phusion reaction (Fu et al., 2014), which required additional bases to be added to the primers with homology to the pET101 vector (Table 16, Appendix). Briefly, 1.5 µl of PCR product was combined with 0.5 µl linearised pET101, 5 µl 2x HotFusion Master Mix (Fu et al., 2014) and water to a final volume of 10 µl. Reactions were incubated at 50 °C for 1 hr, then 45 seconds at 5 °C decreasing temperatures (e.g. 45 °C, 40 °C etc). 1 µl was transformed into electrocompetent cells (EpiCentre) as described above.

Colony PCR (conditions as previously described, but water volume adjusted) with T7 forward and reverse primers from the cloning kit confirmed the ORF had been successfully cloned into the vector. The vector was also sequenced (Macrogen Europe) using primers provided in the Champion Directional Expression kit (T7 forward and reverse) to confirm the insert was in the correct orientation.

All UDP-like ORFs and the positive control expression vector were chemically transformed into the OneShot (InVitrogen) expression cells according to instructions. Briefly, 2 µl UDP DNA or 1 µl control vector DNA was gently mixed with competent cells (25 µl cells and 25 µl cold 10 % glycerol) and incubated on ice for 30 mins. Cells were shocked at 42 °C for 30 seconds, then held briefly on ice before being transferred to 250 µl room temperature SOC media. Cells were incubated for 30 minutes at 37 °C at 180 rpm, then added to 10 ml LB broth with AMP (50 mg/L) and incubated overnight. This culture was used for the initial disc diffusion assays. Freezer stocks were made as described above, and cultured from two consecutive single colony and overnight cultures before being used for E test assays.

### 5.2.6. MIC/Co-selection testing

MIC testing of the resistant clones was performed using Iso-sensitest agar, using the two 5 µl spot method of overnight culture diluted to OD 600nm 0.25 (Andrews, 2001). EC100 pcf430 (host with empty vector) was used as a negative control. This was performed to determine BKC MIC for all the fully sequenced BKC resistant original inserts and the cloned UDP-like ORFs.

For assessing significance between the numbers of clones resistant to BKC or CTAB, first the total numbers across all plates were corrected for library coverage. Coverage for RB, SC and GL are 302 µl, 7.42 µl and 306 µl, respectively. Library coverage was determined by dividing the amount of library plated across the 5 plates (500 µl for RB and GL, and 50 µl for SC) by the volume of a single coverage. Total numbers of resistant clones for each library and each treatment (BKC or CTAB) were divided by this library coverage and rounded to the nearest whole value.

The entire RB and SC libraries were also screened on AMP and TRMP to investigate possible co-resistance of CTAB and antibiotic resistance genes in the insert DNA. Approximately similar numbers of clones (~ 200) were isolated by plating on one plate containing only TET, and another containing BKC (16 mg/L) and TET. These were replica plated onto agar containing AMP or TRMP at the MIC of the host with empty vector (4 and 1.5 mg/L, respectively), approximately 1.5x this MIC (7 mg/L and 2.25 mg/L, respectively) and at clinical breakpoint concentrations for *Enterobacteriaceae* (8 and 4 mg/L, respectively (EUCAST, 2014)), as the host was *E. coli*.

Original BKC resistant inserts and cloned UDP-like ORFs were tested for increased antibiotic resistance compared to the empty vector control (pcf430 for original inserts, or pET101 for UDP-like ORFs) with disc diffusion assays (Fisher-Scientific). Antibiotic susceptibility disc diffusion assays were performed for Doxycycline hydrochloride (30 µg) and Minocyline (30 µg) for the UDP-like ORFs only (as all other vectors contained TET resistance); and AMP (10 µg) for the original inserts only (as UDP-like ORF containing vectors contained an AMP resistance gene). Both UDP-like ORFs and original inserts were also screened on TRMP (2.5 µg), Cefotaxime (‘TAX’ 5 µg), Imipenem (‘IMP’ 10 µg), Colistin (‘COL’ 10 µg) and Sulfamethoxazole (‘SMX’ 25 µg). Testing was performed according to EUCAST standards (Matuschek et al., 2014), with Mueller Hinton

(MH) agar amended with TET for vector maintenance for the original inserts; or AMP (50 mg/L) for vector maintenance and IPTG (0.025 µM final concentration) to induce expression for the UDP-like ORFs. It is noted there may be some problems with the reliability of colistin disc diffusion assays (EUCAST, 2016).

Negative control expression plates without IPTG were included, as well as the positive control expression vector plated as above on plates also containing X-Gal (to allow for visual confirmation of expression through blue/white screening). Disc diffusion assays were performed in triplicate for all three expression vectors and for the expression vector control. Inhibition zone sizes were averaged, and inhibition zone sizes were compared using an ANOVA and Tukey post-hoc test for significance.

E tests (Biomerieux) for SMX, TRMP and SMX / TRMP were also performed on UDP-like ORFs with appropriate controls following EUCAST standards, using MH agar supplemented as above.

### 5.2.7. Statistics

Statistical analyses were performed in R Studio (RStudio, 2015).

## 5.3. Results

### 5.3.1. QAC exposed libraries had higher QAC, TRMP and AMP resistance

Contaminated libraries (RB, SC) had significantly higher numbers of resistant clones compared to the ‘pristine’ environment (GL, all $p < 0.001$, ChiSq Test). Numbers of clones resistant to BKC or CTAB were significantly different in the RB library ($p < 0.001$, Chi-Sq Test) but there were no significant differences between treatments (BKC or CTAB) in the SC or GL libraries (Table 14).

Table 14. Numbers of BKC or CTAB resistant colonies from each of the three metagenomics libraries from the initial screen, and corrected for per Gb of library,

rounded to the nearest whole number. The library coverages are 0.63, 1.59 and 1.53 Gb for RB, SC and GL, respectively.

| Library | No. BKC resistant | No. CTAB resistant | No. BKC resistant per Gb library | No. CTAB resistant per Gb library |
|---|---|---|---|---|
| **RB** | 397 | 648 | 151 | 260 |
| **SC** | 160 | 240 | 38 | 57 |
| **GL** | 1 | 5 | 1 | 5 |

An initial screen for co-selection for antibiotic resistance found greater than 80 % of all CTAB resistant clones in the RB and SC libraries also had reduced susceptibility to both TRMP and AMP (compared to the empty vector control MIC, Figure 59). Clinical resistance was extremely rare (1 CTAB resistant clone was also clinically resistant to TRMP).

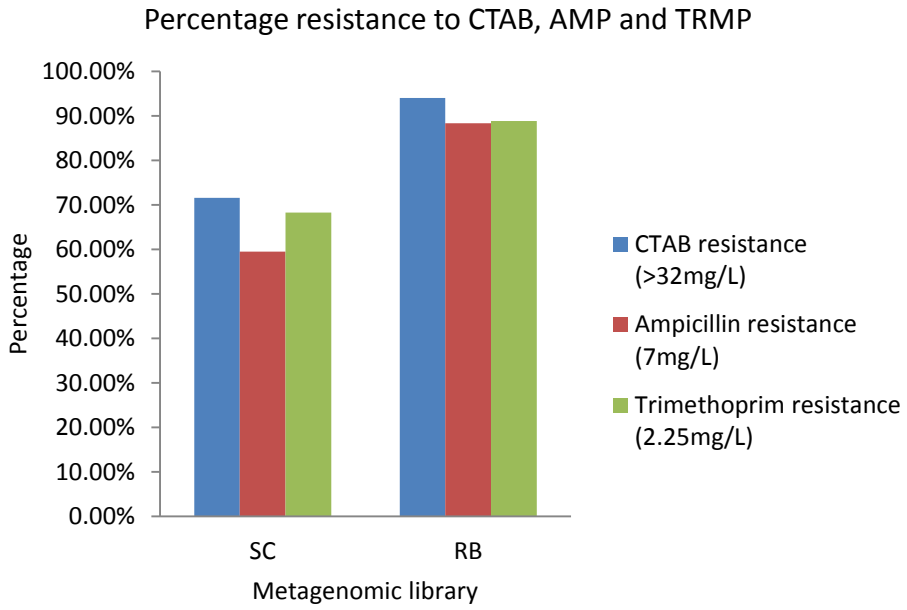


Figure 59. Percentage of the entire sewage cake ('SC') and reed bed ('RB') libraries resistant to CTAB, or with reduced susceptibility to Ampicillin, and Trimethoprim compared to the empty vector control (MIC 32 mg/L, 4 mg/L and 1.5 mg/L respectively).

### 5.3.2. Full insert sequencing discovered many co-resistance and cross-resistance genes

To identify the genes responsible for BKC resistance, 8, 6, and 1 unique BKC resistant clones were identified for the RB, SC and GL libraries respectively, via

restriction digestion. Following transposon mutagenesis, 20 and 10 potentially unique knock out clones of interest were sequenced for the RB and SC libraries, respectively; however, only three of each of the original 8 (RB) and 6 (SC) plasmids were recovered in this initial mutagenesis. Subsequent mutagenesis with individual plasmids was performed, but this was still unable to identify a knock out mutant for all unique inserts. Therefore, all inserts were fully sequenced by primer walking to account for the possibility there could be multiple resistance genes within the insert.

Full sequencing of the 'unique' inserts identified some of the inserts as identical (based on ~ 2,000 bp in forward and reverse having 100 % homology), so sequencing of these was discontinued. The full sequences for the remaining unique inserts were examined for genes that could potentially confer antimicrobial resistance, through ORF Finder and BLASTp searches.

