Selection for antibiotic resistance in complex microbial communities

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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

Measureable concentrations of antibiotics are released into the environment from anthropogenic sources. Environmental risk assessment investigates the risks these concentrations pose to aquatic life but does not determine whether selection for resistance is occurring. Recent studies suggest environmental concentrations of tetracycline, ciprofloxacin and cefotaxime may be able to select for resistance in complex microbial communities.

The aims of research presented in this thesis were to determine whether selection for resistance occurs at environmentally relevant concentrations of macrolide antibiotics; to understand how mixtures of antibiotics affect selective endpoints; to understand the effect of temperature on selective endpoints, and, finally, to compare the methods used with previously published methods.

Selective endpoints of macrolide antibiotics were found to be 1,000 µg/L of azithromycin and erythromycin and 750 µg/L of clarithromycin which is significantly higher than current environmental concentrations.

Mixing of antibiotics produces at least an additive, if not a synergistic, effect. The selective endpoints of sulfamethoxazole and trimethoprim decrease over 11 times and by a half, respectively, when used in combination. The selective endpoint of macrolides is reduced by a third when they are found in combination.

Selective endpoints of individual genes are affected by temperature but, as only preliminary data has been produced, overall effect concentrations have not been determined across the entire experimental resistome. Alternative gene

targets associated with taxa favoured at low temperatures may be under selection.

Finally, the phenomenon of increased persistence has been described and the minimal increased persistence concentration has been defined for the first time.

Data presented here can be used by policy makers in environmental risk assessments, in conjunction with other ecotoxicological endpoints to determine safe release levels of antibiotic residues in wastewater. This will help minimise selection for antibiotic resistance in the environment and, therefore, exposure of humans to resistant bacteria through environmental transmission.

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Author’s Declaration

All of the work presented in this thesis is the author’s own work under the supervision of named supervisors. Work undertaken as part of a collaboration, by other members of the research team or with the assistance of visiting students has been highlighted in the appropriate section.

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Definitions

Term	Definition
Active pharmaceutical ingredient(s)	The component (or components) of a pharmaceutical that produces the effect
Anthropogenic pollution	Pollution that originates from human activity
Antibiotic	An antimicrobial that inhibits or kills bacteria
Antibiotic resistance	The ability of a bacteria to grow in the presence of an antibiotic
Antibiotic resistance gene	A gene that encodes a protein which is able to prevent the activity of an antibiotic
Antimicrobial	A drug used to inhibit or kill a microorganisms such as bacteria, fungi, viruses or protozoa (used interchangeably throughout this thesis with “Antibiotic”)
Antimicrobial resistance	The ability of a microorganism to grow in the presence of an antimicrobial (used interchangeably throughout this thesis with “Antibiotic Resistance”)
Assessment factor	A value applied to results from ecotoxicology experiments to account for a degree of uncertainly
Bacteria	A diverse group of unicellular, microscopic microorganisms which lack organelles
Bactericidal	An antibiotic that kills bacteria
Bacteriostatic	An antibiotic that inhibits the growth of bacteria
Biofilm	A thin layer of a bacterial community (and other microorganisms) that has adhered to a solid surface

Commensal bacteria	Bacteria that live within another organism
Co-selection	The selection by an antibiotic of multiple resistance genes from different antibiotic classes
Ecotoxicology	The study of the potential harmful effects of chemicals on the biological organisms found in the environment
Emerging contaminant	Contaminants that enter water environments from anthropogenic pollution (such as wastewater treatment plants or agricultural runoff)
Fitness advantage	The advantage to an organism's ability to survive by harbouring a particular gene or trait
Fitness cost	The disadvantage to an organism's ability to survive by harbouring a particular gene or trait
Horizontal Gene Transfer (HGT)	The transfer of genetic material between bacterial cells and species that is not parent to offspring transmission
Integron	A mobile genetic element that are found in bacteria and are defined by an *intI* gene, a recombination site and a promotor
In vitro	Experimental work that is undertaken outside of a natural organism.
Lowest Observed Effect Concentration (LOEC)	The lowest concentration of a pharmaceutical tested at which a response is observed
Minimal Increased Persistence	The lowest concentration of an antibiotic required to see an increase of persistence (or differential rates

Concentration (MIPC)	of negative selection) of resistance genes
Minimal Selective Concentration (MSC)	The lowest concentration of an antibiotic required to see an increase in positive selection
Minimum Inhibitory Concentration (MIC)	The lowest concentration of an antibiotic that is required to produce a visible reduction in growth of bacteria
Mobile Genetic Element (MGE)	Genetic material that is able to move around within the genome and can be passed between different cells and species
No Observed Effect Concentration (NOEC)	The highest concentration of a pharmaceutical where no effect is seen or the concentration tested below the LOEC
Pathogenic	Disease causing
Plasmid	A genetic element that is capable of replicating independent of the chromosome. It is typically circular and small and is often found in bacteria
Polymerase Chain Reaction (PCR)	An experimental technique that allows the amplification of a DNA target in which multiple rounds of heating and cooling are used to denature the DNA, to anneal DNA primers to the DNA strands and to extend and copy the DNA template using nucleotide bases and a polymerase enzyme.
Predicted Environmental Concentration (PEC)	The expected environmental concentration of a substance. Predictions take into account the concentration present and the concentration being inputted in the environment, the spread of the

	substance through the environment and removal and/or degradation rate of the substance.
Predicted No Effect Concentration (PNEC)	The concentration of antibiotic at which no adverse effect is expected to be seen. In this case, where an assessment factor is applied to the No Observed Effect Concentration.
Quantitative Polymerase Chain Reaction (qPCR)	An experimental technique based on the PCR technique that is able to monitor the amplification of DNA in real time
Resistome	All antibiotic genes found in a given area in both pathogenic and non-pathogenic bacteria
Risk Quotient (RQ)	An estimation of risk that is calculated from the ratio of predicted environmental concentration to the predicted no effect concentration
Selective endpoint	The concentration at which selection is first observed. This can be a LOEC when using statistical means or an MSC when defining using a selection coefficient graph.

Abbreviations

Abbreviation	Definition
AMR	Antimicrobial resistant
ANOVA	Analysis of variance
API(s)	Active pharmaceutical ingredient(s)
ARB	Antibiotic resistant bacteria
ARG(s)	Antibiotic resistance gene(s)
ASRIT	Activated sludge respiration inhibition test
AZ	Azithromycin
bp	Base pairs
CDC	Centers for Disease Control and Prevention
CLA	Clarithromycin
EMEA	European Medicines Agency
epicPCR	Emulsion, Paired, Isolation and Concatenation polymerase chain reaction
ERY	Erythromycin
GLM	Generalised Linear Model
HGT	Horizontal Gene Transfer
IPCC	Intergovernmental Panel on Climate Change
LOEC(s)	Lowest Observable Effect Concentration(s)

MEC(s)	Measured Environmental Concentration(s)
MEC_{max}	Maximum measured environmental concentration
MEC_{mean}	Mean measured environmental concentration
MEC_{median}	Median measured environmental concentration
MGE(s)	Mobile Genetic Element(s)
MIC(s)	Minimum Inhibitory Concentration(s)
MLS gene(s)	Macrolide, lincosamide and streptogramin resistance genes
MIPC(s)	Minimal Increased Persistence Concentration(s)
MSC(s)	Minimal Selective Concentration(s)
NOEC(s)	No Observed Effect Concentration(s)
$NOEC/LOEC/MSC_i$	NOEC/LOEC/MSC of the individual compound
$NOEC/LOEC/MSC_m$	NOEC/LOEC/MSC of compound in mixture
OD	Optical Density
OECD	Organisation for Economic Co-operation and Development
OIE	World Organisation for Animal Health
PEC(s)	Predicted Environmental

	Concentration(s)
Phage(s)	Bacteriophage(s)
PNEC(s)	Predicted No Effect Concentration(s)
$PNEC_R$(s)	Predicted No Effect Concentration(s) for the selection of resistance
$PNEC_{SW}$	Predicted No Effect Concentration of surface water
qPCR	Quantitative/Real-time polymerase chain reaction
Rpm	Rotations per minute
RQ	Risk Quotient
SMX	Sulfamethoxazole
TET	Tetracycline
TRMP	Trimethoprim
TRMP-SMX	Combination of trimethoprim and sulfamethoxazole
UBA	Umwelt Bundesamt (German Environment Agency)
UN	United Nations
WHO	World Health Organisation
WWTP(s)	Wastewater Treatment Plant(s)

Genes and encoded products

Gene	**Encodes**
16s rRNA	30S subunit of bacterial ribosome
intI1	Class 1 integron
Confers beta-lactam resistance	
bla-CEP-04, bla-CTX-M	ß – lactamase enzyme
Confers ciprofloxacin resistance	
ΔacrR	Deletion of HTH-type regulator gene *acrR*
gyrA1	DNA gyrase subunit A
gyrA2	DNA gyrase subunit A
Confers macrolide resistance	
ereA, ereB	Esterase enzyme
erm(33), erm(35), erm(TR), ermA, ermB, ermC, ermF, ermG, ermT, ermX	rRNA methylase enzyme
macA, macB	Subunit of an efflux pump (ABC transporter)
mef family (*mefA, mefE, mefI, mefO)*	Efflux pump (major facilitator)
mphA, mphC	Phosphortransferase enzyme
msrA, msrC, msrD	Efflux pump (ABC transporter)
Confers sulfamethoxazole resistance	
sul1, sul2, sul3	Dihydropteroate synthase enzyme
Confers tetracycline resistance	
tet32, tetM, tetO, tetQ, tetW	Ribosomal protection protein
tetA, tetG	Efflux pump (major facilitator)

tetJ	Efflux pump
tetL	Efflux pump
Confers trimethoprim resistance	
dfr	Dihydrofolate reductase enzyme

Chapter 1: Introduction

1.1 Background

The discovery of antibiotics during the 20th century has transformed modern medicine (Aminov 2010). None of the classes of antibiotics that have been introduced, however, have escaped the emergence of resistance genes to combat them (Levy & Marshall 2004), (Figure 1). This is not a recent problem: even before penicillin was introduced in the clinic in 1945 (Gould 2016) observations in the laboratory suggested that certain bacterial species had the ability to degrade the compound enzymatically (Aminov 2010; Abraham & Chain 1940).

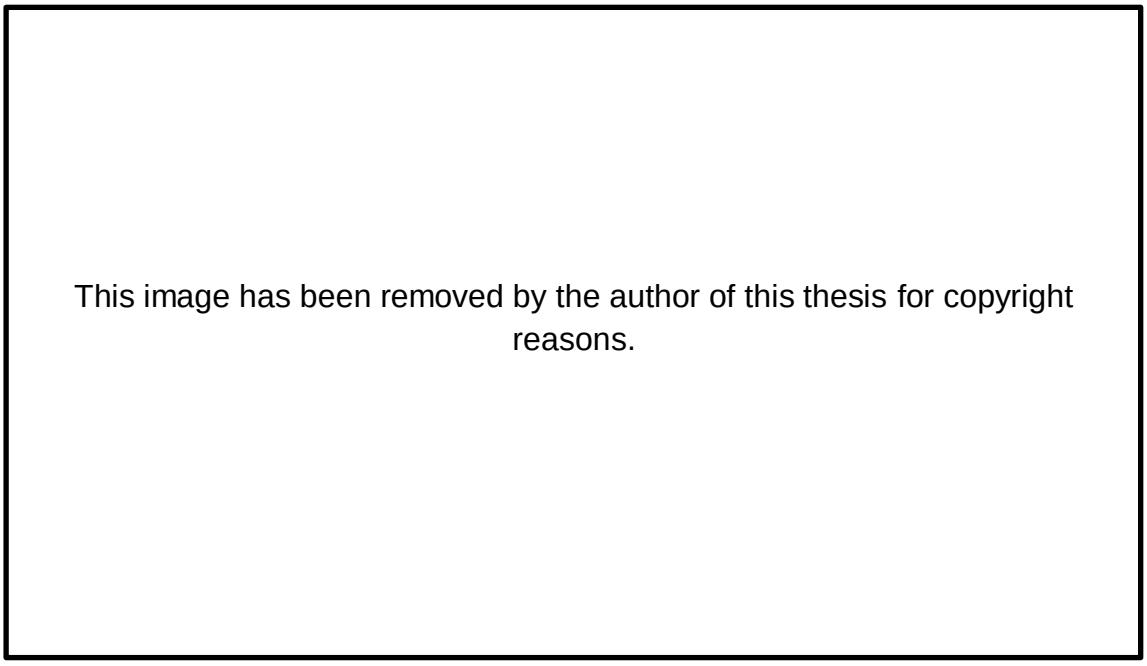


Figure 1: Timeline showing the introduction of an antibiotic class in the clinic and when subsequent resistance arose to that antibiotic class. Figure is reproduced from Clatworthy, Pierson and Hung, 2007.

There has been a rapid increase of the rate of emergence of resistance accompanied by a resulting increase in morbidity and mortality caused by resistant infections (Livermore 2009; Finley et al. 2013; Wellington et al. 2013). Currently, most bacterial infections are still treatable, but resistance has now developed even to "last resort" antibiotics, such as colistin (Thi Khanh Nhu et al. 2016). It is feared that infections that are currently easily treated may become life threatening in the future as the range of effective antibiotics reduces further (Finley et al. 2013; Livermore 2009; Wellington et al. 2013). In 2014, the World Health Organisation (WHO) claimed that antimicrobial resistance (AMR) had become a public health crisis and stated that *"a post-antibiotic era – in which common infections and minor injuries can kill – far from being an apocalyptic fantasy, is instead a very real possibility for the 21st Century"* (WHO 2014). In addition, in work commissioned by the UK Government in 2014 (The Review on Antimicrobial Resistance), it was predicted that AMR will become the leading cause of death by 2050, totalling 10 million deaths per year and a total GDP loss of $100 trillion between 2014 and 2050 (O'Neill 2014).

Antibiotics are important in a wide range of medical and non-medical applications. For example, in addition to the treatment of bacterial infections, there is heavy reliance on these pharmaceuticals for the prevention of infections arising from medical procedures such as organ transplants or when using immunosuppressive drugs in cancer management. In addition, food security will be impacted as global agricultural and aquacultural practices rely heavily on effective antibiotics for disease prevention and treatment in livestock and for growth promotion (Livermore 2009).

1.1.1 The overuse and misuse of antibiotics

Although the relationship between antibiotic usage and the development of antibiotic resistance is complex, extensive and inappropriate use of antibiotics is one of the main drivers for the development of resistance (Friedman & Whitney 2008).

Inappropriate use of antibiotics includes over-prescription, for example prescribing antibiotics when a patient has a respiratory illness caused by a viral infection (Friedman & Whitney 2008). The Longitude Prize charity has conducted surveys within the United Kingdom and found that 55% of GPs feel pressure from patients to prescribe antibiotics, 44% of total GPs surveyed prescribing them to make a patient leave the appointment and 45% prescribing for viral infections when they were aware that the antibiotics will have no effect (Cole 2014). Furthermore, some countries such as China and India are reported to have high and inappropriate use of antimicrobials (Cui et al. 2017; Van Boeckel et al. 2014). In 2006, approximately 210,000 tons of antibiotics were produced in China and only 30,000 tons of this was exported; the remainder was consumed, with approximately 70% of hospital inpatients treated with antibiotics (Cui et al. 2017).

In addition to inappropriate prescribing by doctors, over the counter availability of antibiotics in some countries inevitably leads to overuse and misuse by a public lacking diagnostic and other specialist knowledge (Aminov 2010). Even in countries with regulation, problems persist: for example, although it is illegal to sell antibiotics without prescription in Spain, they are available over the counter in some pharmacies (Llor & Cots 2009) and there has been a trend in the

United States for consumers to purchase antibiotics from other countries online (Mainous et al. 2009).

1.1.2 Current trends in clinical resistance

The effect of use and overuse of antibiotics is already visible. The extensive use of these drugs clinically and in agriculture in the last 75 years has increased the selective pressure on both disease-causing and non-pathogenic bacteria (Laxminarayan et al. 2013). There are many different surveillance programmes currently being undertaken such as “ResistanceMap” (The Center for Disease Dynamics Economics & Policy 2018) and the Global Antimicrobial Resistance Surveillance System (or GLASS) (WHO 2018) to fully understand the global scale of this problem. Programmes such as these are, however, highly dependent on the number of countries participating and reporting: GLASS, for example, had only 71 countries enrolled in its scheme in December 2018 (WHO 2018) and, therefore, outputs from these programmes may be an underestimation of the true extent of the crisis.

The report commissioned by the UK government (see Section 1.1 above) predicted that, globally, 10 million deaths will occur each year from AMR by 2050, rising from 700,000 in 2014 (O’Neill 2014). One study investigated the effects of resistant infections in the European Union and the European Economic Area. Results showed that, in 2015, there were 671,689 cases of resistant infections reported which caused 33,110 deaths and 874,541 disability adjusted life years. The main groups of people affected were those younger than 1 and older than 65 (Cassini et al. 2019). Another study investigated the change in incidence of resistant blood stream infections in children in Malawi from 1998 to 2017. Over that period, they observed an increase in resistant

infections to all first line antibiotics from 3.4 to 30.2% and for *Klebsiella* spp. from 5.9 to 93.7% (Iroh Tam et al. 2019). In India, where the death rate from infectious diseases is double that of the United States, resistance to antibiotics is high. *E. coli* isolated in the community between 2004 and 2007, was found to have high levels of resistance to ampicillin (75%), naladixic acid (73%) and to trimethoprim and sulfamethoxazole used in combination (59%). An increase in high levels of resistance was also observed in *E. coli* to third-generation cefalosporins (70 to 83%) and fluoroquinolones (78 to 85%) between 2008 and 2013 (Laxminarayan & Chaudhury 2016).

Pandrug resistant infections, which are defined as infections with "*non-susceptibility to all agents in all antimicrobial categories*" (Magiorakos et al. 2012), have also emerged. In August 2015, a female in Nevada, United States, who had recently been hospitalised a number of times during an extended stay in India, was diagnosed with a *Klebsiella pneumoniae* infection. This particular bacterium was resistant to 26 antibiotics tested and resistant intermediately to tigecycline. The only antibiotic that the infection was susceptible to was fosfomycin. As this was only approved for uncomplicated cystitis in the United States, however, it could not be administered and the patient died of septic shock approximately one month after being admitted to hospital (Chen et al. 2017).

All these studies show that there is a trend of increasing resistance in clinical settings which will, ultimately, lead to an increased death rate as more resistance elements emerge and it becomes more difficult to treat multidrug and pandrug resistant infections. Although only a small number are presented here,

there are many other similar studies that have observed an increase in antibiotic resistant infections.

1.1.3 Current strategies to mitigate resistance

An obvious way to overcome the problem of resistant bacteria is to develop new antibiotics (O'Neill 2015b). However, in the past 30 years only 2 new classes of antibiotics (oxazolidinones and cyclic lipopeptides) have been developed (Gupta & Nayak 2014). The investment of time and money needed to bring an antibiotic to market is high (O'Neill 2015b). For pharmaceutical companies, it is, therefore, difficult to produce an economic return given that antibiotics are generally taken for short periods of time, unlike drugs used to treat chronic diseases and treatments for cancer (Braine 2011). This makes the development of new antibiotics an unattractive proposition for pharmaceutical companies (O'Neill 2015b). As a result, focusing on mitigating the development of resistance is an important strategy as it is imperative to protect the current available antibiotics. Governments and non-government organisations have and are still setting out strategies for the control of AMR. The WHO, for example, has proposed a range of strategies which, if implemented, should slow the development of resistance. Policies include reduction of the use of antibiotics in agriculture and antibiotic stewardship initiatives for both community and hospital environments (Finley et al. 2013; Leung et al. 2011). In 2019, the UK government produced its 5 year plan to tackle resistance. Their policies came under three headings "*Reducing need for and unintentional exposure to antimicrobials," "Optimising use of antimicrobials"* and *"Investing in innovation, supply and access to tackle AMR"* (UK Government 2019).

Recently, the environment has been implicated in the dissemination of antibiotics, antibiotic resistant bacteria and resistance genes. Mitigation strategies to limit dissemination of antibiotics into these compartments, and in turn limiting their ability to select for antibiotic resistant bacteria, have been identified in two key documents: the O'Neill report on "Antimicrobials in Agriculture and the Environment: reducing unnecessary use and waste" and a report published by the United Nations (UN) in 2017 entitled "Antimicrobial Resistance: Investigating the Environmental Dimension." Both documents discuss the impact of antibiotics in the environment, the selection for resistance in these areas and potential ways to mitigate this. Whilst the first document focuses on the use of antibiotics in agriculture and aquaculture, the latter goes further than this and also focuses on the release of pharmaceutical residues into the environment (O'Neill, 2015a; Gaze and Depledge, 2017). The UK government's 5 year action plan also discusses ways of reducing the spread of resistance through the environment; the need to support additional research in this area and the need to increase public awareness of the risk to the environmental resistome (UK Government 2019). The role of the environment in the spread of antibiotics, the selection and spread of antibiotic resistant bacteria and genes and the potential for these bacteria to become clinical problems are discussed in more detail below.

1.2 The role of the environment in antibiotic resistance

Antibiotics and antibiotic resistance genes (ARGs) are found throughout environmental settings (Kümmerer 2003; Kümmerer 2004). Antibiotics occur naturally in the environment as they are produced by bacteria, such as the soil dwelling *Actinomycetes,* in order to gain a competitive advantage, for example, in relation to nutrients or space (D'Costa et al. 2006). Bacteria are also able to

utilise antibiotics as a sole carbon source (Dantas et al. 2008) and as signalling molecules (Romero et al. 2011). As antibiotics target a variety of bacterial processes, however, species with the ability to produce them must carry some form of resistance element (intrinsic resistance) as a “self-protection” method to avoid self-destruction by the compounds they are secreting (D’Costa et al. 2006).

As well as natural production of antibiotics and resistance, pollution from anthropogenic sources can lead to the dissemination of resistance genes, alongside detectable concentrations of antibiotics, throughout the natural environment.

1.2.1 The Environmental Resistome

The environmental resistome is defined as “*all ARGs including those circulating in pathogenic bacteria, antibiotic producers, and benign non-pathogenic organisms found either free living in the environment or as commensals of other organisms*” by Wright, 2010.

Resistance genes can be mobilised from the chromosome and are able to move through a diverse range of species by a process called horizontal gene transfer (HGT) (Barlow 2009). HGT of ARGs does not only occur in clinical bacteria (Lerminiaux & Cameron 2019), but also has a role in the spread of ARGs in the environment (X. X. Zhang et al. 2009). HGT occurs by one of three key mechanisms that transfer genes. These are conjugation, transformation and transduction (Thomas & Nielsen 2005). Conjugation is usually considered the key method of HGT in the transfer of ARGs, although more recent evidence suggests that transformation and transduction may play a more important role than originally thought (von Wintersdorff et al. 2016).

Conjugative transfer is a process whereby plasmids are passed directly between two bacterial cells by cell-to-cell junctions and a bacterial cell pore (Thomas & Nielsen 2005). This type of HGT has been known to transfer ARGs since the 1950s (Berglund 2015). After the introduction of antibiotics in the clinic, plasmids bearing ARGs are found to be widespread (Wright 2010). Genes that reside within the chromosome can be mobilised to plasmids, and *vice versa*, by the help of mobile genetic elements (MGEs) and can, therefore, be transferred vertically as bacterial cells divide (Gaze et al. 2013).

MGEs are defined as elements that are able to incorporate DNA from a plasmid into the chromosome, and *vice versa*, or from one genome or plasmid to another. There are four main types of MGEs: integrons; transposons; insertion sequences and integrative and conjugative elements (Partridge et al. 2018). Integrons are defined by the *intI* gene (encoding a recombinase), a recombination site and a promotor. The cassette array found in combination with these genes often includes multiple resistance genes. Integrons can integrate themselves and the associated cassette array via recombination into DNA that contains a recombination site (Partridge et al. 2009). Transposons and insertions sequences are fragments of DNA which are able to insert themselves and resistance genes that are associated with them into new sections of DNA within the host cell (Partridge et al. 2018). Integrative and conjugative elements (ICEs) are also able to excise themselves from a host chromosome and integrate into a new one. These elements have also been found to be associated with the transfer of ARGs (Sultan et al. 2018).

Transformation involves the uptake of DNA from the extracellular environment. This includes uptake into bacterial cells, integration of extracellular DNA into the

host chromosome and the functional expression of that gene within the host bacterial cell. Many human pathogenic bacteria, including *Streptococcus* and *Neisseria*, are able to take up extracellular DNA naturally from the environment (Thomas & Nielsen 2005). Environmentally, the transfer of free DNA may seem unlikely, as DNA is sensitive and can easily degrade. However, stabilisation can occur within the environment by the adhesion to soil and sediment particles (Berglund 2015).

Transduction is the movement of DNA between cells using bacteriophages (phages) as vectors. Phages are viruses that infect bacteria by inserting their own DNA into host bacterial cells and then replicating inside them. Specialised phages are able, when replicating, to produce particles that contain both their own replication machinery and everything they need to survive as well as portions of the bacterial cell chromosome, therefore playing an important role in the evolution of the bacterial species they are infecting. The role of phages in the spread of antibiotic resistance has previously been a relatively neglected area of study. However, recently, metagenome analysis has found virome genes linked to resistance genes for, amongst others, tetracycline and ampicillin (Balcázar 2014).

There are two key types of environmental resistance genes: natural resistance and resistance from anthropogenic pollution. Together, these comprise the environmental resistome.

1.2.1.1 Natural resistance

As stated, environmental microorganisms produce antibiotics for a variety of purposes. This has led to the development of ARGs to provide self-protection for producers and protection for competitors (D'Costa et al. 2006). Antibiotics

were first thought to have been produced naturally by bacteria between 40 million and 2 billion years ago. It would seem to follow, therefore, that ARGs were present and widespread prior to the introduction of antibiotics for the treatment of clinical infections and are able to be found in pristine environments where there is minimal human contact and anthropogenic pollution (D'Costa et al. 2011).

A number of studies have investigated the presence of ARGs in pristine environments. For example, Zeng *et al.*, 2019 used metagenome analysis to investigate presence and diversity of ARGs in animal guts in comparison to two pristine environmental sampling sites (Antarctica soil and Alaskan permafrost). The number of ARGs detected in the pristine environment was significantly lower than in the animal gut microbiomes, although they were always detected (Zeng et al. 2019). Van Goethem *et al.*, 2018 also used a metagenomic approach to investigate ARG presence and diversity in Antarctic soil. Over 17 sites they identified 177 ARGs which typically encoded efflux pumps for both single and multiple drugs. Common inactivation elements were also seen to be effective against aminoglycosides, ß-lactams and chloramphenicol. The authors were not able to identify any mobile genetic element regions associated with the genes found and instead identified the presence of antibiotic biosynthesis genes. This, therefore, leads to the assumption that these genes are harboured by antibiotic producers and are not readily transferable to their competitors (Van Goethem et al. 2018). Finally, D'Costa *et al.*, 2011 used qPCR to investigate ARGs in 30,000 year old, Beringian permafrost. The ARGs found were diverse but commonly conferred resistance to ß-lactams, glycopeptide and tetracycline antibiotics. They concluded, therefore, that antibiotic resistance is an ancient phenomenon (D'Costa et al. 2011).

1.2.1.2 Anthropogenic introduction and spread of ARGs into the environment

In contrast to natural resistance, ARGs can enter the natural environment through anthropogenic pollution. Figure 2 shows details of some of the many ways ARGs can spread. This section will discuss the different ways ARGs can enter the environment from different sources.

Figure 2: The spread of antibiotics and antibiotic resistance through the environment. The figure shows where antibiotics are deployed (indicated by the bottles with pills) and routes antibiotic resistant bacteria can travel through the environment (red arrows). A large proportion of the red arrows are also applicable to ways in which antibiotic residues can pass into the environment. Missing from this diagram is the introduction of antibiotics and antibiotic resistance from pharmaceutical manufacturing effluent. Image reproduced from: https://www.antimicrobial-resistance.biomerieux.com/popup/bacteria-and-the-environment/

Bacterial contaminants, in contrast to chemical pollutants (which can degrade, be diluted or sorb to environmental particles), can multiply and spread through the environment and can, subsequently, spread their genes (such as ARGs) to other, unrelated, bacterial hosts via HGT (Berendonk et al. 2015).

Water environments are critical and underappreciated routes for the spread of ARGs (Finley et al. 2013). They are prime areas where environmental and clinical bacterial species are mixed and able to share their genes through HGT (Perry & Wright 2013). Wastewater treatment plants (WWTPs) are one of the major locations where ARGs are introduced into the environment. Patients are treated with antibiotics and those antibiotics, subsequently, exert a selective pressure on commensal gut bacteria to become resistant. These bacteria are then excreted and can enter WWTPs (Finley et al. 2013). Treatment of waste in a WWTP may remove detectable ARGs and antibiotic resistant bacteria (ARB) from treated waste, may reduce the load or may actually increase the load. A study in 2011 investigated whether five Michigan based WWTPs, using different treatment methods, had detectable ARGs and ARB in the five plants' final effluent and biosolids. A comparison of effluent and biosolids suggested that biosolids contribute more greatly to the release and introduction of ARGs and ARBs into the environment. A significant reduction in ARGs and ARBs in the final effluent occurred, in comparison to the influent. Across the different plants tested ARGs and ARBs were still detected in a number of the effluent and biosolid samples. Resistance genes and bacteria were subsequently released into the environment in some of the effluent and all of the biosolids (Munir et al. 2011). Another study, conducted in 2009, and, again, in a Michigan WWTP, used disk diffusion methods to test the antibiotic susceptibility of *Acintobacter* spp. This study investigated the percentage of resistance to eight antibiotics

tested in wastewater influent and effluent and downstream of the WWTP. Four of the eight antibiotic resistance classes tested saw a significant increase in prevalence at effluent in comparison to influent and a subsequent further increase downstream of the WWTP. This suggests that the treatment at this plant was selecting for ARB (Y. Zhang et al. 2009). A study published by Amos *et al.*, 2018 investigated the impact of a WWTP, on ARGs, in the receiving river. This study targeted the class 1 integron using qPCR. The class 1 integron has been frequently described as a good marker for pollution from human sources as it is often associated with ARGs (Gillings et al. 2015). It was determined that there was a significant increase in the prevalence of the class 1 integron at three downstream sites after the WWTP, in comparison the samples taken at three upstream sites. The class 1 integron prevalence was four times higher in the river after receiving effluent than prior to the WWTP. These results suggest that this WWTP is directly inputting or selecting for ARGs in the natural environment (Amos et al. 2018).

All of the above studies investigated WWTPs that mainly deal with household waste. A study by Bengtsson-Palme *et al.*, 2014 investigated the impact of pharmaceutical waste on a lake in India. Using a non-impacted, healthy Swedish lake as a comparison, they found 7,000 times more ARGs in the polluted Indian lake. The authors provide two hypotheses for why this might be: either the high concentrations of antibiotics being released by the pharmaceutical production facility select for resistance in environmental bacteria or the resistance is a result of direct input from the manufacturing facility (Bengtsson-Palme et al. 2014).