The full list of ORFs, their predicted function and identity is shown in Table 18 (Appendix). Three inserts contained ORFs with varying similarity to known antibiotic genes. Insert S4 contained a tetracycline resistance MFS efflux pump (100 % ID) and multidrug resistance protein mdtB (42 % ID); R17 contained a HTH MrM multiple antibiotic resistance protein (52 % ID); and R3 contained a penicillin binding protein (30 % ID). Intriguingly, S4 also contained part of a transposase (100 % ID but only 42 % coverage), suggesting recent mobilisation of the tetracycline MFS efflux pump and the potential for further transfer. Insert R10 also contained a transposase, suggesting co-localised genes such as the ABC transporter protein and UDP-galactose-4-epimerase also within this insert would be potentially mobilisable. In terms of biocide resistance, the only notable ORF was found in insert R24, which contained a sulfatase, (up to 96 % ID) capable of degrading anionic surfactants (Jovcic et al., 2010).

Other key ORFs identified could be loosely grouped into 3 types based on predicted resistance mechanism strategy. For membrane / transporter / efflux pumps, there were hits for ABC transporters, MFS transporters, a FIST-domain containing protein (involved in transport and binding of small ligands) and other membrane proteins. For preventing cell lysis / penetration there were hits for a predicted capsular polysaccharide biosynthesis protein and cell wall / membrane synthesis proteins, including the UDP-like ORFs and a UDP-galactopyranose-mutase. To combat cell damage, there were hits for an oxidoreductase and a divalent ion tolerance protein. In one of the inserts, there was high sequence

identity and coverage for 16S rRNA and several other genes (including an ABC transporter) from *Pseudomonas veronii*, which has been shown to have potential bioremediation capabilities (Nam et al., 2003, Onaca et al., 2007).

### 5.3.3. Transposon mutagenesis revealed QAC resistance was mediated by a diverse set of previously uncharacterised genes

For the transposon mutagenesis knock outs, the ORF name, sequence similarity and number of mutant clones containing knocked out ORFs for both RB and SC libraries are shown in Table 19 (Appendix). UDP-galactose-4-epimerase-like genes were present in the majority of knock outs; these have been found in previous functional metagenomic studies, referred to as *galE* genes which confer increased resistance to tetracyclines (Kazimierczak et al., 2009) and increased salt tolerance (Culligan et al., 2012). *GalE* mutants have also been shown to exhibit increased susceptibility to clinically important antibiotics such as TAX, IMP, and vancomycin (Nakao et al., 2006, Nayak et al., 2006).

Alignment of these UDP-galactose-4-epimerase like ORFs with the ORFs published in the paper by (Kazimierczak et al., 2009) found limited sequence similarity (Table 20, Appendix). A brief phylogenetic analysis (Figure 60) was conducted to visualise the similarity of UDP-galactose-4-epimerase like ORFs between knock outs from this study. This identified 7 distinct groups of UDP-like proteins. One UDP-like ORF from 3 of the identified subgroups which grouped closely with the reference gene were selected to be sub-cloned to screen for cross-resistance; namely R11, R161 and S78. These were sub-cloned into the pET101 expression vector and screened for reduced susceptibility to a range of antibiotics, alongside the original BKC resistant clones.

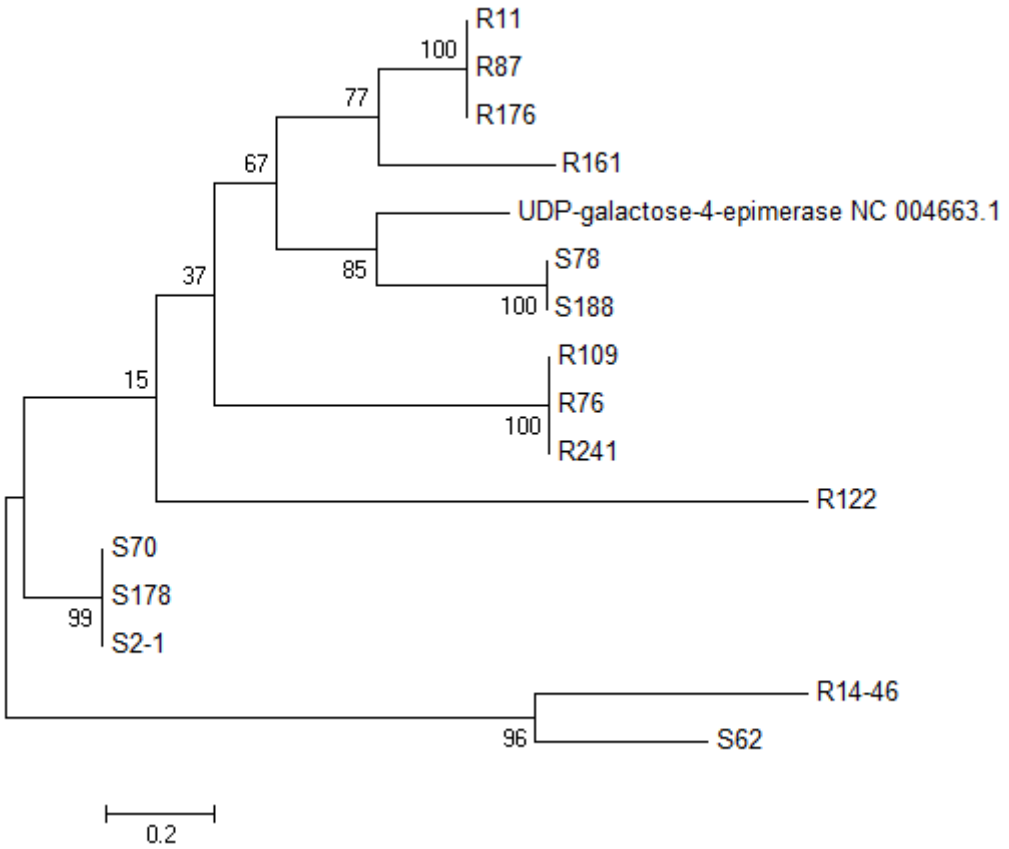


Figure 60. Maximum likelihood tree of the UDP-galactose 4-epimerases identified in this study, with a reference strain from GenBank (Accession NC 004663.1). Bootstrap values based on 500 bootstrap replicates. Sequences beginning with R are from the RB library, and with S from the SC library.

Antibiotic disc diffusion assays were conducted to determine the antibiotic susceptibility profiles of the original BKC resistant clones and the three sub-cloned UDP-like ORFs. For all the original BKC resistant clones, there was a slight decrease in susceptibility to SMX, which was marginally significant for insert S2 ($p$ = 0.066, ANOVA with Tukey post-hoc test), and insignificant for all other inserts. Insert S2 also showed a decrease in susceptibility to TAX and a slight decrease in susceptibility to TRMP, both of which were insignificant. R10 showed a minimal decrease in susceptibility to COL but this was not significant. For all other compounds, susceptibility increased for the inserts compared to the empty vector control, which was significant for all inserts to IMP and for S4 to AMP ($p$ < 0.05, ANOVA with Tukey post-hoc test, Figure 61).

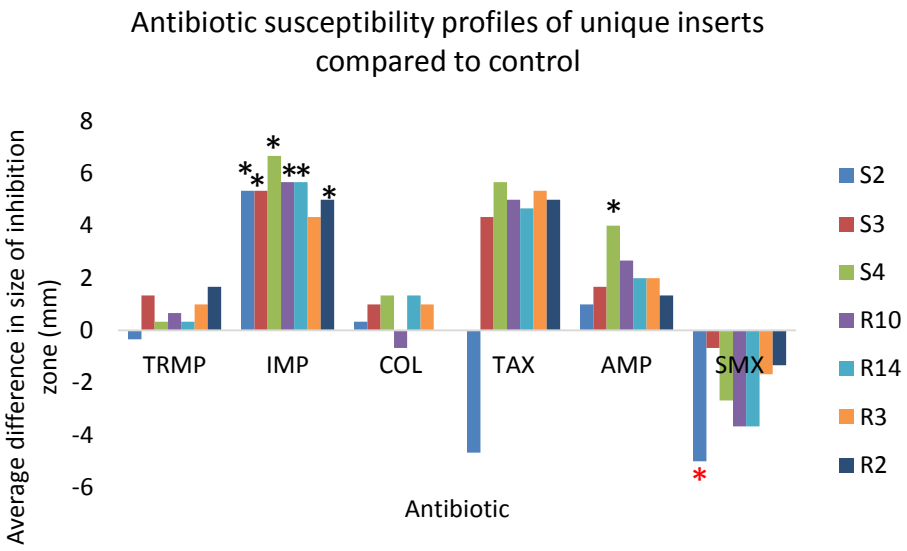


Figure 61. A reduction in the average inhibition zone indicates decreased susceptibility compared to the empty vector control. Values are calculated by averaging (n=3) the inhibition zone sizes, and subtracting the control zone size from the zone size of the inserts (S# = from the SC library; R# = from the RB library). TRMP = Trimethoprim, IMP = Imipenem, COL = Colistin, TAX = Cefotaxime, AMP = Ampicillin, SMX = Sulfamethoxazole. * indicates significant difference in size compared to control ($p < 0.05$, ANOVA and Tukey post-hoc test. Red * = ($p < 0.1$).