The high use of antibiotics in livestock production selects for antibiotic resistance in the guts of the treated animals (Allen et al. 2010). Manure from farm animals is often used as a fertiliser for crops, particularly in organic farming. This allows the spread of antibiotic resistant bacteria to both crops and soil. A study by Udikovic-Kolic *et al.*, 2014 investigated whether coliform bacteria and antibiotic resistant bacteria were significantly higher in soil fertilised with manure in comparison to an inorganic fertiliser used as a control. They determined that both total colony forming units and resistant colony forming units were significantly enriched with use of the manure in comparison to the control. Using qPCR, they also determined that the *bla-$_{CEP-04}$* (encoding a ß-lactamase enzyme) was significantly higher (Udikovic-Kolic et al. 2014).

There are questions as to whether it is a risk to human health if ARB and ARGs are entering the environment and whether these genes and bacteria pass back into humans and become a clinical problem. Obtaining conclusive evidence that clinically important resistance genes originated from the environment, however, is difficult as these genes may undergo many rounds of selection prior to being identified in clinical bacteria (Perry & Wright 2013). Previous data has shown, however, through metagenome sequencing, that a clinical resistance gene can have up to 100% sequence identity to resistance genes found in environmental bacteria (Fosberg et al. 2012). For pathogenic bacteria to acquire resistance from the environmental settings, close contact of both donor and recipient bacteria is key (Perry & Wright 2013). Exposure of the human microbiome to environmental bacteria frequently occurs, for example, (Perry & Wright 2013) through the food chain (WHO 2017a) (as a result of the high use of antibiotics in agriculture (O'Neill 2015a)) or coastal waters that have been contaminated with sewage effluent (Finley et al. 2013).

Examples of exposure to antibiotic resistant bacteria through the food chain include: supermarket pork products found to be contaminated with livestock-associated MRSA (Hadjirin et al. 2015); 11.4% of *E. coli* isolated from lettuce and a lettuce farm (soil and irrigation water) were resistant to one or more antibiotics (Holvoet et al. 2013) and 72.3% of 205 bacterial isolates from lettuce, spinach and alfalfa sprouts were found to be resistant to at least one antibiotic whilst over 90% of these resistant strains were resistant to ampicillin and cephalothin (Bezanson et al. 2008). Although these studies show that humans are exposed to environmental ARGs and ARBs they do not show that subsequent colonisation of resistant bacteria from these sources occurs. One study, however, investigated antibiotic resistance carriage in the guts of surfers who swallow the greatest volume of potentially contaminated coastal waters of all recreational water users. The study determined that surfers were four times more likely to be colonised by *E. coli* possessing a *bla-$_{CTX-M}$* gene (encoding a ß lactamase gene) than non-surfers thus suggesting a direct link between environmental exposure and human colonisation by ARB and ARGs (Leonard et al. 2018). This, however, does not show how long these ARB and ARGs are able to persist in the gut after exposure. Work investigating persistence of resistant *E. coli* in international travellers found two months after travel, a majority of participants were no longer colonised. However, 18% of participants were still colonised six months after travelling (Kennedy & Collignon 2010). Length of gut colonisation has not, however, been investigated after environmental exposure and it is not clear, therefore, if the same would be true after this type of exposure.

1.2.2 How antibiotics enter and spread through the environment

Figure 2 (Section 1.2.1.2) shows the variety of ways in which antibiotic resistant bacteria can be disseminated throughout the environment. This diagram can, however, also be applied to antibiotic residues and their passage from use through to their subsequent entry into environmental compartments.

Following clinical treatment, antibiotics are often excreted, fully intact and in an active form (Kümmerer 2009), and enter WWTPs (Kümmerer 2003). Some antibiotics persist at low concentrations throughout the environment and are not readily biodegradable. The fate of these pharmaceuticals is dependent on their water solubility, their ability to persist in the environment and on their polarity (Wellington et al. 2013). In WWTPs antibiotics have three possible fates: they may biodegrade to undetectable concentrations; be released fully intact or as metabolites into rivers and other receiving waters via effluent or may adsorb to sewage sludge (Wellington et al. 2013; Singer et al. 2016). In a paper published in 2011, studies investigating removal rates of emerging contaminants by investigating the mean concentration in WWTP influent and effluent were reviewed. The compiled dataset contained, amongst other emerging contaminants, 13 antibiotics. Removal rates, between influent and effluent, ranged from 95.1% removal of tetracycline to 1.4% removal of trimethoprim. An average removal rate for all antibiotics tested was 51.4%. All antibiotics tested were above detection limit in the final effluent and were subsequently released into the environment (Deblonde et al. 2011).

As stated previously, antibiotics are also an integral part of agricultural and aquacultural processes for both the treatment and prevention of disease and growth promotion in livestock (Chang et al. 2015). Whilst the use of antibiotics

as growth promotors has been banned by the European Union the practice still persists in many other parts of the world (Levy & Marshall 2004, O'Neill 2015a). Implementing these drugs in agriculture is often described as one of the major contributors to the clinical problem of antibiotic resistance and hence an immediate reduction in the unnecessary use (prophylactically and for growth promotion) is needed to help reduce the global impact of resistance (Chang et al. 2015). It was estimated that in 2013 global consumption of antibiotics by food animals was approximately 131,109 tons. This is set to increase to over 200,000 tons by 2030 (Schar et al. 2018). In 2010, antimicrobial use in agricultural practices accounted for 71% of total antibiotic use in Denmark (Finley et al. 2013)

Both active antimicrobial compounds and gut bacteria from treated animals are excreted. The excreta is frequently used in crop production as a fertiliser, therefore exerting a selective pressure on environmental bacteria associated with crop plants and soil (Aminov 2010; Allen et al. 2010). There is also the potential, through the use of manure as a crop fertiliser, that these veterinary antibiotics may leach into surface waters and groundwater (Blackwell et al. 2009).

Antimicrobials are also used for treatment and prevention of disease in global aquaculture. They are usually administered into aquacultural practices as part of feed or directly to the water (Heuer et al. 2009; Cabello et al. 2013). In this way, they are used for metaphylactic treatment (treating both healthy and diseased individuals) as well as being used for the prevention of disease outbreaks (Cabello et al. 2013; Santos & Ramos 2018). The use of antimicrobials in aquaculture is frequently unregulated (Heuer et al. 2009).

Regulation is reported to be particularly poor in South America and parts of Asia (Conti et al. 2015). As China is the leading global fish supplier (over 60% of aquaculture) this is of particular concern (Henriksson et al. 2018). Antimicrobials that are not consumed, and those that are excreted, can accumulate in the sediment of the aquaculture tanks or under cages and can leach into surrounding sediments and water bodies (Cabello et al. 2013). This does not need to be the case, however. In Norway, use of antibiotics in aquacultural practices has been reduced drastically (from 48 tonnes to 1 tonne) through the implementation of a rigorous vaccination programme (Midtlyng et al. 2011).

Finally, another potential route of entry into the aquatic environment is through effluent from pharmaceutical manufacturing plants during the production and formulation of antibiotics. Concentrations of antibiotics have been observed to be over 1 mg/L in such effluents and their receiving waters (Larsson 2014).

1.2.3 Environmental concentrations of antibiotics

Concentrations of antibiotics in the environment vary considerably depending on the location and the local anthropogenic pressures exerted. Generally, concentrations of antibiotics in the environment are within the ng/L to µg/L range (Andersson & Hughes 2012). Concentrations of mg/L are known to occur in environments such as hospital and pharmaceutical waste but ng/L is more typical of ground and surface waters (Homem & Santos 2011).

Many surveillance studies have been carried out to investigate the antibiotic concentrations in a variety of environmental settings. An example of this includes a study conducted in two WWTPs along the River Thames in the UK which investigated concentrations of antibiotics in both the influent and effluent of the plants. The highest concentration of 11 antibiotics tested was 2.32 µg/L of

ofloxacin in influent and 0.224 µg/L of erythromycin in effluent (Singer et al. 2014). An example of extremely high concentrations from anthropogenic pollution was described in a study by Larsson, de Pedro and Paxeus, 2007. This study investigated the concentrations of antibiotics in the effluent of a pharmaceutical production plant in India. Even after treatment of the waste from this facility, concentrations of 28,000 and 31,000 µg/L of ciprofloxacin was detected on the two days on which sampling occurred. These concentrations were then, presumably, discharged freely into the environment. The authors note that these are unusually high concentrations. The concentration of 31,000 µg/L is equivalent to 45 kg of ciprofloxacin being discharged from the manufacturing plant on a daily basis (Larsson et al. 2007).

In 2016 the Umweltbundesamt (UBA – German Environment Agency) developed a database, collating a significant number of these surveillance studies from around the world. The database includes a range of environmental concentrations of different pharmaceuticals, including antibiotics, in a number of different matrices. Matrices ranged from natural bodies of water, such as lakes and rivers, to wastewater from sources such as urban, hospital and pharmaceutical manufacturing plant waste. Concentrations shown on the database range from undetectable to 200,000 µg/L of sulfamethoxazole found in untreated wastewater from a pharmaceutical production facility in Kenya. This is, as with Larsson, de Pedro and Paxeus, 2007, an unusually high concentration and would presumably have been lower after the waste had been treated. Concentrations on the UBA database typically range from ng/L to µg/L although occasional higher concentrations are seen in pharmaceutical and hospital waste (Umweltbundesamt 2016).

1.2.4 Do environmental concentrations of antibiotics select for antibiotic resistance?

There is a question as to whether there could be a selective pressure from antibiotic residues entering the environment and whether those concentrations of antibiotics are high enough to select for resistance, thus exacerbating the problem.

It is clear that the introduction of antibiotics into the clinic has led to detectable concentrations of antibiotics in the environment (Kümmerer 2003) and to increased levels of ARGs (Finley et al. 2013). For example, soil samples taken in the Netherlands were 2 to 15 times more likely to contain ARGs in 2008 compared to soil collected in 1970 (Knapp et al. 2010). It is unclear, however, whether these resistance genes have been selected for by high concentrations of antibiotics in the gut of the treated patients (van Schaik 2015) or animals (Marshall & Levy 2011) and subsequently released into the environment, or if selection for ARGs is happening at environmental concentrations.

As discussed in Section 1.2.3, typical environmental concentrations of antibiotic are low in comparison to those used to treat infections, ranging from ng – µg/L (Kümmerer 2009). These concentrations are significantly lower than minimum inhibitory concentrations (MIC) and it was, therefore, assumed that selection for resistance genes did not occur in environmental settings (Andersson & Hughes 2012). If selection occurs at these low concentrations it is important, therefore, to determine at what concentration an antibiotic, or any co-selective agent, results in increasing resistance gene prevalence (Andersson & Hughes 2012). In this way it will be possible to determine if mitigation strategies need to be implemented to minimise the selective risk posed by these compounds.

Experimental work undertaken by Gullberg et al. 2011 and Gullberg et al. 2014 used single species competition assays to investigate whether selection may occur at much lower antibiotic concentrations than previously thought. These concentrations are more representative of concentrations found within an environmental setting (Gullberg et al. 2011; Gullberg et al. 2014; Kümmerer 2009).

A diagram, published in Gullberg et al. 2011 and shown in Figure 3, describes the theory behind selection for resistance at lower concentrations. Here, the "*Traditional selective window*" can be seen in red which shows the traditional view of selection from a clinical perspective. Selection was believed only to occur between the MIC of susceptible and of resistant bacteria. Growth of the susceptible bacteria (here indicated by the blue line) is no longer occurring and therefore positive selection for the resistant bacterium is seen.

Below the traditional window, however, it was hypothesised that selection would be seen in a "*Sub-MIC selective window*," depicted here in yellow. This occurs between the minimal selective concentration (MSC) – defined as the concentration of antibiotic "*where the resistant mutant is enriched over the susceptible strain*" (Andersson & Hughes 2011) - and the MIC of the susceptible bacteria. As the growth rate of susceptible bacteria (blue line) decreases below the growth rate of resistant bacteria, there is less competition from susceptible bacteria for resources and, therefore, positive selection for resistant bacteria is seen.

It is hypothesised, therefore, that at concentrations lower than those used clinically, and in the range of those concentrations found in the environment, positive selection for resistant bacteria may occur.

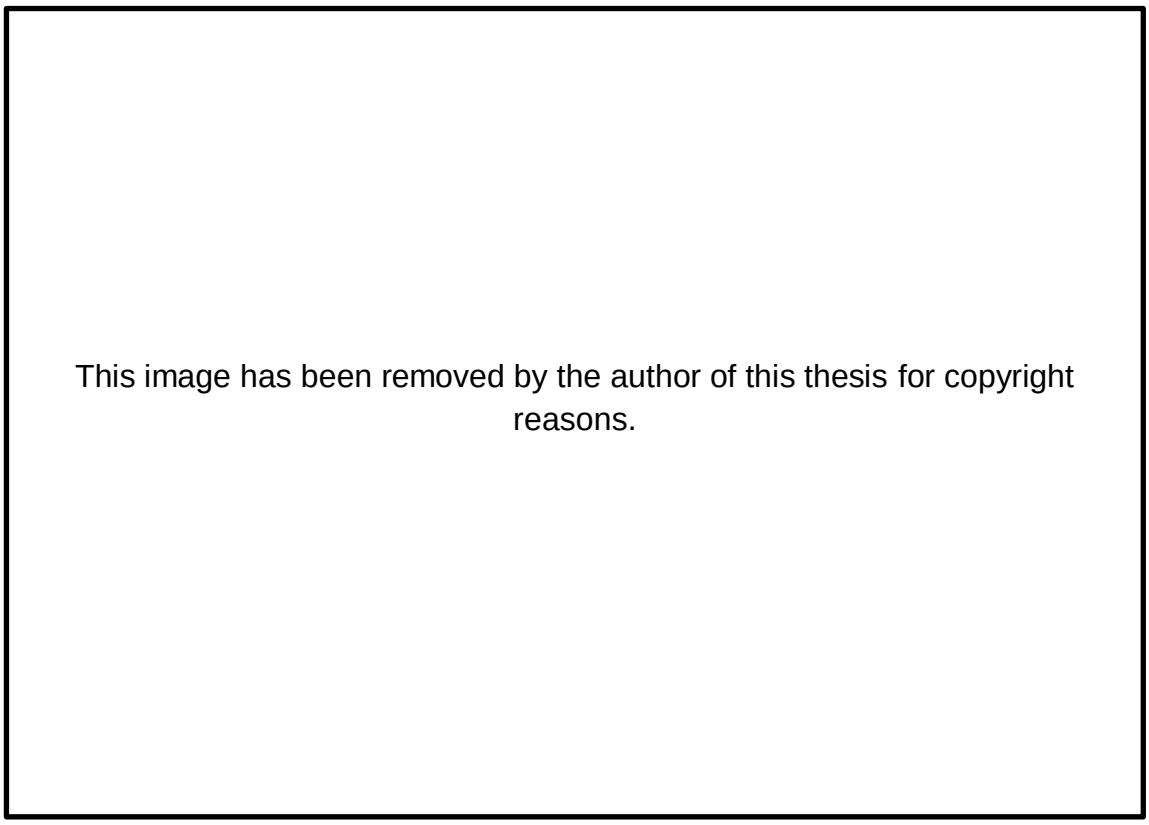


Figure 3: Antibiotic concentration affects growth rates of susceptible and resistance bacteria differently. As antibiotic concentration increases, growth rate of the susceptible bacteria (blue line) decreases. This means there is less competition for resources for the resistant bacteria (red line). This allows for an increase in positive selection to occur. The MSC is defined as where the growth rate of the susceptible drops below that of the resistant. Graph reproduced from: Gullberg *et al.*, 2011.