For the UDP-like ORFs, Figure 62 shows the difference in size of inhibition zone compared to the empty vector control, with significance determined by One-way ANOVA with Tukey post-hoc test. UDP-like ORF R11 and S78 showed significant increases in susceptibility to TAX ($p < 0.01$ and $p < 0.05$, respectively), whereas R161 showed a significant decrease ($p < 0.05$). All three showed significant decreased susceptibility to SMX (all $p < 0.01$), and S78 also showed a significant decrease in susceptibility to IMP ($p < 0.05$) and TRMP ($p < 0.05$).

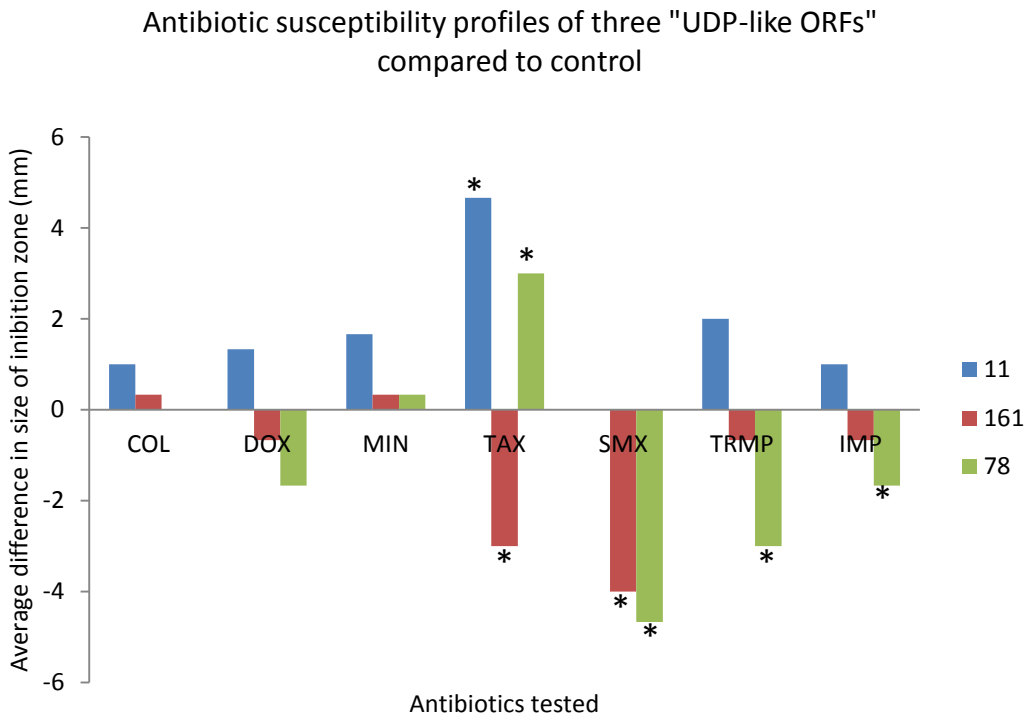


Figure 62. Values are calculated by averaging the inhibition zone sizes (n=3), and subtracting the control zone size from the zone size of the inserts. COL = Colistin, DOX = Doxycycline, MIN = Minocycline, TAX = cefotaxime, SMX = sulfamethoxazole, TRMP = trimethoprim, IMP = imipenem. Average difference = average size of inhibition zone (mm) for 'UDP-like' ORF (11, 161 or 78) – average size of inhibition zone (mm) for the empty vector control. Biological replicates n=3. Significance according to ANOVA and Tukey test, p value < 0.05 = *. (Unable to screen on AMP as used for vector maintenance).

To verify these results and determine the difference in MIC, MIC strip (E test) assays were performed (Table 15). Only UDP R11 showed an increase in TRMP MIC compared to the control; meanwhile the MIC for UDP R161 was the same and for UDP S78 there was a slight decrease. For SMX, the increase in MIC was more pronounced, with the MIC of UDP R11 and UDP S78 tripling that of the control. As TRMP and SMX are commonly administered in combination therapeutically (Sirotori et al., 2010), MICs using the TRMP / SMX E test combination strip were also determined. The average MICs (n=2) were slightly higher for all cloned UDP inserts than the control.

Table 15. E test MIC results for cloned UDP-like ORFs. TRMP = Trimethoprim, SMX = Sulfamethoxazole, TRMP / SMX = Trimethoprim / Sulfamethoxazole combination strip. '^' represents increase in MIC, '-' represents no difference in MIC, 'v' = decrease in MIC (all compared to empty vector control pET101).

| Antibiotic | Empty vector control pET101 MIC (µg/ml) | R11 MIC (µg/ml) | R161 MIC (µg/ml) | S78 MIC (µg/ml) |
|---|---|---|---|---|
| TRMP | 0.19 | 0.25 ^ | 0.19 - | 0.125 v |
| SMX | 2 | 6 ^ | 2 - | 6 ^ |
| TRMP / SMX | 0.019 | 0.0255 ^ | 0.0275 ^ | 0.032 ^ |

Cloned UDP like ORFs also had their BKC MIC's determined again in the expression vector to confirm BKC resistance. BKC MIC for the expression control was > 2 and < 4 mg/L. For UDP R11 and UDP R161 the BKC MIC was > 4 and < 6mg/L; and for UDP S78 it was > 6 and < 8 mg/L.

## 5.4. Discussion

Several studies have utilised a functional metagenomic approach to investigate the environmental resistome (Allen et al., 2009, Amos et al., 2014b, Wichmann et al., 2014, Su et al., 2014), as it allows definitive identification of the gene responsible for resistance whilst not being subject to culture bias (Garcia-Armisen et al., 2013). However, as an un-replicated field study, the findings may only be applicable to the environments studied. Additionally, this method is not without difficulty. In this study, to ensure biocide resistance genes for all unique inserts were knocked out, transposon mutagenesis was performed on unique vectors individually. With an insert size of 15 kb and allowing for gene sizes of around 500 bp, one in 30 mutagenised clones were expected to be a BKC knock out. However, after screening over 300 clones generated from some unique clones, no knock out mutants could not be obtained. It is possible that these inserts contained more than one gene conferring resistance to BKC and as the EpiCentre Kan-25 kit is optimised for single insertion events only, complete loss of phenotypic resistance was observed. Therefore, these inserts were sequenced fully by primer walking.

This simultaneously allowed for screening for co-resistance candidates; i.e. QAC and antibiotic resistance genes clustering within the same insert. There

was some evidence of this, including a mobilised tetracycline resistance MFS efflux pump (100 % ID) co-located with a transposase, and a sulfatase. This is particularly significant given the small insert size and the limited number of inserts sequenced. Other known resistance genes were identified but these had low sequence similarity to the database entries. However, a recent study found predicted ORFs with sequence similarity as low as 55 % (unpublished data, Lihong Zhang) can still produce the same function.

Many ORFs had varying levels of similarity to proteins in the database, including a variety of possible efflux pumps (MFS / ABC transporter), genes involved in cell wall / polysaccharide capsule synthesis; or involved in reducing reactive oxygen species (oxidoreductase, nitrilase). These genes would need further characterisation to confirm a definite functional role in either biocide or antibiotic resistance. Some proteins had very low sequence similarity to any entries in the GenBank database (< 40 %), and these could have been captured from unculturable bacteria, which would be largely underrepresented in the database. Research should continue to focus on isolating these proteins and functionally verifying their resistance mechanism(s), and ideally their host background and genetic context, in order to better understand co-selection of antibiotic resistance in the environment and potential for HGT into pathogens. Together, these results show there is a large environmental resistome in these anthropogenically impacted sites, consisting of a diverse range of resistance mechanisms, most of which have not been fully characterised as in previous studies (Su et al., 2014, Amos et al., 2014b).

On a phenotypic level, reduced susceptibility to AMP and TRMP was both observed and common to CTAB resistant clones in both ‘contaminated’ libraries. Reduced susceptibility to either antibiotic was greater in the RB library, which is likely to have a higher QAC exposure than the SC library. There were no WWTP or agricultural farms near the RB site which may have resulted in exposure to antibiotics, antibiotic resistant bacteria or antibiotic genes. This suggests that more co-selection for antibiotic resistance occurred in the RB environment (as opposed to direct selection, which is more likely in SC as it will be exposed directly to antibiotics). Though clinical resistance was extremely rare, this does not render the findings of this study clinically insignificant as it is possible the resistance mechanisms in their current genetic context are under low level expression, and form part of the proto-resistome (Perry et al., 2014). Genetic context has been

shown to greatly influence MIC previously (Amos et al., 2014a), so with mobilisation into a new genetic context allowing higher levels of expression (which with some of the inserts would be possible, due to the presence of a transposase); or with higher gene copy numbers MIC could increase via the gene dosing effect (Martinez and Baquero, 2000).

In fact, most of the ORFs identified in the study have not been reported previously as conferring resistance; only genes encoding a hydrolase (Su et al., 2014) and UDP-galactose-4-epimerases (Nayak et al., 2006, Nakao et al., 2006, Kazimierczak et al., 2009) have been reported previously as conferring antibiotic resistance. To investigate their cross-resistance potential, a subgroup of UDP-like ORFs were sub-cloned into a new vector to allow for definitive assignment as resistance determinants.