The study published in 2011 investigated resistance elements found within the chromosome (Gullberg et al. 2011) and the 2014 research investigated mobile genes on plasmids (Gullberg et al. 2014). MSCs were determined, in both studies, to be significantly lower than MICs of a number of antibiotics. For example, MICs were found to be 10, 230 and 10 times higher than MSCs (determined by plotting selection coefficients) for the resistance mutations *gyrA2* (encodes DNA gyrase subunit A)*, gyrA1* (encodes DNA gyrase subunit

A) and *ΔacrR* (deletion of HTH-type regulator gene *acrR*), respectively, for ciprofloxacin (Gullberg et al. 2011).

Although the work from these two studies suggests that positive selection may be occurring at environmental concentrations of antibiotics, a limitation of this method is that only single species were studied. This is not representative of the mixed communities of bacterial species found in the environment or in human and animal microbiomes. Subsequent studies have aimed to determine MSCs for a variety of antibiotic compounds using complex communities from environmental sources which is, therefore, more representative of the environment.

Two studies have been published investigating the selective potential of two compounds, tetracycline (Lundström et al. 2016) and ciprofloxacin (Kraupner et al. 2018), in a complex biofilm system. The first study attempted to determine a MSC for tetracycline at low concentrations. Tetracycline was the chosen compound as it is a broad spectrum antibiotic that is commonly used and "*up to 75% of consumed tetracyclines are excreted in their active form,*" meaning that it is, therefore, often found in the environment (Lundström et al. 2016). Prevalence of the tetracycline resistance genes, *tetA* and *tetG* (which both encode efflux pumps), was determined in an experimental biofilm mixed community system at the end of the experiment. Prevalence of these genes were significantly higher at the tetracycline concentration of 1 µg/L in comparison to the no antibiotic control. In their second study, the team investigated the effects of ciprofloxacin at low concentrations. Again, a biofilm model was used but this time alongside a test tube system for comparative purposes. In the test tube system, a significant difference in phenotypic

resistance was observed at 5 µg/L in comparison to the no antibiotic control. In the biofilm system, a significant difference was determined for both genotypic and phenotypic resistance in *E. coli* compared at 10 µg/L in comparison to the no antibiotic control (although 5 µg/L was not tested in the biofilm experiment) (Kraupner et al. 2018).

A study published in 2018 used a microcosm method, more similar to the method used in Gullberg et al. 2011 and Gullberg et al. 2014, but with a wastewater influent bacterial community as the inoculum instead of single species. QPCR was used to determine the prevalence of $bla_{CTX\text{-}M}$ over the 7 day experimental period. The MSC for cefotaxime was 0.4 µg/L (Murray et al. 2018).

All of these data sets show an effect of antibiotics at environmentally relevant concentrations and the importance of the environment as a place where selection for AMR can occur. These studies, and their limitations, are discussed in more depth in Chapter 6.

Sub-MIC concentrations also have effects on other bacterial processes, which can subsequently exacerbate the carriage of resistance genes. For example, studies have shown that sub-MIC concentrations of antibiotics can increase growth rate (Migliore et al. 2013), biofilm formation (Waack & Nicholson 2018), the rate of spontaneous mutation (Gillespie et al. 2005; Henderson-Begg et al. 2006; Cortes et al. 2008), the occurrence of HGT (Jutkina et al. 2018; Shun-Mei et al. 2018) and, finally, recombination rate (López & Blázquez 2009). The ability to increase these processes, as well as selecting for ARGs, means that concentrations below the MIC could enhance the spread of these genes

through environmental bacterial populations and, subsequently, lead to a greater prevalence of ARGs (Sandegren 2014).

Selection at sub-MIC concentrations may pose a greater risk than selection occurring above the MIC of susceptible bacteria (Khan et al. 2017; Andersson & Hughes 2012). The fitness cost tends to be high when bacteria evolve resistance under concentrations that are above MIC of susceptible strains. At sub-MIC concentrations there is a lower fitness cost of adaptation. This means that when the selective pressure of the antibiotic is no longer present, the resistance that has developed under sub-MIC conditions is less likely to revert back to its susceptible state (Sandegren 2014; Andersson & Hughes 2010).

The studies discussed previously, including those specifically looking at selection for resistance at low concentrations and others investigating related factors like HGT and mutation rate, provide compelling evidence that selection can and does occur at low antibiotic concentrations in the environment. A study published in 2019, however, suggested that ARGs found in the environment were associated with faecal pollution and not selection from antibiotic residues being released into the environment concluding that there were "*no clear signs of selection in the environment*" (Karkman et al. 2019). This study analysed publically available metagenome databases searching for crAssphage (a bacteriophage that has been associated with faecal metagenomes from humans). It determined that ARGs and crAssphage abundances correlated well in sewage polluted environments but that there was no correlation between the two in human faecal metagenomes. The authors concluded that this meant that ARGs in sewage polluted environments are largely explained by faecal pollution and that no selective effect may be seen at low environmental antibiotic

concentrations. This data appears contradictory, as there is no correlation observed in human faecal metagenomes, and the study appears to makes claims that are not fully supported by the data. Whilst the study has no published data to disprove that selection is occurring in the environment, in the introduction to the paper the authors state that "*concentrations of selective agents in a sewage-impacted environment might not be sufficient to cause selection,*" citing the Lundström *et al.*, 2016 and Kraupner *et al.*, 2018 studies carried out by other members of the same research group. These publications clearly do show an effect at environmental concentrations. Their claims appear to be an unjustified view of only one subset of the data they have produced. Faecal contamination probably does play a substantial, even dominant, role in accounting for abundance of ARGs and ARB observed in sewage polluted environments. This, however, does not negate the possibility that extremely important selection is occurring at environmental concentrations of antibiotics as shown by Lundström *et al.*, 2016, Kraupner *et al.*, 2018 and Murray *et al.*, 2018. This, to the best of the author's knowledge, is the only published study to present data with the aim of demonstrating that selection does not play a role in the environmental dimension of AMR.

1.2.5 Issues with current environmental risk assessments

Antibiotics, often released into the environment from excretion by patients via WWTPs, were only identified as a risk to the environment in the 1990s. Since then, there has been a steady increase in the number of monitoring studies. Currently, there are several hundred active pharmaceutical ingredients (APIs) in aquatic environmental settings such as ground water, surface water and sewage treatment plants. Guidelines on conducting environmental risk assessments have, therefore, been developed by the European Medicines

Agency (Ågerstrand et al. 2015). These guidelines currently include determining the risk of the compound by using the ratio of the predicted environmental concentration (PEC) to the predicted no effect concentration (PNEC). This relies on environmental testing which includes, for example, growth inhibition tests on algae, toxicity tests on early life stage fish and reproduction testing on *Daphnia spp*. (European Medicines Agency (EMEA) 2006). Tests for the effect of antibiotics on microbes are limited. Tests include: the ASRIT (activated sludge respiration inhibition test), which determines the effect of antibiotics of microbial communities in WWTPs (OECD 2010); tests on cyanobacteria which investigate the effect antibiotics have on growth rate (OECD 2011) and tests on the effect on nitrification of soil bacteria (OECD 2000). These tests and guidelines have their limitations. The microbial communities tested are very specific to each test. As all microbial communities may react differently in terms of their sensitivity to antibiotics, tests may not be applicable to all community compositions (Brandt et al. 2015). Also, current guidelines do not include studying selection for antibiotic resistance in environmental bacteria from the release of antibiotics and other potentially co-selective pharmaceuticals into the environment (Ågerstrand et al. 2015). Developing an ecotoxicological test for determining the concentration at which antibiotics and other co-selective agents will select for antibiotic resistance was suggested by Ashbolt *et al.*, 2013 although currently no standardised test exists.

Ågerstrand et al. 2015 have recommended that investigating the selection for antibiotic resistance should be made a requirement of studying the effects of antibiotics in the environment, as they suggest it will *"provide a more accurate picture of the risks connected to the environmental occurrence of antibiotics.*" These recommendations have been made because it is believed that

persistence of antibiotic compounds in natural environments will exert a selective pressure on environmental, non-pathogenic, bacteria. This could potentially select for novel resistance genes, which can then in turn be mobilised into clinically relevant pathogenic bacteria (Ågerstrand et al. 2015).

A study in 2016 used a mathematical approach to set guidelines for the release of antibiotics into the environment based on their selective nature. This study determined PNECs for the selection of resistance ($PNEC_RS$) by using the EUCAST database to determine the lowest 1% observed MIC found per antibiotic. They then adjusted this concentration for the number of test species and applied an assessment factor of 10. Whilst this approach has been useful for setting initial guidelines for the safe release of antibiotic compounds (as all antibiotics and combinations of antibiotics on the EUCAST database were able to be given selective endpoints), the authors do note the limitations of this method which needs to be validated using experimental methods to determine $PNEC_RS$ (Bengtsson-Palme & Larsson 2016).

Developing a straightforward experimental test to determine the selective potential of antibiotics in mixed microbial communities, that is relevant to environmental conditions and which can, therefore, screen many APIs for ecotoxicology testing, is critical for testing antimicrobial agents and other potential co-selecting compounds before release into the environment. The data produced by such a test will give a better understanding for regulators as to the concentrations of these APIs that are safe to release from WWTPs.

1.3 Aims

The aim of this the work reported in this thesis was to investigate whether environmental concentrations of antibiotics select for antibiotic resistance. All

experiments undertaken used a week long experimental evolution assay similar to those used in Gullberg *et al.*, 2011 and 2014 and used complex microbial communities, instead of single species, to better mimic the microbial communities in the environment. QPCR was used to target different ARGs, *intI1* (encoding the class 1 integron) and 16S rRNA (encoding the 30S subunit of the bacterial ribosome) to determine the change in prevalence of resistance over time. Metagenome analysis was also used during some of the experiments to provide additional data. The study had 4 main objectives. These were:

1. Investigating whether environmental concentrations of three macrolide antibiotics (azithromycin, clarithromycin and erythromycin) select for resistance.
2. To investigate the effect of combinations of antibiotics on selective concentrations.
3. To investigate whether temperature affects the selective endpoint of an antibiotic.
4. To investigate whether the experiments used for this study are comparable to previously published, more “environmentally relevant” methodology by investigating the MSC of tetracycline as in Lundström *et al.*, 2016.

1.4 Co-authored papers

The two following papers were outputs from a collaboration with the Water Research Institute – National Research Council, Italy. The author supervised a visiting PhD student on a summer placement, helped to plan projects and taught experimental skills needed, such as qPCR, which the PhD student employed to produce some of the data included in the papers listed below. The

author was also sent samples for qPCR analysis following the placement and reviewed and edited the scientific content for the qPCR sections and the wording of the entire manuscripts.

Rauseo J., Ademollo N., Cardoni M., Di Lenola M., Gaze W.H., **Stanton I.C.**, Grenni P., Pescatore T., Spataro F., Patrolecco L. Dissipation of the antibiotic sulfamethoxazole in soil amended with anaerobically digested cattle manure. *Journal of Hazardous Materials*. 2019. 378. 120769. doi: 10.1016/j.jhazmat.2019.120769

Grenni P., Patrolecco L., Rauseo J., Spataro F., Di Lenola M., Aimola G., Zacchini M., Pietrini F., Baccio D., **Stanton I.C.,** Gaze W.H., Caracciolo A.B. Sulfamethoxazole effects on river water microbial community, on the spread of antibiotic resistance and on the aquatic plant *Lemna minor.* Accepted - *Microchemical Journal* Special edition.

The author has also participated in work that investigates using a growth rate assay to determine MSC. Final week long selection experiments for the three macrolide antibiotics presented in Chapter 3 have been included in this publication along with the preliminary trimethoprim work from Chapter 4 (Section 4.4.2.1).

Murray A.K., **Stanton I.C.**, Zhang L, Snape J., Gaze W.H. A novel experimental assay to determine effect concentrations of antibiotics which select for antimicrobial resistance. In prep. Submitting to *Environmental Health Perspectives.*

Chapter 2: Materials and Methods

The commonly used methodologies that apply to most of the experiments in this thesis are presented here. Any deviation from these will be stated in chapter specific method sections. Specifics, such as antibiotic, antibiotic concentration and resistance genes, are also presented in chapter specific methods sections, alongside any experiments not common to all chapters of this thesis.

2.1 Complex community sampling methods

Wastewater influent was used as the bacterial inoculum for all experiments. This was predominately collected from a treatment plant serving Falmouth and Penryn, Cornwall, UK. This plant serves a population of approximately 43,000 and two grab samples were taken in October 2015 and October 2017. One experiment, undertaken by a placement student Jasmin Rauseo, presented in Chapter 4, used wastewater influent from a treatment plant serving Camborne and Redruth, Cornwall, UK. That plant serves approximately 34,000 people, and was collected in December 2016. Table 1 shows the experiments undertaken presented in this thesis and the corresponding inoculum. Figure 78 (Page 300 - Appendix) shows the sampling sites. The two sites were similar to each other, serving predominantly domestic waste and one community hospital each.

Table 1: Details of location and sampling date of wastewater inoculum used, corresponding thesis section and experiment

Inoculum	Thesis Section	Experiment description
Falmouth/Penryn October 2015	Chapter 3	All selection experiments undertaken in this chapter. This includes both preliminary

		experiments and final experiments for compounds AZ, CLA and ERY.
Falmouth/Penryn October 2015	Chapter 4 Section 4.4.1.1	Preliminary macrolide mixing experiment.
Falmouth/Penryn October 2017	Chapter 4 Sections 4.4.1.2 - 3	Subsequent macrolide mixing and validation experiments.
Falmouth/Penryn October 2015	Chapter 4 Section 4.4.2.1: Trimethoprim	Trimethoprim MSC experiment completed by Jessica Wright, Aimee Murray and Isobel Stanton.
Camborne December 2016	Chapter 4 Section 4.4.2.1: Sulfamethoxazole	Sulfamethoxazole MSC experiment completed by visiting PhD student, Jasmin Rauseo, under the supervision of Isobel Stanton.
Falmouth/Penryn October 2017	Chapter 4 Sections 4.4.2	TRMP/SMX mixing experiments including preliminary growth rate experiment.
Falmouth/Penryn October 2017	Chapter 5	All experiments in this chapter including growth rate experiments, chemical degradation and investigations into the effect of temperature on the MSC of AZ.
Falmouth/Penryn October 2015	Chapter 6 Section 6.4.1	Repeat of Lundström et al. 2016 tetracycline experiment for validation

		of method.
Falmouth/Penryn **October 2017**	Chapter 6 Section 6.4.1	Tetracycline MSC.

Wastewater samples were frozen at -80 °C in 40 ml aliquots that consisted of 20 ml of wastewater and 20 ml of 40% glycerol (Fisher Scientific). For growth rate experiments, smaller volumes (4 ml) were frozen in a 1:1 ratio with 40% glycerol, to prevent freeze-thawing or wastage of large volumes. Aliquots from the same wastewater treatment plant taken during the same sampling session were presumed to be pseudo-replicates for subsequent experiments.

2.2 Selection experiments

Wastewater samples were processed to remove existing chemicals and other non-bacterial substances, such as potentially selective compounds. This was undertaken by centrifuging samples at 2,730 xg for 10 minutes to pellet the bacteria. The supernatant was then removed and the pellet was resuspended in 0.85% saline solution of the same volume. This was repeated once. This method was used as it was advised from the industrial partner, Jason Snape.

Iso-sensitest broth (Oxoid) was inoculated with the washed wastewater at a 10% v/v and with an appropriate concentration of antibiotic. Iso-sensitest was used as it is used for antibiotic susceptibility testing and therefore does not bind to the antibiotics. This meant that the concentration of antibiotic spiked is the concentration that is bioavailable to the bacteria. Antibiotics and their concentrations used can be found in relevant chapters. All experiments were run alongside a no antibiotic control consisting only of washed wastewater and iso-sensitest broth.

Samples most commonly consisted of 5 replicates per concentration of antibiotic (of 5 ml each), although, in some preliminary studies, 3 replicates were used. This is stated in the relevant chapters. Samples were incubated shaking at 180 rpm for 24 hours (unless otherwise stated). For the majority of the experiments, samples were incubated at 37 °C. This was to allow for a relatively rapid experiment as this was hoped that this could be regularly used as an assay for the risk assessment of antibiotics. Where a different temperature is used, this is indicated in the relevant section. After 24 hours, 50 µl of culture was passaged into fresh iso-sensitest broth and appropriate concentrations of antibiotic. Passaging was repeated once a day over 7 day period. This was to allow a large number of generations of the bacteria to be exposed to the appropriate antibiotic concentration and, therefore, provide the most evident dose response.

At the beginning and the end of the selection experiment, two 1 ml samples were centrifuged at 21,000 xg for 3 minutes, the supernatant was removed and the pellet was resuspended in 1 ml of 20% glycerol. Samples were frozen at -80 °C until further processing. The day 0 samples were frozen as quickly as possible on the same day, usually within 2 hours. Samples were kept on ice until frozen to minimise growth, change in population structure and change in antibiotic resistance genes.

2.3 DNA extraction

DNA was extracted, as per the manufacturer's instructions, from frozen bacterial samples sampled at day 0 and day 7 using the MO Bio UltraClean® Microbial DNA Isolation Kit (now the QIAGEN DNeasy UltraClean Microbial Kit). DNA was frozen at -20 °C until use.

2.4 Real-time PCR (qPCR)

2.4.1 Genes, primers and gBlocks

A variety of antibiotic class specific resistance genes were quantified using qPCR. The antibiotic class specific genes chosen and reasons for choosing the various genes can be found in the relevant chapters. The class 1 integron-integrase gene (*intI1)* was also targeted. Class 1 integrons have the ability to integrate a wide range of antibiotic resistance genes, and may be located on the host genome or plasmid. For this reason, they have frequently been described as a good indicator both for anthropogenic pollution and for the presence of AMR genes in the environment (Gillings et al. 2015; Kotlarska et al. 2015; Gaze et al. 2011; Abella et al. 2015; Jechalke et al. 2014). Finally, the 16S rRNA gene was amplified. This encodes the 16S ribosomal RNA in the small unit of the ribosome of prokaryotic cells and is primarily used as the target housekeeping gene for identification and taxonomy of bacterial species (Clarridge 2004). In the experiments presented in this thesis, the 16S rRNA gene was used as a proxy for bacterial cell count (Lundström et al. 2016; Murray et al. 2018). Molecular prevalence was calculated by dividing resistance gene and the *intI1* gene copy number by 16S rRNA copy number.

Primers for all genes were ordered from IDT Technologies and are set out in Table 2.

Table 2: List of genes targeted by qPCR, corresponding forward and reverse primers, target region in gene and reference. Degenerate base codes: H = A or C or T, K = G or T, M = A or C, R = A or G, W = A or T, Y = C or T.

Gene	Forward (5' – 3')	Reverse (5' – 3')	Target region in gene (bp)	Reference/ Designed by
ermB	TTGGATATTCACCGAACACTAGGG	ATAGACAATACTTGCTCATAAGTAACGG	403 to 701	Jung et al. 2009
ermF	TCTGGGAGGTTCCATTGTCCT	ACTTTCAGGACCTACCTCATAGA	372 to 504	Lihong Zhang
***mef* family**	GGTGTRYTAGTGGATCGTCA	GMHCCAGCTGCTGCKATAAT	193 to 266 (aligned to *mefA* gene)	Isobel Stanton
mphA	TGGTGCATGGCGATCTCTAC	GACGCGCTCCGTGTTGTC	587 to 642	Isobel Stanton
msrD	CAAGCTGCARAATACGAACAATTT	CCGCAGCCCTYTCCAAT	610 to 667	Isobel Stanton
intI1	GCCTTGATGTTACCCGAGAG	GATCGGTCGAATGCGTGT	526 to 724	Barraud et al. 2010
16S rRNA	CGGTGAATACGTTCYCGG	GGWTACCTTGTTACGACT	1367 to 1508	Suzuki et al. 2000
***macB* subtype 1**	CTGCCGTCTCGCAAAACCT	GCACTGGCAGCAACATCAAC	1049 to 1106	Isobel Stanton
***macB* subtype 2**	TGTTGTGGGCGTGGTGGAAG	CCCACACTCGAAGCGCTTTA	1299 to 1363	Isobel Stanton
macB	TGAACCAGC	TGAAGTACAG	1028 to 1144	Isobel Stanton

subtype 3	TGTACTACGTCG	GTGGCTGACGC		
***macB* subtype 4**	GCAGCAACACCATCGACATCTA	ACAACACCAGGGTTTCAATCG	935 to 1012	Isobel Stanton
tetG	CGCTAACGAGCCTCACCAAT	TGCGAATGGTCTGCGTAGTA	1010 to 1164	Lundström et al. 2016
tetM	GGTTTCTCTTGGATACTTAAATCAATCR	CCAACCATAYAATCCTTGTTCRC	1389 to 1476	Peak et al. 2007
tetA (1)	GGCACTCATGCTCGGAAT	GCAGGCAGAGCAAGTAGAGG	909 to 1222	Lundström et al. 2016
tetA (2)	CTCACCAGCCTGACCTCG	CACGTTGTTATAGAAGCCGCATAG	1102 to 1172	Zhu et al. 2017

Primers developed for this work were designed by obtaining a number of sequences of the target gene from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and using the primer design program Primer Express 3.0.1. This program generated many primer options and these options were tested by aligning potential primer sequences with a number of gene variants in the web program MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/) to determine if the primers were in conserved regions of the gene. If no conserved primer sequences were determined, degenerate bases were used (as per IUPAC codes) (Johnson 2010). Genbank sequences that were used to design these primers in this study can be found in Table 3.

Table 3: Genbank sequence references used to design primers

Gene	Genbank sequences
***mef* family**	**mefA:** AY071835.1, AY064722.1, AY064721.1, NG_04959.1, AY319932.1, NG_047976.1, DQ304773.1, KJ809088.1, NG_047961.1, NG_047957.1, AF227521.1, AF227520.1 **mefE:** EU870854.1, EU870853.1, U83667.1 **mefI:** HG965092.1, EU870852.1 **mefO:** DQ016305.1 **unclassified mef genes:** DQ445271.1, AY355405.1
mphA	JQ824049.1, NC_019375.1, JN233704.1, AB262968.1, AY522923.1, AF188331.1, KU665641.1, GQ402463.2, HE616910.2, CYCH01000073.1, AB261016.2, KP453775.1, CYFJ01000056.1, CYFT01000096.1, NC_009981.1, KP987215.1, CU928163.2, NC_014615.1, D16251.1, AB366440.1, JX442974.1, CP006663.1, NC_021576.1
msrD	AJ715499.2, KJ809088.1, NG_048005.1, KU180707.1, KM194596.1, KC669392.1, KX077898.1
macB subtype 1	AB071146.1, KY689638.1
macB subtype 2	KN150735.1, KN150733.1
macB subtype 3	KT613918.1, JQ309921.1
macB subtype 4	JQ309920.1

Standards for qPCR were designed for each gene and ordered as gBlocks from IDT technologies. These were designed by aligning primers to a sequence of the gene and taking the region they amplify together with a number of bases (at least 10 bp) on each end. Sequences for these can be found in Table 4.

Table 4: List of genes and associated gBlock sequences used as standards in qPCR assay, with reference

Gene	gBlock sequence (5' – 3')	Reference /Designed by
ermB	CCTTGGATATTCACCGAACACTAGGGTTGC TCTTGCACACTCAAGTCTCGATTCAGCAAT TGCTTAAGCTGCCAGCGGAATGCTTTCATC CTAAACCAAAAGTAAACAGTGTCTTAATAAA ACTTACCCGCCATACCACAGATGTTCCAGA TAAATATTGGAAGCTATATACGTACTTTGTT TCAAAATGGGTCAATCGAGAATATCGTCAA CTGTTTACTAAAAATCAGTTTCATCAAGCAA TGAAACACGCCAAAGTAAACAATTTAAGTA CCGTTACTTATGAGCAAGTATTGTCTATTT	Lihong Zhang
ermF	TCTGATGCCCGAAATGTTCAAGTTGTCGGT TGTGATTTTAGGAATTTTGCAGTTCCGAAAT TTCCTTTCAAAGTGGTGTCAAATATTCCTTA TGGCATTACTTCCGATATTTTCAAAATCCTG ATGTTTGAGAGTCTTGGAAATTTTCTGGGA GGTTCCATTGTCCTTCAATTAGAACCTACA CAAAAGTTATTTTCGAGGAAGCTTTACAATC CATATACCGTTTTCTATCATACTTTTTTTGAT TTGAAACTTGTCTATGAGGTAGGTCCTGAA AGTTTCTTGCCACCGCCA	Lihong Zhang
***mef* family**	AGTATCATTAATCACTAGTGCCATCTTGC AAATGGCGATTATTTTTTACCTTACAGAAA AAACTGGATCCGCGATGGTCTTGTCTATG GCTTCACTATTAGGTTTTTTACCCTATGCG GTCTTTGGACCTGCAATTGGTGTGCTAGT GGATCGTCATGATAGGAAGAAGATAATGA TTGGTGCTGATTTAATTATCGCAGCAGCT GGTTCGGTGCTTACTATTGTTGCATTCTA TATGGAGCTACCTGTCTGGATGGTTATGA	Isobel Stanton

	TAGTATTGTTTATCCGTAGCATTGGAACA GCTTTTCACACCCCGGCTCTCAATGCGGT TACGCCACTTTTAGTACCAGAAGAA	
mphA	TGCCGCAGGACTCGGAGGTCTTCGCGGA GAGCTTCGCGACCGCGCTCGCCGCCCTG CATGCCGTCCCCATTTCCGCCGCCGTGG ATGCGGGGATGCTCATCCGTACACCGAC GCAGGCCCGTCAGAAGGTGGCCGACGA CGTTGACCGCGTCCGACGCGAGTTCGTG GTGAACGACAAGCGCCTCCACCGGTGGC AGCGCTGGCTCGACGACGATTCGTCGTG GCCAGATTTCTCCGTGGTGGTGCATGGC GATCTCTACGTGGGCCATGTGCTCATCGA CAACACGGAGCGCGTCAGCGGGATGATC GACTGGAGCGAGGCCCGCGTTGATGACC CTGCCATCGACATGGCCGCGCACCTTAT GGTCTTTGGTGAAGAGGGGCTCGCGAAG CTCCTCCTCAC	Isobel Stanton
msrD	CCTTATCGGCACAGGTTCATGGTATTTTA GCGGATGAACCTACGAGCCATTTAGACC GTGAAGGAATTGATTTTCTAATAGGACAG CTAAAATATTTTACAGGTGCACTGTTAGTT ATTAGCCATGACCGCTATTTTCTTGATGA AATAGTAGATAAAATATGGGAACTGAAAG ATGGCAAAATCACTGAGTATTGGGGAAAC TACTCTGATTATCTTCGTCAGAAAGAGGA AGAACGTAAGAGACAAGCTGCAGAATAC GAACAATTTATTGCGGAACGTGCTCGATT GGAGAGGGCTGCGGAGGAAAAGCGAAAA CAGGCTCGTAAAATAGAACAGAAGGCAAA AGGTTCTTCAAAGAAAAAAAGTACTGAAG GCGGAGGGCGTTTAGCTCATCAAAAATCA ATAGGAAGTAAGGAAAAAAAGATGCATAA TGCCGCTAAATCCCTAGAGAACAGGATTG	Isobel Stanton

	CGGCATTAGGAAAAGTAGAAGCTCCGGA AGGC	
tetG	AACAAGCAAGGGGCTTTGCAAGGAACGC TAACGAGCCTCACCAATCTAAGCTCTATC GCAGGACCGCTTGGCTTCACAGCACTCT ATTCTGCCACCGCCGGGGCATGGAACGG TTGGGTTTGGATTGTCGGCGCGATCCTCT ATTTAATATGTCTGCCAATACTACGCAGA CCATTCGCAAATCCGTAGCTGAGACT	Isobel Stanton
tetM	GTATGAGAGCTCGGTTTCTCTTGGATACT TAAATCAATCATTTCAAAATGCAGTTATGG AAGGGATACGCTATGGTTGTGAACAAGG ATTGTATGGTTGGAATGTGACGGACTGTA AAATCTGTTTTAAGTATGGCTTATACTATA GCCC	Lihong Zhang
tetA	CTCGGCGAAAGGCGGGCACTCATGCTCG GAATGATTGCCGACGGCACAGGCTACAT CCTGCTTGCCTTCGCGACACGGGGATGG ATGGCGTTCCCGATCATGGTCCTGCTTGC TTCGGGTGGCATCGGAATGCCGGCGCTG CAAGCAATGTTGTCCAGGCAGGTGGATG AGGAACGTCAGGGGCAGCTGCAAGGCTC ACTGGCGGCGCTCACCAGCCTGACCTCG ATCGTCGGACCCCTCCTCTTCACGGCGA TCTATGCGGCTTCTATAACAACGTGGAAC GGGTGGGCATGGATTGCAGGCGCTGCCC TCTACTTGCTCTGCCTGCCGGCGCTGCG TCGCGGGC	Isobel Stanton
intI1	GGCCTTGATGTTACCCGAGAGCTTGGCAC CCAGCCTGCGCGAGCAGCTGTCGCGTGCA CGGGCATGGTGGCTGAAGGACCAGGCCG AGGGCCGCAGCGGCGTTGCGCTTCCCGAC GCCCTTGAGCGGAAGTATCCGCGCGCCGG GCATTCCTGGCCGTGGTTCTGGGTTTTTGC	Lihong Zhang

	GCAGCACACGCATTCGACCGATCC	
16S rRNA	ACGGTGAATACGTTCCCGGGCCTTGTACA CACCGCCCGTCACACCATGGGAGTGGGTT GCAAAAGAAGTAGGTAGCTTAACCTTCGGG AGGGCGCTTACCACTTTGTGATTCATGACT GGGGTGAAGTCGTAACAAGGTAACCG	Lihong Zhang

2.4.2 qPCR protocol

QPCR was undertaken on the Applied Biosystems StepOne™ machine.

Each well consisted of:

- 10 µl of Brilliant III Ultra-Fast Sybr® Green QPCR Master Mix (Agilent Technologies)
- 1 µl of each of forward and reverse primer pairs (IDT Technologies) at a concentration of 10 µM for all primer sets except 16S rRNA which was used at a concentration of 9 µM
- 0.6 µl of diluted ROX dye (provided with the master mix – Agilent Technologies)
- 5 µl of template (standards, water or experimental DNA)
- Nuclease Free Water (Ambion) up to a final volume of 20 µl

Experimental DNA was diluted 5 times before addition of 5 µl to the well. A total of 1 µl of neat DNA, therefore, was added but pipetting error was minimised.

The cycling conditions consisted of an initial cycling step of 95°C for 20 seconds, followed by 50 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Melt curves were also initially run to determine that the primers were specific to the gene. Quality of the qPCR results was checked by the Efficiency

being between 90 and 110 % and an RQ value of greater than 0.99; only data meeting these requirements underwent further analysis.

2.5 Metagenome sequencing and analyses

Metagenome sequencing was undertaken on a subset of samples from selection experiments in Chapters 3 and 6. Details of subsets of samples selected for sequencing and the rationale behind choosing those samples can be found in the relevant chapter.

DNA was extracted as described in Section 2.3. To purify DNA, an initial RNase step was undertaken using 2 µl of 20 mg/ml RNase A (QIAGEN). Subsequently, AMPure XP beads (Beckman Coulter) were used. A 1:1 volume of AMPure XP beads were added into the extracted DNA sample and mixed thoroughly by pipetting and incubated for 10 mins at room temperature. Samples were then placed on a magnetic stand to attract the beads. Supernatants were discarded and beads were washed with ethanol. Purified DNA was eluted in 10 mM Tris-HCL by incubating the beads for 10 mins at 50°C before placing samples back on the magnetic stand and allowing for beads to pellet. The pellet was discarded and purified DNA (supernatant) was tested with the QUBIT BR assay or HS assay (Thermo Fisher Scientific) to check for a suitable concentration of DNA for sequencing. This was subsequently frozen at -20 °C until sent for sequencing.