MIC assays were performed for TRMP, SMX and TRMP / SMX in combination on the cloned UDP-like ORFs. TRMP and SMX are often used in combination to treat urinary tract infections (UTIs); but TRMP is not metabolised fully within the body (Brolund et al., 2010), nor is it biodegradable (Sirtori et al., 2010). Therefore, concentrations of TRMP in waste water treatment plant effluent range from 20 – 1340 ng/L (Le-Minh et al., 2010), and could be selective (Gullberg et al., 2014). Therefore, though it is possible TRMP may have co-selected for QAC resistance rather than *vice versa*, the presence of the UDP-like ORFs in the RB library (presumed to have no direct exposure to antibiotics) suggests it is again QACs co-selecting for TRMP resistance, at least in the RB library.

Two of three tested UDP-like ORFs conferred significantly decreased susceptibility to SMX, equating to a 50 % increase in MIC compared to the empty vector control according to E test assays. Slight increases in TRMP / SMX were also observed for all three tested UDP-like ORFs, but only UDP R11 showed an increase in TRMP MIC. None of these MICs were clinically significant; but as discussed above, genetic context, gene copy number and so on could all influence MIC. There was also a decrease in BKC MIC when the UDP-like ORFs were cloned into the expression vector (a decrease from > 64 mg/L to 4 – 6 mg/L). However, the BKC MIC was still increased compared to the empty vector control, demonstrating these ORFs do indeed confer low level resistance to BKC. It also suggests the genetic context within the insert; or other genes present in the

inserts were contributing to the elevated BKC MICs in the original metagenomic libraries.

UDP-galactose-4-epimerases were present in all sequenced inserts, including the single BKC resistant clone from the GL library. While this indicates this gene is likely to be located in an intrinsically resistant organism, in one case a UDP-like protein was co-localised with a transposase suggesting it had recently been, or could potentially be, mobilised. If mobilised into a human pathogen, these findings indicate certain UDP-like gene variants have the potential to confer multidrug resistance / reduced susceptibility to BKC, SMX and TRMP (and potentially other antimicrobials not screened in this study). It is likely UDP-like proteins confer this resistance through modifying the bacterial exopolysaccharide, thereby interfering with antimicrobial interaction and penetration (Santander et al., 2013, Wu et al., 2014).

If any selection for QAC resistance is observed, it is likely that other resistance genes are selected for which may confer cross-resistance. This could promote co-resistance by selecting (for example) *qacE* on integrons, which are known to often carry antibiotic resistance genes such as *sul1* and be found at relatively high abundance in the environment including the environments used to generate these libraries (Gaze et al., 2005, Gaze et al., 2011). Intriguingly, no *qacE* genes or its variants were identified in this study, which is unusual as they are common in natural, contaminated environments. Lack of *qac* gene detection may also be a result of the small library insert size, but also suggests the resistance determinants are also more common in these environments than the *qac* genes. However, this also demonstrates the necessity of functional metagenomic studies to identify novel resistance determinants, shown in this study to be diverse and conferring higher levels of resistance than known genes. For example, the BKC MIC of clones in this study was more than double (≥ 64 mg/L) of that conferred by the *qacE* gene (32 mg/L, unpublished data).

## 5.5. Conclusions

This study suggests that anthropogenic activities are directly impacting microbial resistance to biocides and some clinically important antibiotics. The resistance determinants identified were diverse, largely uncharacterised and conferred a higher level of QAC resistance than the well-known *qacE* gene, justifying the

continued used of functional metagenomic studies. In the case of the common ORFs (“UDP-like proteins”), this is the first time these have been shown to functionally confer cross-resistance to QACs and reduced susceptibility to clinically important antibiotics.

# Chapter six: Thesis discussion

## 6.1. Environmental implications

This thesis comprises four novel research studies which together, indicate that selection and co-selection for antibiotic resistance is likely to occur in the environment.

Selection endpoints for several different antibiotics spanning different drug classes have been determined, forming the most comprehensive dataset of MSCs to date. Critically, these endpoints were determined in complex bacterial communities which are more environmentally representative and so provide more realistic endpoints for ERA than MSCs determined in single species systems (Berendonk et al., 2015, Brandt et al., 2015). Previous work using single species, isogenic host competition experiments (Gullberg et al., 2014, Gullberg et al., 2011) showed that MSCs determined for the same antibiotic in the same experimental system can be highly different, depending on the resistance determinant present. For example, the tetracycline MSC for the chromosomal insertion mutation Tn*10d*Tet was 15 µg/L (Gullberg et al., 2011) but for the plasmid-borne *tetRA* resistance gene the tetracycline MSC was 45 µg/L. In this study, single species and community assays were directly compared for the first time. This revealed that single species MSCs, at least for cefotaxime, are poor predictors of MSCs in complex communities, with a huge disparity (a four order of magnitude difference) between MSCs in a complex community compared to single species experiments. Further research should focus on community-derived endpoints, and to validate these experimental systems as being representative of natural environments (e.g. by exploring lower nutrient and temperature conditions).

Co-selection for antibiotic resistance by both antibiotics and QACs was observed in chapters two, four and five. In chapter two, metagenome analyses showed cefotaxime co-selected for genes conferring resistance to many different antibiotic classes. The FUN-PCR method coupled with PacBio sequencing in chapter four showed antibiotics and the QAC BKC selected for increased numbers of resistance genes to unrelated antibiotic classes in the first position in class 1 integron arrays. The co-selected genes would therefore be more highly expressed due to proximity to the Pc promoter (Partridge et al., 2009).

Additionally, in chapter five, co-resistance (or reduced susceptibility) to antibiotics, CTAB and BKC was observed in functional metagenomic libraries generated from QAC-impacted environments in an unreplicated field experiment. This is a cause for concern particularly in the SC library, as QACs are commonly present at high concentrations in WWTPs (Buffet-Bataillon et al., 2012). If the SC was spread directly onto agricultural land as fertiliser, these resistance mechanisms would have direct contact with the environmental resistome, potentially allowing HGT. Additionally as WWTPs are a HGT hotspot (Rizzo et al., 2013), hospital sterilisation and human health may be compromised if QAC resistance is transferred into opportunistic human pathogens (Gillings et al., 2009). A clade of novel genes (UDP-galactose-4-epimerases) was identified as conferring cross-resistance to BKC and reduced susceptibility to antibiotics. Similar genes, UDP-4 glucose-epimerases, have been reported to confer biocide resistance once before (Tansirichaiya et al., 2017) but cross-resistance to antibiotics was not assessed. These UDP-4 glucose-epimerases were cloned from the human oral metagenome rather than the environment, where selection for biocide resistance may be less expected. Together, these findings necessitate further study that should also consider the co-selective potential of a wide range of compounds to fully protect the environment. Selection experiments such as those performed in chapters two, three and four can be used for this purpose. These can be combined with metagenome analyses as in chapter two; or even undergo massively parallel qPCR quantification of diverse resistance determinants, as the studies in chapters two and three confirm qPCR as being the more sensitive method for MSC determination (Lundstrom et al., 2016).

Understanding persistence of integrons in microbial populations is crucial for determining the potential for fixation or maintenance of antimicrobial resistance within the environment, and potential for future capture of antimicrobial resistance gene cassettes. Class 1 integrons can persist in soil, albeit at low levels, even 24 months after application of sludge (Gaze et al., 2011). Following on from the work in chapter four, it would be interesting to track integron prevalence and diversity during antimicrobial exposure, but also integron persistence after the exposure has ended. FUN-PCR coupled with PacBio sequencing could also be used to investigate more communities exposed to other antimicrobials at more environmentally relevant concentrations, to elucidate the selective and co-selective potential of different antimicrobials. These results

should be compared to the MSCs determined using the assay in chapter two, to reveal if these MSCs are still protective in an integron / gene cassette selection context. While chapter four studied class 1 integron arrays directly, chapter three showed antibiotics can select for increased class 1 integron prevalence. Enrichment of integrons provides not only a platform for the selection of resistance genes within a host, but also mobilisation to different hosts; some of which could be pathogenic, having significant impacts on human and animal health.

## 6.2. Towards improved ERA

A range of different community-based endpoints were determined for the first time (MSCs, LOECs, and empirical PNEC$^{R}$s) in chapters two and three for antibiotics all deemed as 'critical' or 'highly important' to human medicine, based on WHO recommendations (WHO, 2015). Therefore, improved stewardship of these antibiotics is essential. Long term experiment LOECs gave the highest selection endpoint values, followed by growth based LOECs and then MSCs (in all but one case, where the clarithromycin growth based LOEC was higher than the long term LOEC). The standard ecotoxicological risk assessment approach of applying a safety factor of 1000 to emulate acute exposure (Straub, 2013) was applied to LOECs to generate empirical PNEC$^{R}$s. These endpoints were compared to previously derived ecotoxicological PNECs (see chapter three) and estimated PNEC$^{R}$s (Bengtsson-Palme and Larsson, 2016). In some cases the empirical PNEC$^{R}$s were more protective; in others, the estimated PNEC$^{R}$s were more protective. However, for all antibiotics tested, with the exception of trimethoprim, the ecotoxicological PNECs were protective of both estimated and empirical PNEC$^{R}$s. It is reassuring that ecotoxicological PNECs are also protective of resistance selection, but the case of trimethoprim indicates work should continue to determine empirical PNEC$^{R}$s to ensure complete protection. Estimated PNEC$^{R}$s could be used to guide these experiments, as recommended previously (Bengtsson-Palme and Larsson, 2016).

The unadjusted endpoints (i.e. MSC and LOECs based on long term or growth experiments) for all the antibiotics studied were highly comparable, but further data generation is required to determine firstly, which endpoint should be used for ERA to afford greatest protection against selection for resistance; and

secondly, simply more data on the selective potential of more antimicrobials is necessary (i.e. including, but not limited to, antibiotics). There are 22 different classes of antibiotics approved for clinical use (Coates et al., 2011), and to date only six classes have an MSC or selective endpoint determined (including four which were generated as part of this thesis (Gullberg et al., 2014, Gullberg et al., 2011)). Based on the findings in chapter three, these MSCs or LOECs will need to be determined on a case by case basis. There is still the possibility that there may be some similarity in MSCs or LOECs within the same antibiotic class or the same type of resistance mechanism, which should be explored as it could facilitate more rapid risk assessment.

The highest available MECs in aquatic systems for antibiotics investigated in this study were used to calculate RQs, and in the majority of cases the RQs were unacceptably high suggesting selection for resistance may occur (in many cases, irrespective of the selective endpoint used). However, these are 'worst case' scenarios, and many environmental compartments with lower MECs will not be under risk for resistance selection based on the endpoints determined in this thesis. More thorough, coordinated monitoring of MECs is necessary for adequate ERA and environmental protection.

Chapters three and four described novel tools that can generate further data to quantify the risk posed by different selective compounds. The next step will be to determine the selective effect of mixtures of different antimicrobials in complex bacterial communities, or additive effects of mixtures of antibiotics within the same class. The growth rate assay described in chapter three provides real potential to test these effects, as well as further, rapid data generation and the ability to test environmental applicability. Ultimately this growth rate assay can be used to test the selective potential of effluent and surface water samples. Whilst this is still an *in vitro* assay, it may be preferable to *in situ* experiments as experimental conditions can be closely controlled to discern individual biological effects (Jansen et al., 2014). Though *in situ* experiments will provide the most realism, they will be subject to sampling bias and a potentially vast number of confounding interactions that may be difficult to disentangle. For example, the research conducted previously in biofilms (Lundstrom et al., 2016) is subject to such issues, as uncharacterised communities were being constantly introduced into the experimental system. Therefore, the observed effects could be due to the composition of the community, and not the controlled experimental conditions

(i.e. antibiotic concentration). Replication would no doubt be another issue for *in situ* experiments, and may mask biological effects due to large variation between replicates as observed in chapters two and three; indeed, increases in prevalence were observable at concentrations below the LOEC for all compounds tested but these were not statistically significant. MSCs and $PNEC^R$s were determined as an attempt to mitigate this potential underestimation of risk. Higher replication and / or an estimated endpoint (i.e. MSC) could mitigate these issues for *in situ* experiments. Finally, *in situ* experiments may be subject to other biases (such as sampling biases), and will inevitably take much longer to gather results due to the lower nutrient levels and lower temperatures there will be in the environment, which will increase bacterial generation times. This in turn will greatly inflate experimental costs. Particularly if selection or resistance is cumulative over time *in situ,* as shown in chapter two, the time required for an effect to be observed *in situ* could exceed experimental feasibility. The growth rate assay can be used to explore environmental conditions much more simply, and at lower cost. Time taken to observe biological effects (i.e. selection) could be determined under environmental conditions using the growth rate assay, and used to inform *in situ* experiments, if required.

## 6.3. Clinical implications

Novel research presented in this thesis has direct relevance to evolution of antibiotic resistance in the clinic, as well as the environment. It has been suggested that longer courses of antibiotics may select for antibiotic resistance (Day et al., 2015). In chapter two, increase in $bla_{CTX\text{-}M}$ prevalence was shown to be cumulative over time, both within 24 hours and over several days. Also in chapter two, indirect selection was shown to occur at very high antibiotic concentrations (32 mg/L, much higher than the clinical breakpoint concentration of 2 mg/L). This means indirect selection is likely to occur during antibiotic chemotherapy. Further work should investigate these implications. Particular resistance genes could be identified which confer ‘high risk’ of indirect selection, and patients could be screened for presence / absence of these genes in order to personalise treatment. Efforts should be made to determine if such individually tailored treatment plans improve patient outcome and reduce selection for (or enrichment of pre-existing) resistance *in vivo*.

There has been considerable research investigating the optimal dosage of antibiotics in the body, although recent data suggest that the current, standard (intermittent) dosing regimens do, at times, allow for antibiotic concentrations to become suboptimal (i.e. sub-inhibitory). This is expressed in pharmacokinetic / pharmacodynamics terms as a percentage of the amount of time ('*T*') serum concentrations are below or above the MIC of the target organism (e.g. *T* > MIC 40 % is serum concentrations exceed the target organism MIC only 40 % of the time (Van Herendael et al., 2012)). In chapter two, selection for resistance plateaued at sub-inhibitory concentrations, meaning that *bla*$_{\text{CTX-M}}$ genes were selected just as strongly at sub-inhibitory concentrations as those exceeding the clinical breakpoint. Therefore *T* < MIC of antibiotics during chemotherapy may be even more crucial than previously described. If a similar situation occurs for β-lactamase inhibitor (βLI) concentrations, this could result in increased selection for β-lactamases as a result of preferential binding to extracellular β-lactamases, as hypothesised in chapter two. Furthermore, no work has investigated the MECs of βLIs, and the potential role these could play in selecting for β-lactamase mediated resistance in the environment, despite previous work showing increased selection for resistance at sub-inhibitory antibiotic concentrations and very low βLI concentrations (Yurtsev et al., 2013).

Antibiotic resistance has been described as the 'quintessential' One Health issue (Robinson et al., 2016). The One Health approach is described by the American Veterinary Medicine Association (AVMA) as "...the collaborative effort of multiple disciplines – working locally, nationally, and globally – to attain optimal health for people, animals and our environment…" (AVMA, 2008). Therefore, it is crucial to consider the role of the environment in human and animal health.

It has been shown that clinically important, highly mobile resistance genes, such as the *bla*$_{\text{CTX-M}}$ genes, originated in environmental bacterial species (Humeniuk et al., 2002, Cantón et al., 2012, Olson et al., 2005). Therefore, isolated mobilisation events in the environment do occur, with clinical implications. There have been limited studies which have investigated transmission between the natural environment, humans and animals. For example, the risk of MRSA colonisation in pig farm workers increases by 760x compared to the general population (Voss et al., 2005), and recreational use of different, natural environments can result in exposure to resistant pathogens

(Leonard et al., 2015, Jones et al., 2017). This thesis has shown that MECs of some antibiotics have the potential to select for resistance. Increased prevalence of resistance in the environment results in increased probability of 'isolated' mobilisation events; and / or contact between people or animals and the environmental resistome. It seems reasonable then, to consider environmental protection against resistance selection synonymous to protection of human and animal health against resistant infections.

Much work is still required to determine the conditions under which transmission of antibiotic resistance from the natural environment to humans and animals occurs. Risk of colonisation and infection following environmental exposure should also be determined. Knowledge of this interplay between the environment and human and animal health will greatly impact investment by stake holders towards any mitigation strategies that are required, once the selective risk of antibiotics in the environment has been fully and appropriately assessed.

## 6.4. Concluding remarks

This research suggests there is a risk of selection and co-selection for antibiotic resistance occurring in the environment, for particular compounds and in certain environmental hotspots. A method to rapidly generate further data has been described. The direct implications of this in terms of animal and human health were not investigated in this thesis, but will be essential for convincing stake holders to design and undertake the necessary mitigation strategies. The purpose of this research is a vital one – to prolong the utility of current antibiotics and safeguard novel compounds by delaying resistance emergence for as long as possible. Only time will show if our response has been swift enough, but currently, time is ticking towards the eventuality of a post-antibiotic era.

## Appendix

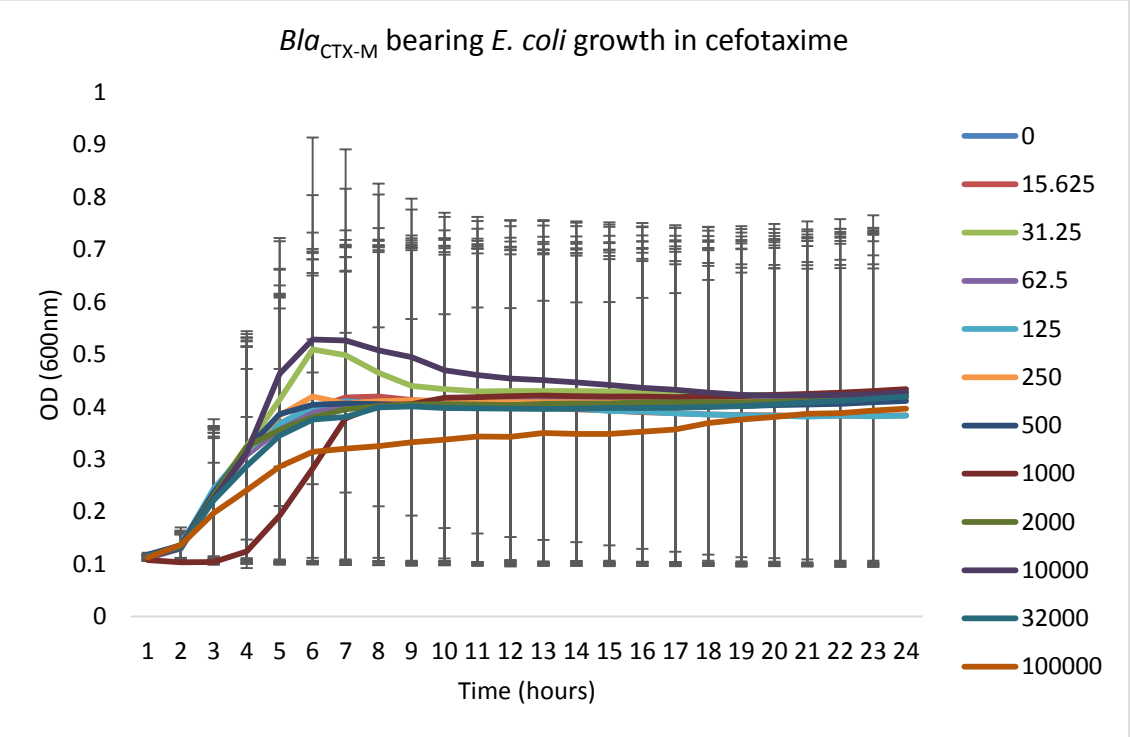


Figure 63. Pilot experiment for determining the dilution of untreated waste water to use for the complex community experiments. Average (n=2) growth of *bla*$_{CTX-M}$ bearing *E. coli* in cefotaxime (µg/L) over 24 hours, shown with standard deviation.