Samples were sent to and processed by Exeter Sequencing Service. The Nextera XT DNA Library Prep Kit was used to prepare the libraries and the libraries were sequenced on either a MiSeq v2 250 PE (Chapter 3) or HiSeq 2500 (Chapter 6). All samples that were sequenced yielded approximately 3GB per sample.

The raw sequence data for Chapter 3 was analysed by Aimee Murray and by the author for Chapter 6 as described in Murray et al. 2018. Both forward and reverse sequence reads were trimmed of adapter sequences using the programme Skewer (H. Jiang et al. 2014) in the paired-end mode.

First, FastQC (Andrews 2010) and MultiQC (Ewels et al. 2016) were used to analyse the raw sequencing data to ensure robustness. This analysis provided information whether adapter sequences had been removed and on sequence quality. Only when sequences had been deemed to be of good quality and adapter sequences had been successfully removed, were subsequently analyses undertaken. “Good quality” was determined if the sequence had not “failed” any section of the analysis performed by FastQC and MultiQC, with the exception of GC content. GC content occasionally did not “pass” the quality check for all sequences. This can often be interpreted as an indication of contamination. However, as the samples here were a mixed microbial community it is expected that GC content might vary.

For data presented in Chapter 3, 16S rRNA gene sequences were extracted from the raw sequence data. FLASH version 2 (Magoc & Salzberg 2011) was used to combine the paired-end reads. Bacterial species were assigned using MetaPhlAn2 (Truong et al. 2015).

For both data from Chapter 3 and Chapter 6, Antibiotic Resistance Gene Online Analysis Pipeline (ARGs-OAP) version 2 was used to identify ARGs in the sequences produced by the metagenome (X. Yin et al. 2018) using default settings (blastx alignment length cut-off = 25, blastx alignment evalue cut-off = 1e-07 and blastx alignment identity = 80).

All subsequent analyses were performed by the author. Statistical analyses were performed on all data sets produced from the metagenome as per Section 2.6.

2.6 Data analysis

All graphs were produced by the author using the ggplot2 package (Wickham 2016) in R studio (RStudio Team 2016) except the 16S community structure graphs (Chapter 3) which were generated by Aimee Murray as part of the metagenome analysis pipeline. This was produced using HClust2 (Segata 2018) (with Bray-Curtis distance measurements used between species. Figure 77 in Chapter 6, which adapted an image from Gullberg et al. 2011, was produced by the author using InkScape 0.92.2.

R Studio (RStudio Team 2016) was used to perform all statistical analyses. Data were tested for normality using the Shapiro-Wilk test. Parametric datasets were analysed using ANOVA and Dunnett's tests (using the multcomp package (Hothorn et al. 2008)) and for non-parametric data, Kruskal-Wallis and Dunn's tests (using the dunn.test package (Dinno 2017)) were used. Dunnett's and Dunn's tests were chosen as they compare each group of replicates (in this case each concentration) to the first group of replicates entered into the data matrix (in this case the no antibiotic control). These tests were performed on day 7 prevalence data unless otherwise stated. For qPCR data, ANOVA/Kruskal-Wallis tests were also undertaken on the prevalence of genes at day 0 to ensure that there was minimal variation in prevalence between replicates. Where day 0 samples were significantly different from each other, post-hoc tests were undertaken on the difference between day 0 and 7, rather than the prevalence at day 7. This was to ensure that a significant change in

prevalence at day 7 was observed because of a dose response rather than difference in starting prevalence. This is indicated where applicable.

If, due to natural variation in the samples, the Dunnett's/Dunn's test did not align with the biological effect observed, Generalised Linear Models (GLM) were performed. Both Gaussian and Gamma families were explored, with various link functions, and the best fit model was selected based on diagnostic plots and AIC values. Using a different statistical approach to determine significance was undertaken to ensure that the most conservative selective endpoint estimate was determined. This would allow the estimate to be the most protective of selection for antibiotic resistance genes in the environment and to, therefore, minimise the risk that antibiotics test pose to the environment.

Where possible, selection coefficient graphs were produced as described in Gullberg et al. 2011. The formula used was: selection coefficient = [LN(prevalence at day 7/prevalence at day 0)]/number of days. Selection coefficients were plotted and the MSC was defined where the line of best fit crosses the x axis. Linear and polynomial models were plotted to the data points using the polynom package (Venables et al. 2019) in RStudio (RStudio Team 2016). To determine which line of best fit to use, a summary of the model fit was obtained. The R^2 value was used to find the model that best correlated with the data and a one way ANOVA was used to determine whether models were significantly different to each other. A limited range of concentrations is occasionally plotted, or concentration values have been transformed (square root transformation), to provide a more accurate MSC value as the spread of concentrations can skew the line of best fit. This will be noted in the appropriate section. For certain replicates, the prevalence at day 0 was 0. This, however,

was not truly the case as prevalence had increased in the same biological replicate by the end of the 7 days. This suggested that the gene was in the initial sample, but was below the limit of detection. Due to the nature of the formula (having to divide by prevalence at day 0, where the prevalence was 0 this was mathematically impossible) and that certain genes were below the limit of detection rather than completely absent, a pseudo value was used. To determine the best estimate for the possible range of values between the limit of detection and zero, the pseudo-value was calculated as half of the detection limit.

2.7 Definitions of selective endpoints

To avoid confusion between selective endpoints determined by different means (either statistically or by selection coefficient), they will, henceforth, be referred to by different names and acronyms. All selective endpoints determined by statistical means will be described as a LOEC (lowest observed effect concentration). This is the lowest concentration where significant positive selection for a gene was observed to 95% confidence using qPCR data. The immediately preceding test concentration (the highest concentration where no selection is seen) is referred to as the NOEC (no observed effect concentration). If a selective endpoint is able to be determined by a selection coefficient, this will be defined as the MSC, as previously described and used in Gullberg et al. 2011, Gullberg et al. 2014 and Murray et al. 2018. If the prevalence of a gene increases over time in the no antibiotic control, it is impossible to determine a MSC for this gene as the line of best fit never crosses the x axis. This will be highlighted in the relevant sections. PNEC (predicted no effect concentration) will be used when an assessment factor has been applied to the NOEC or the MSC. Throughout this thesis an assessment factor of 10 is

applied to all NOEC and MSC values. This is the assessment factor recommended by the EMEA to determine PNEC values for surface water (European Medicines Agency (EMEA) 2018).

Chapter 3: The potential for macrolide antibiotics to select for antibiotic resistance

Author contributions

The author conducted all experimental work in this chapter under the supervision named supervisors. DNA was sent to Exeter Sequencing Service who sequenced the samples. Aimee Murray checked the quality of the sequences, ran them through two pipelines to analyse the 16S and antibiotic resistance gene data. Aimee Murray also produced the 16S diversity graphs. The author analysed the raw data from the antibiotic resistance gene analysis pipeline.

3.1 Abstract

In 2015, the European Commission created the first watch list of 10 compounds, or groups of compounds, that evidence suggests are emerging pollutants in the aquatic environment. The macrolide antibiotics, azithromycin, clarithromycin and erythromycin, were included on this list as one of the ten groups identified as potentially posing a risk to the environment. Currently, there is no requirement to determine, or a standardised test for determining, the selective potential of antibiotics or other co-selective compounds at environmental concentrations. The aim of this investigation was, therefore, to determine the selective potential of the three macrolide antibiotics in a complex community assay, as all previous work has only investigated this in single species experiments.

This chapter includes a literature review of environmental macrolide concentrations; an assessment of resistance gene host diversity; nine week

long selection experiments for three macrolides at a wide range of concentrations; extensive qPCR analysis for key genes; a culture based analysis; metagenome data analyses; identification of additional qPCR gene targets from metagenome data and further qPCR analysis for these targets.

Week-long selection experiments, using wastewater influent as the inoculum, were performed at various concentrations of the three macrolides tested. Samples were taken at the beginning and end of the experiment and qPCR was used to track the change in prevalence of macrolide resistance genes and the class 1 integron gene *intI1* over the 7 day period. Metagenome culture based analyses were used to validate qPCR results. Metagenome analysis also investigated the co-selective properties of the macrolides tested and of any changes in community structure as a result of increasing antibiotic concentration.

A LOEC of 750 µg/L was determined for clarithromycin, with a NOEC of 500 µg/L. LOECs for azithromycin and erythromycin were both 1,000 µg/L with NOECs of 750 µg/L. In addition, a MSC of approximately 514 µg/L was estimated for erythromycin. These values are significantly higher than current measured environmental concentrations and, therefore, based on results from this experimental system, these compounds do not currently pose an environmental risk in terms of selection for resistance. These values should, however, be used in conjunction with ecological endpoints to decide whether these compounds should be removed from the watch list.

3.2 Introduction

Macrolides are a clinically important class of antibiotic. In 2014 they were the third highest prescribed antibiotic in England accounting for 15% of all antibiotic

prescriptions (Public Health England 2015). Erythromycin (ERY) was the original macrolide antibiotic and is a natural product isolated from *Saccharopolyspora erythraea (*formerly known as *Streptomyces* erythraeus) in 1952 by McGuire et al. (McGuire et al. 1952; Shryock et al. 1998; Wright et al. 2014). The term “macrolide” was coined in 1957 as the metabolite produced by *S. erythraea* contained a macrolactone ring (Wright et al. 2014). ERY is comprised of many components with the active ingredient being erythromycin A (Hawkyard & Koerner 2007). Erythromycin A consists of the lactone ring (14-membered) as well as an amino sugar and L-cladinose. Semi-synthetic macrolides that are derived from erythromycin A have 14-membered lactone rings and include clarithromycin (CLA), dirithromycin and roxithromycin. Azithromycin (AZ) has a modified 15-membered lactone ring with the addition of a nitrogen atom and there are also 16-membered lactone ring macrolides (myocamycin, midecamycin, spiramycin, josamycin and tylosin) (Leclercq & Courvalin 2002). Semi-synthetic derivatives, such as AZ and CLA, offer better tolerability and pharmacokinetics due to the alterations of the erythromycin A molecule (Leclercq & Courvalin 1991; Amsden 1996; Leclercq 2002).

Macrolides inhibit bacterial growth by targeting protein synthesis and are bacteriostatic. *In vitro* experiments have shown, however, that AZ, CLA and ERY have bactericidal effects against certain *Streptococcus* spp. (Pankey & Sabath 2004). They achieve bacteriostatic effects by binding to the 23S rRNA of the large subunit of ribosomes (50S). This binding prevents peptides that have been newly synthesised from entering and passing through the tunnel of the ribosome and this, in turn, prevents translation (Kannan & Mankin 2011; Mazzei et al. 1993).

In the clinic, macrolides are used to treat a wide range of infections caused by both Gram-positive and Gram-negative bacteria. For example, AZ is recommended for treatment of shigellosis in children (CDC 2006; DuPont 2009) and as a secondary treatment for adult patients (WHO 2005); and is often considered for use against invasive non-typhoidal salmonella (Sjölund-Karlsson et al. 2011). All three compounds, AZ, CLA and ERY, are used to treat respiratory infections, for example for both post-exposure prophylaxis and treatment of pertussis (CDC 2005). CLA is also used in combination with a proton pump inhibitor and either metronidazole or amoxicillin to treat *Helicobacter pylori* infections (Chey et al. 2017). Finally, the CDC recommends a single dose of 1 g of AZ to treat the sexually transmitted infection, chlamydia (Geisler et al. 2015) and, as recommended by the British Association for Sexual Health and HIV, a single 1 g oral dose of AZ in combination with 500 mg of cefriazone to treat gonorrhoeae (Bignell & FitzGerald 2011).

Macrolides are also used in agricultural and veterinary practices. Tylosin, a 16-membered macrolide, was a feed additive used as a growth promotor in the European Community until it was banned (along with other antibiotics) in 1999 (Hao et al. 2014; Butaye et al. 2003). In a 2015 list by the World Organisation for Animal Health (OIE), the macrolide antibiotics were classed as “Critically Important Antimicrobial Agents” for veterinary practice where they are deployed, for example, to treat both haemorrhagic digestive disease and Mycoplasma infections in pigs. In cattle they are used to treat respiratory infections and liver abscesses (OIE 2015).

As with all antibiotic classes, macrolides have not escaped the rise of antibiotic resistance. A study published in 2016 investigated CLA-resistant *H. pylori* from

4 regions of the United States and found 32.3% of these isolates were resistant (and a range from 23.1-45.8% between different sites) (Park et al. 2016). In 2017 the WHO produced a list of priority pathogens where research and development of new antibiotics was important. These pathogens were categorised into three priority levels; “Priority 1: CRITICAL, Priority 2: HIGH and Priority 3: MEDIUM.” On this list, CLA – resistant *Helicobacter pylori* are classed as “Priority 2: HIGH” (WHO 2017b). A further study in 1991 tracked macrolide resistance in *Streptococcus pneumoniae* in the United States between 1979 and 1987 and determined that 0.3% of *S. pneumoniae* were resistant to ERY (Spika et al. 1991; Lonks 2004). Two surveillance studies published in the early 2000s determined significantly higher rates of resistance (Thornsberry et al. 2002; Jacobs et al. 2003; Lonks 2004). The study published in 2002 evaluated levels of various antibiotic resistant pathogens from 1999-2000 in the United States and suggested that 19.4% of *S. pneumoniae* isolates were now resistant to ERY, with a further 7.2% resistant to both ERY and clindamycin (Thornsberry et al. 2002). The Alexander project, which collected data from a range of countries worldwide, determined that 24.6% of *S. pneumoniae* isolates were resistant to ERY during the study period from 1998-2000 (Jacobs et al. 2003). In addition, between 2004 and 2008, an increase in AZ resistant *N. gonorrhoeae* was seen from 0.3% and 3.9% in Scotland (Palmer et al. 2008). Furthermore, a study published in 2018 retrospectively looked at isolates in the China Gonococcal Resistance Surveillance Programme collected between 2013 and 2016. Here they saw an average AZ resistance prevalence for these isolates of 18.9% (Yin et al. 2018).

Resistance to macrolides can occur by mutations (specifically, base substitutions) in the 23S ribosomal RNA, allowing for macrolide target site

modification (Vester & Douthwaite 2001). There are also a high number of acquired resistance genes that protect bacteria in a variety of ways. These include rRNA methylases, macrolide 2'-phosphotransferases, esterases and efflux pumps (both major facilitators and ATP-binding). Genes that encode rRNA methylases include a variety of *erm* genes (*ermA, ermB, ermC, ermF* etc). These work by adding methyl groups (one or two) to a single adenine within the 23S rRNA. As of 2008, 33 *erm* genes had been identified with this group of genes able to confer resistance to lincosamides and streptogramin B antibiotics, as well as the macrolides (Roberts 2008). Macrolide 2'-phosphotransferases inactivate the macrolides on 14-,15- and 16-membered rings by enabling the transfer of phosphate from ATP onto the 2'-hydroxyl group which include *mphA, B, C* and *D* (Achard et al. 2008; Taniguchi et al. 2004). While esterases, such as *ereA* and *ereB,* are not the most common macrolide resistance mechanism deployed, they can produce extremely high levels of resistance with MICs greater than 1,600 µg/ml. They work by breaking down an ester bond that is key in forming the lactone ring (Wright 2005). Efflux pumps, such as *mefA* and *msrD* (Roberts 2008), work by physically pumping the antibiotic out of the bacterial cell. They can be specific to an antibiotic class or can work against multiple classes (Webber & Piddock 2003).