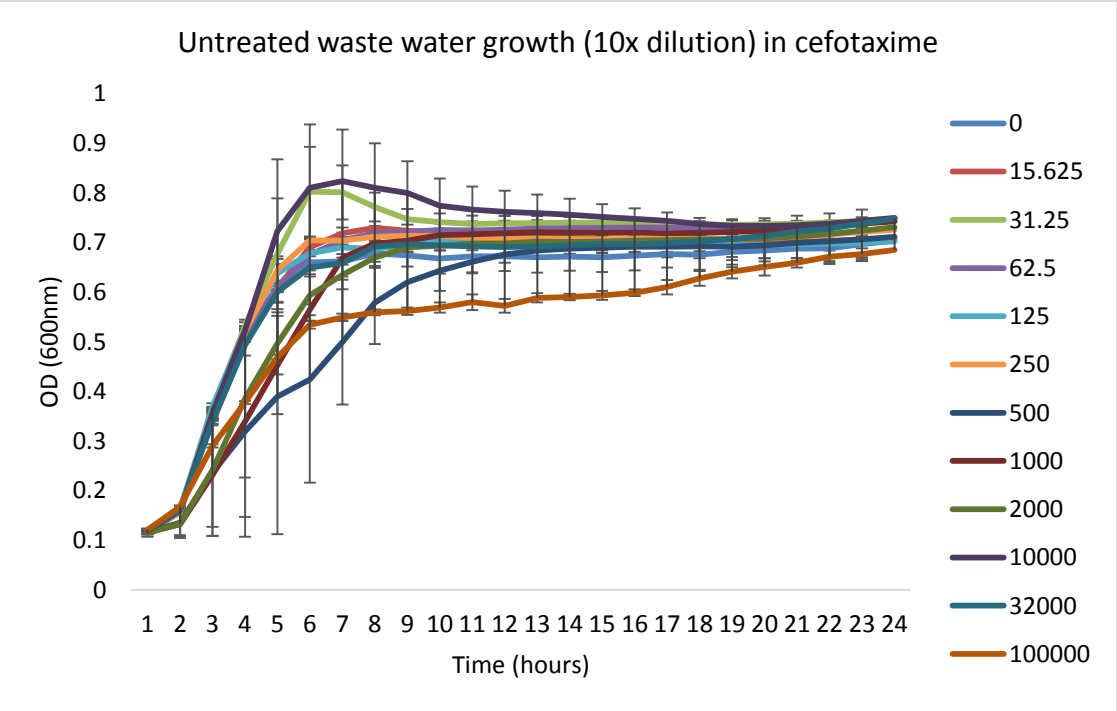

Figure 64. Pilot experiment for determining the dilution of untreated waste water to use for the complex community experiments. Average (n=2) growth of untreated waste water inoculated at a 10x dilution in cefotaxime (µg/L) over 24 hours, shown with standard deviation.

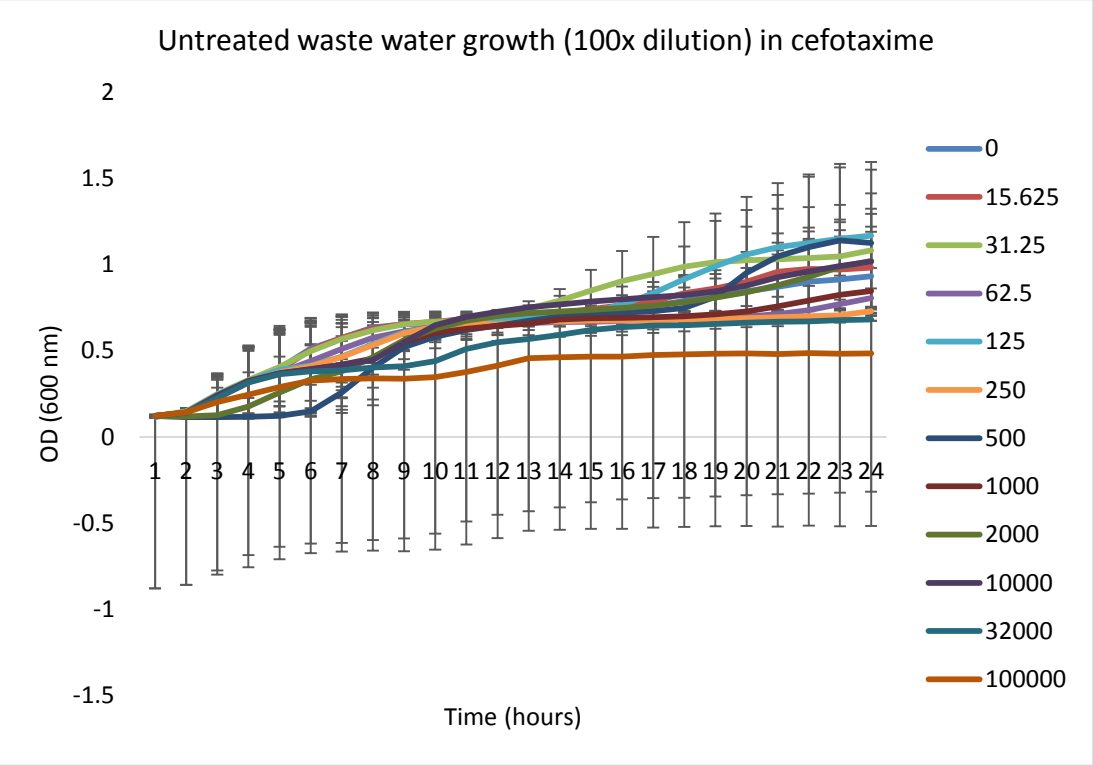


Figure 65. Pilot experiment for determining the dilution of untreated waste water to use for the complex community experiments. Average (n=2) growth of untreated waste water inoculated at a 100x dilution in cefotaxime (µg/L) over 24 hours, shown with standard deviation.

Table 16. Table showing primer sequences used for amplifying UDP-galactose-4-epimerase-like ORFs to be cloned into the pET101 vector.

| Reaction/name | Primer sequence | Product size (bp) |
|---|---|---|
| Cloning/R11F | CACCATGCGTGTGCTCGTC | 1,025 |
| Cloning/R11R | TTACACATACCCTTGCGG | 1,025 |
| Cloning/R161F | CACCATGCGCGTTTTAGTAACAGG | 1,020 |
| Cloning R161R | TTACAAGCCAGCAGCGCCCC | 1,020 |
| HotPhusion/R161F2 | TCAGGAGCCCTTCACCATGCGCGTTTTAGTAACAGG | 1,040 |
| HotPhusion/R161R2 | AATTGAGCTCGCCCTTACAAGCCAGCAGCGCCCC | 1,040 |
| Cloning/S78F | CACCATGAACGGGAAGGTAATAG | 1,043 |
| Cloning/S78R | TCATGATTTCTTGTTTTTCAGTTTGTTTTCCC | 1,043 |
| HotPhusion/S78F2 | TCAGGAGCCCTTCACCATGAACGGGAAGGTAATAG | 1,053 |
| HotPhusion/S78R2 | AATTGAGCTCGCCCTCATGATTTCTTGTTTTTCAGTTTGTTTTCCC | 1,053 |

Table 17. Co-resistance experiment. CTAB resistant clones and a random selection of unscreened clones from RB and SC libraries were replica plated onto ampicillin ('AMP') and trimethoprim ('TRMP') at different concentrations. Percent resistance (n = 200). Increased resistance at 1.5x MIC compared to at MIC could be explained by stress-induced expression of resistance determinants or spontaneous mutation in the host.

| | Percentage resistance | | | | | |
|---|---|---|---|---|---|---|
| | AMP | | | TRMP | | |
| | MIC (4 mg/L) | 1.5x MIC (7 mg/L) | Clinical (8 mg/L) | MIC (1.25 mg/L) | 1.5x MIC (2.25 mg/L) | Clinical (4 mg/L) |
| RB | 82.3 | 94 | 0 | 76.8 | 94.6 | 0 |
| RB-CTAB$^R$ | 41.9 | 94 | 0 | 61.7 | 94.5 | 0 |
| SC | 92.9 | 71.6 | 0 | 90.5 | 89.1 | 0 |
| SC-CTAB$^R$ | 61.7 | 82.6 | 0 | 37.6 | 94.8 | <1 |

Table 18. All the ORFs identified by primer walking all unique BKC resistant inserts. Shown is the name of the predicted protein, the % identity to the entry in the GenBank database, and the accession number for this highest hit. For some ORFs, multiple predicted proteins are shown with coverage in brackets. ORFs of particular interest (antimicrobial resistance genes or genes that logically could have roles in conferring resistance) are marked by '***'.

| Insert | ORF | % ID | Accession |
|---|---|---|---|
| GBKC *** | UDP-glucose 4-epimerase [Chlorogloeopsis fritschii] | 61 | WP_016875182.1 |
| *** | UDP galactose 4-epimerase (EC 1.7.7.12) [Streptomyces lividans] | 52 | AAA26747.1 |
| *** | pyridoxal-dependent decarboxylase [Candidatus Koribacter versatilis] | 54 | WP_011522086.1 |
| *** | pyridoxal-dependent decarboxylase [Candidatus Koribacter versatilis] | 70 | WP_011522086.1 |
| | permease [Acetobacterium dehalogenans] | 38 | WP_035356200.1 |
| | sulfur reduction protein DsrE [Dehalococcoidia bacterium SCGC AB-539-J10] | 28 | WP_029475985.1 |
| | hypothetical protein [Burkholderia gladioli] | 32 | WP_046578597.1 |
| | hypothetical protein [Kitasatospora phosalacinea] | 41 | WP_033256445.1 |
| | hypothetical protein [Streptomyces sp. MspMP-M5] | 39 | WP_018536861.1 |
| | | | |
| R2 *** | peptide ABC transporter substrate-binding protein [Pseudomonas veronii] | 96 | WP_046488955.1 |
| | probable polyvinylalcohol dehydrogenase [Gimesia maris] | 48 | WP_002649604.1 |
| | nitrilase [Candidatus Solibacter usitatus] | 77 | WP_011684136.1 |
| | hypothetical protein [Phyllobacterium sp. UNC302MFCol5.2] with Domiain of Unknown Function (DUF) | 42 | WP_027231546.1 |
| | transcriptional regulator [Pseudomonas veronii] | 100 | WP_046381648.1 |

| | | | |
|---|---|---|---|
| *** | peptide ABC transporter substrate-binding protein [Pseudomonas veronii] | 84 | WP_046381647.1 |
| *** | peptide ABC transporter substrate-binding protein [Pseudomonas veronii] | 88 | WP_017846050.1 |
| *** | UDP-glucose 4-epimerase [Planctopirus limnophila] | 57 | WP_013109608.1 |
| | aldehyde dehydrogenase [Pseudomonas veronii] | 100 | WP_046381649.1 |
| | MULTISPECIES: 50S ribosomal protein L28 [Pseudomonas] | 100 | WP_003176907.1 |
| *** | UDP-glucose 4-epimerase [Planctopirus limnophila] | 68 | WP_013109608.1 |
| | | | |
| R3 | divalent ion tolerance protein CutA [Azoarcus sp. BH72] | 73 | WP_011764057.1 |
| *** | UDP-galactose 4-epimerase [uncultured prokaryote] | 72 | BAL57187.1 |
| | 2-hydroxyhepta-2,4-diene-1,7-dioate isomerase [Anaerolinea thermophila] | 61 | WP_013559783.1 |
| | glutamyl-tRNA(Gln) amidotransferase [Anaerolinea thermophila] | 75 | WP_013558748.1 |
| | 2-hydroxyhepta-2,4-diene-1,7-dioate isomerase [Anaerolinea thermophila] | 60 | WP_013559783.1 |
| | thiol-disulfide interchange protein [Sulfuritalea hydrogenivorans sk43H] | 72 | BAO31229.1 |
| | riboflavin synthase subunit alpha [Sulfuritalea hydrogenivorans] | 85 | WP_041101012.1 |
| | chemotaxis protein [Massilia sp. LC238] | 44 | WP_036215602.1 |
| ***FIST DOMAIN | hypothetical protein AZKH_2025 [Azoarcus sp. KH32C] | 52 | BAL24338.1 |
| | Uncharacterised protein [Bordetella pertussis] | 56 | CFD85428.1 |
| | PREDICTED: probable ribonuclease ZC3H12D isoform X3 [Macaca nemestrina] | 43 | XP_011751866.1 |
| *** | hypothetical protein AW09_003777 [Candidatus Accumulibacter sp. BA-91]<br><br>penicillin-binding protein [Rhodococcus sp. ARP2] | 62<br><br>30 | KFB71096.1<br><br>WP_047889119.1 |
| | DUF1745 domain-containing protein [Methylobacter tundripaludum] | 65 | WP_006893638.1 |
| | | | |
| R10 | glycerol kinase [Perlucidibaca piscinae] | 77 | WP_022956698.1 |
| | hypothetical protein [Citromicrobium bathyomarinum] | 47 | WP_040378562.1 |
| *** | UDP-galactose-4-epimerase [Bacillus selenatarsenatis] | 72 | WP_041963976.1 |
| | UDP-glucose 6-dehydrogenase [Acidovorax delafieldii] | 28 | WP_005794330.1 |
| *** | hypothetical protein [Bacillus thuringiensis]<br><br>transposase [Escherichia coli]<br><br>beta galactosidase alpha [Cloning vector pNOT218] | 62<br><br>68<br><br>67 | WP_013555072.1<br><br>WP_012766388.1<br><br>AAL79196.1 |
| *** | UDP-galactose-4-epimerase [Rhodopirellula sallentina] | 68 | WP_044303185.1 |
| | hypothetical protein [Balneatrix alpica] | 52 | WP_027312693.1 |
| | glucose-6-phosphate isomerase [Psychrobacter cryohalolentis] | 60 | WP_011512493.1 |
| *** | ABC transporter permease [Streptomyces galbus] | 63 | WP_033524975.1 |
| | | | |
| R14<br><br>*** | hypothetical protein [Firmicutes bacterium CAG:646]<br><br>UDP-galactose-4-epimerase [Dasania marina] | 70<br><br>71 | WP_022010496.1<br><br>WP_019530321.1 |
| | putative polysaccharide transport system component signal peptide protein (ragA) [Ralstonia solanacearum PSI07] | 42 | CBJ50510.1 |
| | | | |
| R17 | hypothetical protein [Nocardioides insulae] | 57 | WP_028660028.1 |
| | hypothetical protein [Burkholderiales bacterium GJ-E10] | 60 | WP_045469855.1 |
| | hypothetical protein [Rubrivivax gelatinosus] | 40 | WP_050985547.1 |

| | | | |
|---|---|---|---|
| | hypothetical protein [Aquincola tertiaricarbonis] | 47 | WP_046115559.1 |
| *** | conserved hypothetical protein [Novosphingobium sp. KN65.2] | 52 | CDO35116.1 |
| | Ankyrin repeat and death domain-containing protein 1A [Pteropus alecto] | 51 | ELK05332.1 |
| *** | hypothetical protein [Aphanizomenon flos-aquae]<br><br>UDP-galactopyranose mutase [Haliscomenobacter hydrossis] | 64/65<br><br>51/62 | WP_027402397.1<br><br>WP_013765800.1/ WP_013765800.1 |
| *** | MULTISPECIES: membrane protein [Streptomyces] | 55 | WP_037659172.1 |
| | | | |
| R24 *** | UDP-glucose 4-epimerase [Pseudomonas sp. 11/12A] | 69 | WP_047530443.1 |
| | aminoglycoside phosphotransferase [Streptomyces aureofaciens] | 33 | WP_052838580.1 |
| | protein tyrosine kinase [Pseudomonas sp. 11/12A] | 92 | WP_047530445.1 |
| | hypothetical protein [Pseudomonas sp. 11/12A] | 98 | WP_047530451.1 |
| *** | sulfatase [Pseudomonas sp. 11/12A] | 88 | WP_047530441.1 |
| | protein tyrosine kinase [Pseudomonas sp. 11/12A] | 91 | WP_047530445.1 |
| *** | lipopolysaccharide biosynthesis protein [Pseudomonas sp. 11/12A] | 99 | lipopolysaccharide biosynthesis protein [Pseudomonas sp. 11/12A] |
| *** | sugar ABC transporter substrate-binding protein [Pseudomonas sp. 11/12A] | 97 | WP_047530453.1 |
| *** | sulfatase [Pseudomonas sp. 11/12A] | 96 | WP_047530441.1 |
| | | | |
| S2 *** | MFS transporter family protein [Candidatus Cloacimonas acidaminovorans] | 36 | WP_015425243.1 |
| *** | UDP-glucose 4-epimerase [Anaerolinea thermophila] | 74 | WP_013561327.1 |
| | hypothetical protein [bacterium JGI-5]<br><br>protease [Peptococcaceae bacterium BICA1-7] | 58<br><br>56 | WP_047133258.1<br><br>WP_034123852.1 |
| | | | |
| S3 *** | oxidoreductase [Hassallia byssoidea] | 67 | WP_039746039.1 |
| *** | UDP-galactose-4-epimerase [Draconibacterium orientale] | 66 | WP_038558124.1 |
| | dTDP-glucose 4,6-dehydratase [Thermophagus xiamenensis] | 70 | WP_010526628.1 |
| | UDP-N-acetyl-D-galactosamine dehydrogenase [Adhaeribacter aquaticus] | 68 | WP_026462223.1 |
| | | | |
| S4 | hypothetical protein [Escherichia coli] | 87 | WP_052913615.1 |
| <br><br>*** <br><br>*** | hypothetical protein UUU_05190 [Klebsiella pneumoniae subsp. pneumoniae DSM 30104]<br><br>Multidrug resistance protein mdtB [Erwinia amylovora MR1]<br><br>membrane protein [Thiorhodococcus sp. AK35] | 58<br><br>42<br><br>49 | EJK92533.1<br><br>CCP06064.1<br><br>WP_043755805.1 |
| | FIG00732864: hypothetical protein [Klebsiella pneumoniae IS10] | 55 | CDK62066.1 |
| | hypothetical protein WRSd5_03586 [Shigella dysenteriae WRSd5] | 95 | ESU79657.1 |
| *** | tetracycline resistance protein, class A [Escherichia coli BWH 24] | 93 | ERO93707.1 |
| | hypothetical protein [Salmonella enterica] | 100 | WP_001372230.1 |
| | hypothetical protein HMPREF9551_04418 [Escherichia coli MS 196-1] | 100 | EFI86627.1 |
| *** | UDP-glucose 4-epimerase [Anaerolinea thermophila] | 68 | WP_013561327.1 |
| *** | MULTISPECIES: MFS transporter [unclassified Cloacimonetes] | 42 | WP_029949877.1 |
| *** | UDP-galactose 4-epimerase [Leptolinea tardivitalis] | 76 | GAP20355.1 |
| | lactose operon repressor domain protein [Staphylococcus aureus Lyso 2 2010] | 93 | KEK30836.1 |
| *** | tetracycline resistance MFS efflux pump [Escherichia coli] | 100 | WP_011178609.1 |