Macrolides are frequently detected in a range of aquatic environments at ng/L to µg/L concentrations. A review of typical environmental macrolide concentrations (excluding unusually high concentrations from pharmaceutical effluents, for example) was undertaken during this study and is reported in this chapter. Average and maximum concentrations for AZ, CLA, ERY and erythromycin – H_2O (ERY-H2O) can be seen in the results (Section 3.4.1). Individual concentrations and references can be found in the full Table in the Appendix,

Table 11 (Page 301). The maximum value of all these 4 compounds in typical aquatic environments was 4 µg/L for ERY-H2O which was measured in the surface waters of the Jianhan Plain in China (Tong et al. 2014). ERY-H2O is a metabolite of ERY and is thought to be able to select for resistance (Majer 1981; Fan et al. 2009).

In a study published in 2009, the location of antibiotic resistance genes in environmental bacteria was reviewed. These included a range of *erm* genes and *mphA.* This group of macrolide resistance genes were found in bacteria inhabiting a variety of aquatic environments including activated sludge, wastewater treatment plant (WWTP) effluent and “special wastewater” which included agricultural, aquacultural and hospital wastewater. This review only considered 10 macrolide resistance genes from 4 previous surveillance studies and this, therefore, demonstrates the range of aquatic environments in which bacteria harbouring macrolide resistance genes can be found (X. X. Zhang et al. 2009).

A list of compounds of concern, that required better monitoring in the aquatic environment, was produced by The European Commission in 2015. This list consisted of 10 priority substances or groups of substances. Compounds were chosen for the priority list based on their risk quotient (RQ) values. RQ values were calculated by comparing predicted environmental concentrations (PECs) and measured environmental concentrations (MECs) of compounds to predicted no effect concentrations (PNECs) in freshwater. PNECs for AZ, CLA and ERY were calculated using the test species *Ceriodaphnia dubia, Anabaena flos-aquae* and *Synechococcus leopoldenisis,* respectively. The PECs and MECs of all three macrolide antibiotics were found to have always exceeded the

PNECs, leading to RQs > 1 for each compound. These RQs were 6.48, 4.96 and 3.07 for AZ, CLA and ERY, respectively and these compounds were, therefore, deemed to pose a significant risk to the aquatic environment (European Commission 2015b). In 2018, a review of this priority substances list was undertaken. The macrolide antibiotics remained on the new 2018 list and two further antibiotics, ciprofloxacin and amoxicillin, were added (European Commission 2018).

Work to date on the macrolide antibiotics' effect on the environment has either consisted of single species assays, mathematical predictions of selective endpoints or traditional ecotoxicology testing of acute toxicity to aquatic organisms to calculate PNECs.

Gullberg et al. 2014 determined two MSCs for ERY using a single species competition assay. An *E. coli* wildtype strain was competed in a 1:1 ratio with an isogenic resistant mutant at various concentrations of antibiotic. Susceptible and resistant strains had different fluorescent protein genes inserted to allow identification using flow cytometry, after the competition experiment had been undertaken. In this study, an *mph* cassette was used in an experiment with the cassette on a plasmid and an experiment with the cassette on the chromosome which determined MSCs of 3,000 and 200 µg/L of ERY, respectively (Gullberg et al. 2014).

A study by Bengtsson-Palme & Larsson in 2016 calculated a PNEC for selection of resistance ($PNEC_R$). To do this, a mathematical approach was used. MIC values were obtained for all antibiotics and combinations of antibiotics that were available on the EUCAST database (122 MIC values in total). The lowest 1% of the MICs were identified, adjusted for number of

species tested and an assessment factor of 10 applied to calculate $PNEC_R$ values. For the macrolide antibiotics, $PNEC_R$s of AZ, CLA and ERY were determined to be 0.25 µg/L, 0.25 µg/L and 1 µg/L, respectively (Bengtsson-Palme & Larsson 2016), which are significantly lower than experimental data generated by Gullberg *et al.*, 2014.

A meta-analysis was conducted in 2017 on previously published PNECs for freshwater organisms in surface waters. This took the lowest reliable no observable effect concentration (NOEC) data from a number of studies using a range of different ecotoxicology tests and applied an assessment factor of 10 to derive PNECs. For AZ, the PNEC for surface water was 0.019 µg/L, for CLA it was 0.084 µg/L and finally, for ERY the PNEC was 0.2 µg/L. All of these values were calculated from ecotoxicology tests using cyanobacteria from the studies Vestel et al. 2015, Baumann et al. 2015 and Ando et al. 2007 for AZ, CLA and ERY, respectively (Le Page et al. 2017).

In the European Commission's 2015 report, PNECs were derived when the macrolide antibiotics were placed on the Water Framework Directive's priority watch list, giving PNECs for AZ (0.09 µg/L), CLA (0.13 µg/L) and ERY (0.2 µg/L) using the test species mentioned above (European Commission 2015b).

The aim of the work undertaken and reported in this chapter was to investigate selection and co-selection for resistance and to define selective endpoints (LOECs/MSCs) for the three macrolide antibiotics placed on the European Commission's priority watch list (AZ, CLA and ERY). This was done across a range of antibiotic concentrations in complex microbial communities, rather than using single species assays or mathematical modelling.

3.3 Methods

3.3.1 Antibiotics and antibiotic concentrations

The antibiotics used were AZ (Sigma-aldrich), CLA (Molekula) and ERY (Acros Organics). AZ and ERY were both dissolved in ethanol absolute (Fisher) and CLA was dissolved using acetone (Acros Organics).

Three experiments were undertaken:

- "Preliminary experiment 1 – low macrolide concentrations" – this consisted of concentrations 0.1, 1, 10 and 100 µg/L of each of the three macrolides. These concentrations were based on typical environmental concentrations determined by a literature search presented in Section 3.4.1.
- "Preliminary experiment 2 – high macrolide concentrations" was run at 1,000 and 10,000 µg/L for AZ and CLA and at 1,000, 10,000 and 100,000 µg/L for ERY.
- The "Final selection experiments" which were undertaken across a range of these concentrations (100, 250, 500, 750, 1,000, 10,000 and 100,000 µg/L) for all three compounds.

3.3.2 Macrolide resistance genes for qPCR

The macrolide resistance genes *ermB, ermF, mphA, msrD* and *mef* family (which targeted the *mefA, mefE, mefI* and *mefO* genes) were quantified using qPCR. These specific macrolide resistance genes were targeted as they have been reported in a wide range of bacteria, both Gram-positive and Gram-negative species (Roberts 2008). In terms of specific genes, the *ermB* and *ermF* genes were described by Berendonk et al. 2015 to be genetic marker determinants for assessing macrolide resistance in the environment (Berendonk

et al. 2015). In addition, a study from 2009 found that *mphA* was the most common macrolide resistance gene found in clinical isolates of *E. coli* (Nguyen et al. 2009). *IntI1* (as it has been described as a good proxy for ARGs (Gillings et al. 2015)) and 16s rRNA (as a proxy for bacterial cell count) were also quantified.

3.3.3 Culture-dependent (plating) experiment

Culture dependent methods were also used to capture information potentially missed by qPCR (for example, spontaneous mutations). Overnight bacterial cultures at day 7, that had been grown with 100, 1,000, 10,000 and 100,000 µg/L of AZ, as well as cultures of the no antibiotic control, were plated onto 3 different agars. These were Muller-Hinton agar (Oxoid) to capture all culturable bacteria; Chromocult Coliform Agar Enhanced Selectivity (Merck) enriching for *Enterobacteriacae* spp. and Mannitol-salt agar (HiMedia Laboratories Technical Data protocol: http://himedialabs.com/TD/M118.pdf) to enumerate *Staphylococcus* spp.

Serial dilutions of 100 µl of day 7 bacterial cultures were plated onto the three types of agar, both with and without AZ in the media. In this way, prevalence was calculated for phenotypic resistance (number of colonies on agar with AZ/number of colonies on agar without AZ).

The AZ concentration used in the Chromocult agar to determine phenotypic resistance was 16 mg/L. This is the clinical breakpoint for *Shigella* spp. and *Salmonella* Typhi taken from the EUCAST database (EUCAST, Clinical breakpoints – bacteria (v 7.1)). For Muller-Hinton agar and Mannitol-salt agar, the clinical breakpoint for *Staphylococcus* spp. was used (2 mg/L). This value

was also obtained from the EUCAST breakpoint database (EUCAST, Clinical breakpoints – bacteria (v 7.1)).

3.3.4 Samples sent for metagenome analysis

Three replicates of the day 7 samples from the final selection experiment were sequenced. Concentrations included 250, 750, 1,000, 10,000 and 100,000 µg/L of AZ, CLA and ERY as well as the no antibiotic control. Replicates sent for sequencing were those with the highest *intI1* prevalence, with the aim of capturing the highest variety of resistance genes that had not been targeted using qPCR and of seeing the largest co-selective effect on other antibiotic resistance gene classes.

3.4 Results

3.4.1 Macrolide environmental concentrations

A literature review was conducted to determine an environmental concentration range to test. Studies were found by searching for papers that reported the levels of pharmaceuticals in aquatic environments. Studies were eliminated if they did not test for macrolides or concentrations were below detection limit. As the searches were undertaken to determine typical environmental concentrations of macrolide antibiotics, unusually high concentrations, for example, from pharmaceutical plant effluents were excluded as not being indicative of the general pattern of macrolide concentrations in the environment. Instead, the search focused on surface waters, ground waters, WWTP influent and WWTP effluent. A total of 29 studies were used to calculate the mean and maximum concentrations. The results of this search are summarised in Table 5 and the full list of concentrations and references can be found in Table 11 (Page 301, Appendix).

Table 5: Measured environmental concentrations of macrolide antibiotics where detected. Concentrations are in µg/L. 1 = Number of studies used to calculate the mean concentration. 2 = Number of studies where antibiotic was below the limit of detection. N.B. Some studies are included in both of these categories if multiple sites were tested and/or multiple antibiotics were tested.

Antibiotic	Mean	Maximum	Number of studies (1)	Number of studies (2)
AZ	0.193	1.5	27	9
CLA	0.140	1	42	8
ERY	0.225	2.42	50	12
ERY – H_2O	0.412	4	34	1

3.4.2 Preliminary experiment 1 – low macrolide concentrations

Based on the review of environmental macrolide concentrations undertaken (see Section 3.4.1) an initial range finding experiment was conducted to encompass both the mean and maximum concentrations seen in the environment. The concentrations 0.1, 1, 10 and 100 µg/L were chosen for all three macrolides and were run alongside a no antibiotic control. QPCR was used to quantify 5 macrolide genes (*ermB, ermF, mef* family, *mphA* and *msrD*) and the 16S rRNA gene was a proxy for bacterial cell count allowing a molecular prevalence to be calculated at the beginning and at end of the 7 day experiment.

Figure 4A-E shows the effect AZ has at low concentrations. No significant positive selection was seen for any of the 5 macrolide specific genes at any of the concentrations tested compared to the no antibiotic control. A significant increase in prevalence was observed at 100 µg/L (p = 0.0142, Dunn's test) of AZ for *mef* family (Figure 79, Page 309 - Appendix). The error bar seen here is

large and overlaps with the error bar of the corresponding day 0 prevalence. One high outlier replicate was observed in this dataset and when this was removed, no significant increase was seen at any concentration (Figure 4C). No significant increase in prevalence, in comparison to the no antibiotic control, was observed for any gene tested at any AZ concentration tested.

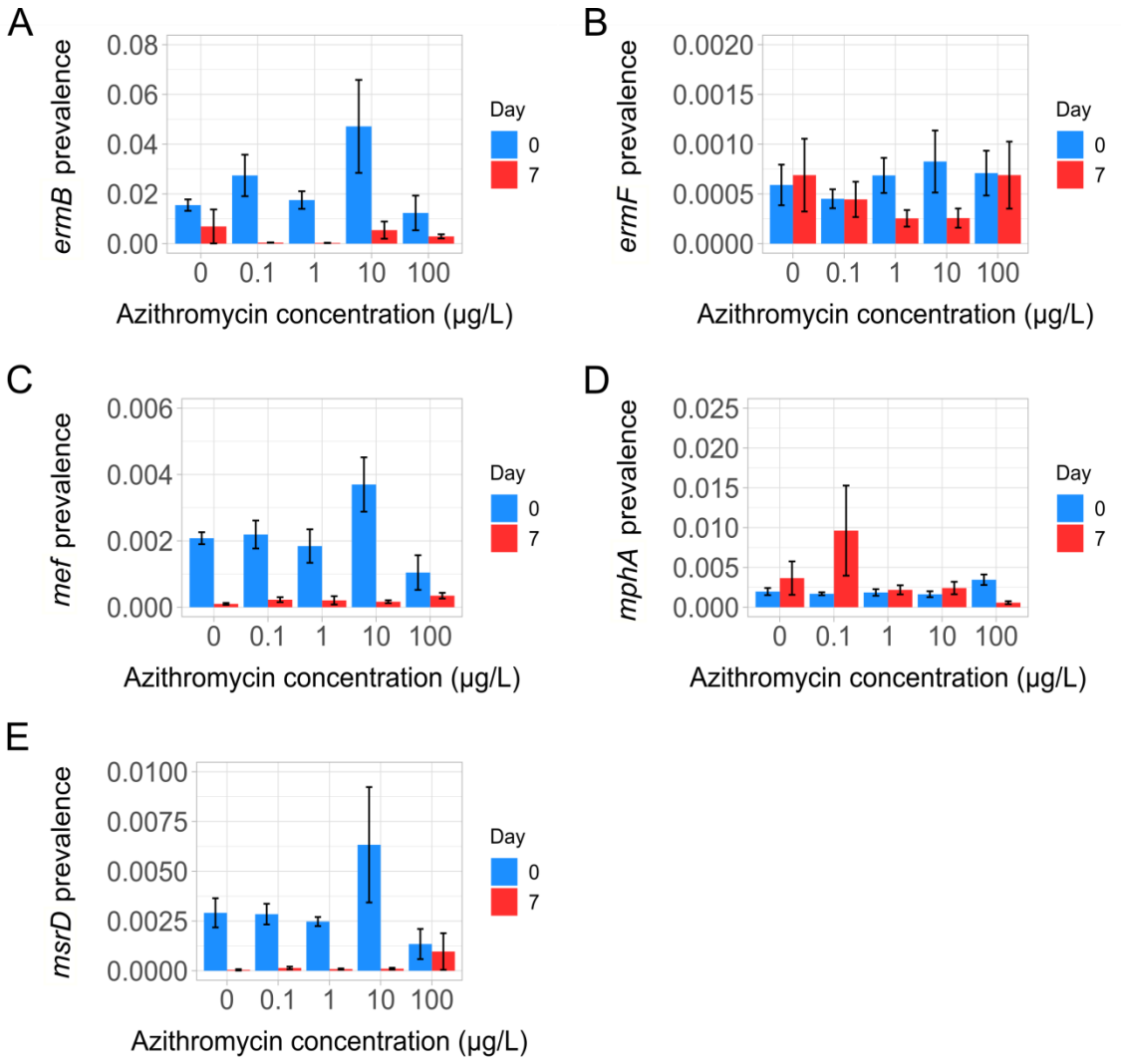


Figure 4A: e*rmB* as a function of low azithromycin concentrations. 4B: e*rmF* as a function of low azithromycin concentrations. 4C: *mef* family as a function of low azithromycin concentrations. 4D: *mphA* as a function of low azithromycin concentrations. 4E: *msrD* as a function of low azithromycin concentrations. Standard error is represented by the error bars.