| | | | |
|---|---|---|---|
| *** | putative transposase [Escherichia coli 3-020-07_S4_C1] | 100 (42% cover) | KEJ62634.1 |
| | transcriptional regulator AraC [Broad host range vector pMLBAD] | 99 | AAM63382.1 |
| | MULTISPECIES: hypothetical protein [Enterobacteriaceae] | 100 (86% cover) | WP_000028208.1 |
| | hypothetical protein [Escherichia coli] | 96 (44% cover) | WP_012644004.1 |
| <br><br>*** | Transcriptional regulator AraC [Shigella sonnei]<br><br>zinc ABC transporter ATPase [Salmonella enterica subsp. enterica serovar Enteritidis str. EC20121178] | 96<br><br>67 | CSP96607.1\|<br><br>AHO12049.1 |
| *** | uncharacterized membrane protein [Longilinea arvoryzae] | 66 | GAP15674.1 |
| *** | tetracycline repressor protein class A transposon 1721 [Klebsiella pneumoniae] | 95 | KMH64585.1 |
| | hypothetical protein HPMG_01967 [Helicobacter pullorum MIT 98-5489] | 60 | EEQ64510.1 |
| | fumarate hydratase [delta proteobacterium MLMS-1] | 50 | WP_007295173.1\| |
| *** | tetracycline repressor protein class A [uncultured bacterium] | 96 (80% cover) | AFR44371.1 |
| | conserved hypothetical protein [Escherichia coli 042] | 100 | CBG36934.1\| |
| | LacOPZ-alpha peptide from pUC9; putative [unidentified cloning vector] | 93 (84% cover) | AAA75561.1 |
| | hypothetical protein [Methanosarcina mazei]<br><br><br><br>pyruvate formate lyase-activating enzyme 1 [Escherichia coli KO11FL] | 72 (96 cover)<br><br>74 (62 cover) | WP_048049241.1<br><br><br><br>AFH18616.1 |
| | hypothetical protein [Achromobacter arsenitoxydans]<br><br>putative acetyltransferase YhhY [Salmonella enterica subsp. enterica serovar Typhimurium str. DT104] | 100 (40% cover) | WP_008166363.1<br><br>CQA99509.1 |
| *** | UDP-glucose 4-epimerase [Anaerolinea thermophila] | 68 | WP_013561327.1 |
| | PREDICTED: E3 ubiquitin-protein ligase synoviolin-like [Nicotiana sylvestris] | 34 | \|XP_009786500.1\| |
| *** | MFS transporter family protein [Candidatus Cloacimonas acidaminovorans] | 36 | WP_015425243.1 |
| *** | UDP-galactose 4-epimerase [Leptolinea tardivitalis] | 76 | GAP20355.1 |
| *** | uncharacterized membrane protein [Longilinea arvoryzae] | 66 | GAP15674.1 |
| | titin2 [Bombyx mori] | 36 | NP_001091843.1\| |
| | | | |
| S21 | ferrochelatase [Photobacterium damselae] | 48 | WP_036764187.1 |
| | UDP pyrophosphate phosphatase [Thalassolituus oleivorans] | 63 | WP_025266051.1 |
| | tyrosine protein kinase [Pseudomonas sp. GM49] | 51 | WP_007999450.1 |
| | phosphotyrosine protein phosphatase [Pseudomonas pelagia] | 52 | WP_022964347.1 |
| *** | MULTISPECIES: UDP-galactose-4-epimerase [Alcanivorax] | 70 | WP_035458552.1 |
| | hypothetical protein [Pseudomonas nitroreducens] | 55 | WP_017521157.1 |
| *** | capsular polysaccharide biosynthesis protein [Pseudomonas fluorescens] | 53 (97 cover) | WP_003211361.1 |
| *** | capsular polysaccharide biosynthesis protein [Pseudomonas sp. M1] | 44 (67) | ETM67913.1 |
| | | | |

Table 19. Predicted ORFs' function and amino acid identity, and the numbers of knock outs containing this ORF.

| No. knock outs | ORF name | % amino acid identity |
|---|---|---|
| 10 | UDP-galactose-4-epimerase | 66 - 99 |
| 4 | 'Hypothetical protein' | 33 - 98 |
| 2 | Drug/metabolite transporter permease/PecM-like protein | 96 - 99 |
| 2 | KAP family P-loop domain protein | 87 - 98 |
| 2 | Putative MFS transporter protein | 36 - 40 |
| 1 | Multidrug transporter/transporter permease/PecM-like protein/integral membrane protein | 96 - 98 |
| 1 | Putatative membrane-associated metal-dependent hydrolase | 78 |
| 1 | Phage T7 exclusion protein | 78 |
| 1 | Oxidoreductase | 69 |
| 1 | Putative polysaccharide transport system component signal protein | 46 |
| 1 | Quinone/putative oxidoreductase | 34 |

Table 20. Table showing sequence similarity between UDP-galactose-4-epimerases identified in this study and in the study by Kazimierczak et al. (2009) ('Clone 9' and 'Clone 15'). M = Megablast, n = blastn. The first number is the sequence identity, the second number following the / is the percentage coverage. Alignment was performed with NCBI blastn for alignment of two sequences.

| | R76 | R87 | R109 | R147 | R161 | R241 | S59 | S62 | S70 | S78 | S167 | S178 | S188 | S2-1 | Rb14-46 | Clone 9 | Clone 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| R11 | 94 N/3 | 99 M | 66N/10 | 93N/2 | 66N/38 | 66N/10 | 90N/1 | 100 N/1 | 100 N/1 | 67N/10 | 93N/1 | 100 N/1 | 67N/9 | 76N/2 | 68N/30 | NO ID | NO ID |
| R76 | | 94 N/2 | 98M | 93N/0 | 86N/2 | 99M | 100 N/1 | 97N/8 | 100 N/0 | 100 N/1 | 100 N/0 | 91N/9 | 100 N/1 | 100 N/0 | 86N/1 | 94N/4 | NO ID |
| R87 | | | 66N/10 | 93N/1 | 66N/44 | 66N/10 | 90N/2 | 100 N/2 | 93N/2 | 67N/9 | 93N/2 | 100 N/2 | 67N/9 | 76N/3 | 68N/31 | NO ID | NO ID |
| R109 | | | | 100 N/1 | 86N/1 | 98M | 100 N/2 | 97N/7 | 100 N/1 | 100 N/1 | 100 N/1 | 91N/7 | 100 N/1 | 100 N/0 | 86N/0 | 81N/7 | 74N/6 |
| R147 | | | | | 100 N/2 | 100 N/2 | 93N/3 | 100 N/1 | 100 N/1 | 93N/1 | 100 N/1 | NO ID | 93N/1 | 100 N/1 | 100N/2 | NO ID | 100N/1 |
| R161 | | | | | | 86N/2 | NO ID | NO ID | NO ID | 100 N/1 | NO ID | NO ID | 100 N/0 | NO ID | 98M | NO ID | 70N/25 |
| R241 | | | | | | | 100 N/2 | 97N/7 | NO ID | 100 N/1 | NO ID | 91N/9 | 100 N/1 | NO ID | 86N/1 | 80N/7 | NO ID |
| S59 | | | | | | | | 100 N/2 | 100 N/1 | 79M | 100 N/0 | 100 N/2 | 78M | 100 N/0 | NO ID | 81N/2 | 100N/1 |
| S62 | | | | | | | | | 98M | 94N/4 | 98M | 95M | 100 N/1 | 98M | 100N/0 | 93N/1 | NO ID |
| S70 | | | | | | | | | | 94N/2 | 99M | 95M | NO ID | 98M | 83/3 | 93N/1 | NO ID |
| S78 | | | | | | | | | | | 94N/2 | 94N/2 | 98M | 94N/2 | 100N/0 | 66N/55 | NO ID |
| S167 | | | | | | | | | | | | 95M | 100 N/0 | 98M | 93N/0 | 93N/1 | NO ID |

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