There was also no significant selection seen at low concentrations for CLA (see Figure 5A-E) or ERY (see Figure 6A-E). A significant increase, in comparison to the no antibiotic control, was seen for *ermB* to 90% confidence at 0.1 and 10 µg/L of CLA (p = 0.0721 and 0.0855, respectively, Dunn's test) and for the *mef* family to 95% confidence at 100 µg/L of CLA (p = 0.0414, Dunn's test). This was, however, not an increase over the starting prevalence of these genes. One high outlier replicate has been removed from the no antibiotic control sample at day 0 for *ermB, ermF, mef* family and *msrD* genes.

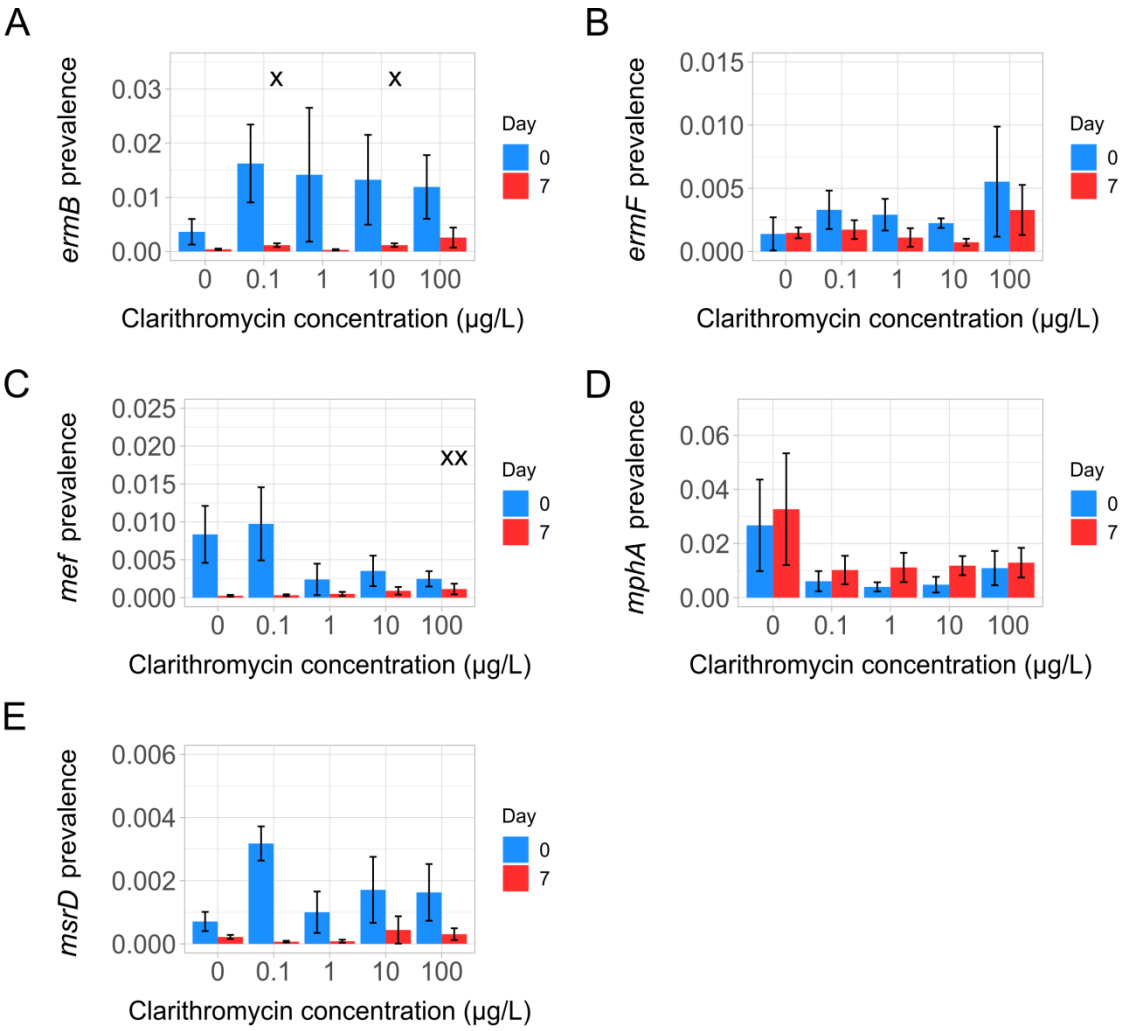


Figure 5A: e*rmB* as a function of low clarithromycin concentrations. 5B: e*rmF* as a function of low clarithromycin concentrations. 5C: *mef* family as a function of low clarithromycin concentrations. 5D: *mphA* as a function of low clarithromycin concentrations. 5E: *msrD* as a function of low clarithromycin concentrations. x = significant increase in comparison to the control to 90% confidence. xx = significant increase in comparison to the control to 95% confidence. Standard error is represented by the error bars.

A similar response was seen for *ermB* in the presence of ERY. A significant increase in comparison to the no antibiotic control was seen to 95% confidence at 0.1 µg/L (p = 0.0233, Dunn's test) and to 90% confidence at 100 µg/L (p = 0.0603, Dunn's test). Again, this was not higher that the starting inoculum of

ermB. One high outlier replicate has been removed from the no antibiotic control at day 0 for *mphA* and at 1 µg/L at day 0 for *mef* family.

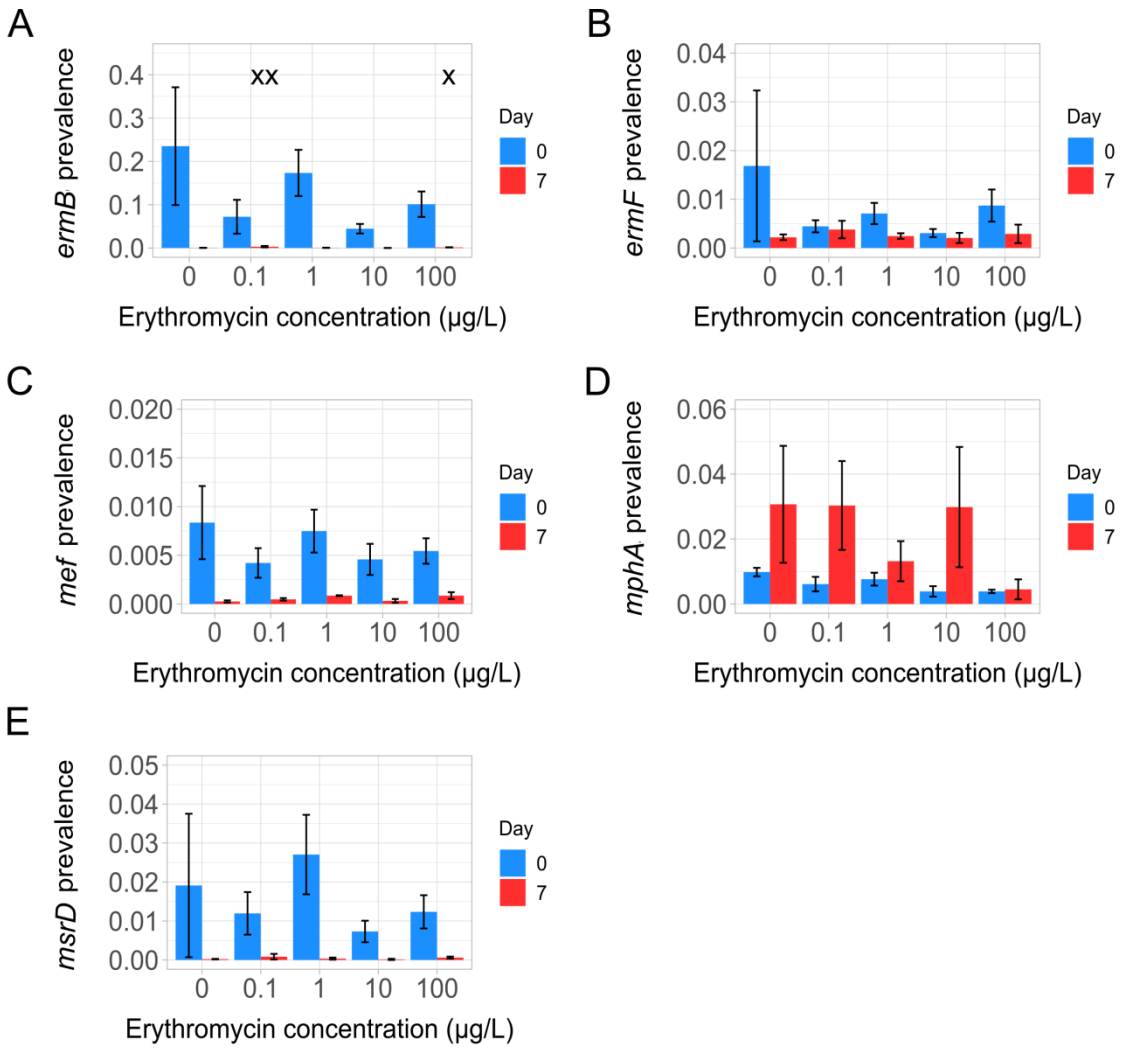


Figure 6A: e*rmB* as a function of low erythromycin concentrations. 6B: e*rmF* as a function of low erythromycin concentrations. 6C: *mef* family as a function of low erythromycin concentrations. 6D: *mphA* as a function of low erythromycin concentrations. 6E: *msrD* as a function of low erythromycin concentrations. x = significant increase in comparison to the control to 90% confidence. xx = significant increase in comparison to the control to 95% confidence. Standard error is represented by the error bars.

Higher, more clinically relevant concentrations were, therefore, tested (Section 3.4.3.)

3.4.3 Preliminary experiment 2 – high macrolide concentrations

As no significant positive selection for any gene was seen at a range of typical environmental concentrations for any of the three macrolide compounds (see Section 3.4.2), a higher range of concentrations was tested. For AZ and CLA, 1,000 and 10,000 µg/L were tested and for ERY 1,000, 10,000 and 100,000 µg/L were tested. A 10-fold higher concentration was used for ERY as it has been suggested that it is less potent than some semi-synthetic derivatives (Jelić & Antolović 2016).

Results showing the effect of AZ on all 5 macrolide resistance genes can be seen in Figure 7. Again, no significant selection was seen for the *mef* family or for *msrD.* Significant positive selection was seen for *ermF* and *mphA.* For *ermF*, significant selection was seen to 90% confidence at 1,000 (p = 0.0784, Dunn's test) and to 95% confidence at 10,000 µg/L (p = 0.0153, Dunn's test) compared to the no antibiotic control. For *mphA,* a significant selective effect was not seen until 10,000 µg/L (p = 0.0368, Dunn's test), however a stronger response was seen in terms of increase in prevalence in comparison to the response of *ermF*. For *ermB,* however, there was a significant difference between prevalence at 10,000 µg/L (p = 0.007, Dunn's test) in comparison to the no antibiotic control although this does not increase above the starting prevalence of *ermB*.

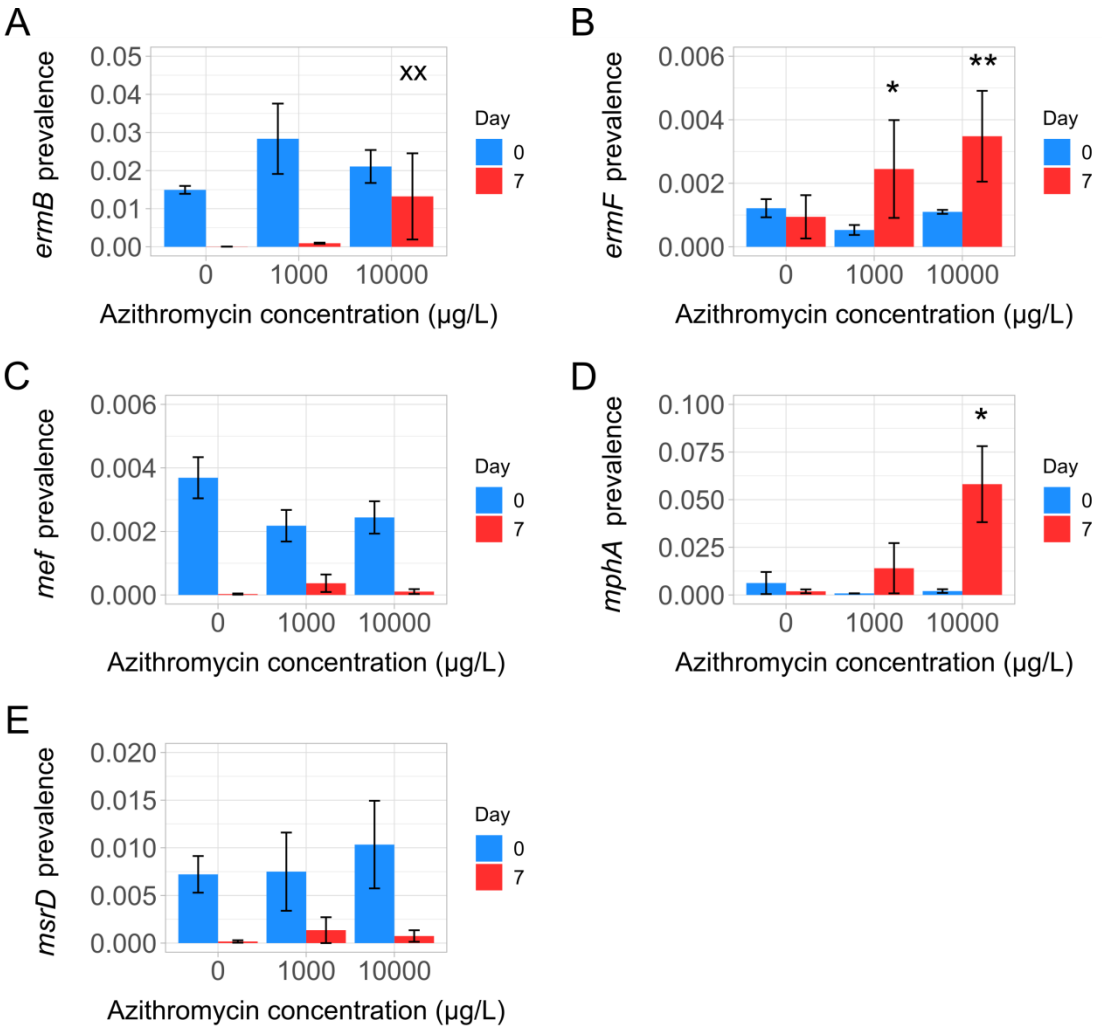


Figure 7A: e*rmB* as a function of high azithromycin concentrations. 7B: e*rmF* as a function of high azithromycin concentrations. 7C: *mef* family as a function of high azithromycin concentrations. 7D: *mphA* as a function of high azithromycin concentrations. 7E: *msrD* as a function of high azithromycin concentrations. xx = significant increase in comparison to the control to 95% confidence. * = significant positive selection to 90% confidence, ** = significant positive selection to 95% confidence. Standard error is represented by the error bars.

CLA showed a similar pattern to AZ. Graphs for this data can be seen Figure 8A - 8E for *ermB, ermF, mef* family, *mphA* and *msrD*, respectively. Again, no positive selection was seen for *ermB*, *msrD* and *mef* family. A significant increase was observed in comparison to the no antibiotic control for *ermB* at 10,000 µg/L (p = 0.0104, Dunn's test) but, as with AZ, this did not increase above the starting prevalence.

For *ermF,* the Dunnett's test did not match with biological effect seen. No significant selection was observed at any concentration. A Generalised Linear Model (GLM) (Gamma, link = log) was performed. This aligned better with the biological effect. Significant selection for *ermF* was seen at 1,000 µg/L (p = 0.00953, GLM (Gamma, log)) and at 10,000 µg/L (p = 0.00375, GLM (Gamma, log)).

Significant selection for *mphA* by CLA was observed to 90% confidence at 10,000 µg/L only (p = 0.0680, Dunn's test), although a biological effect can be seen at 1,000 µg/L.

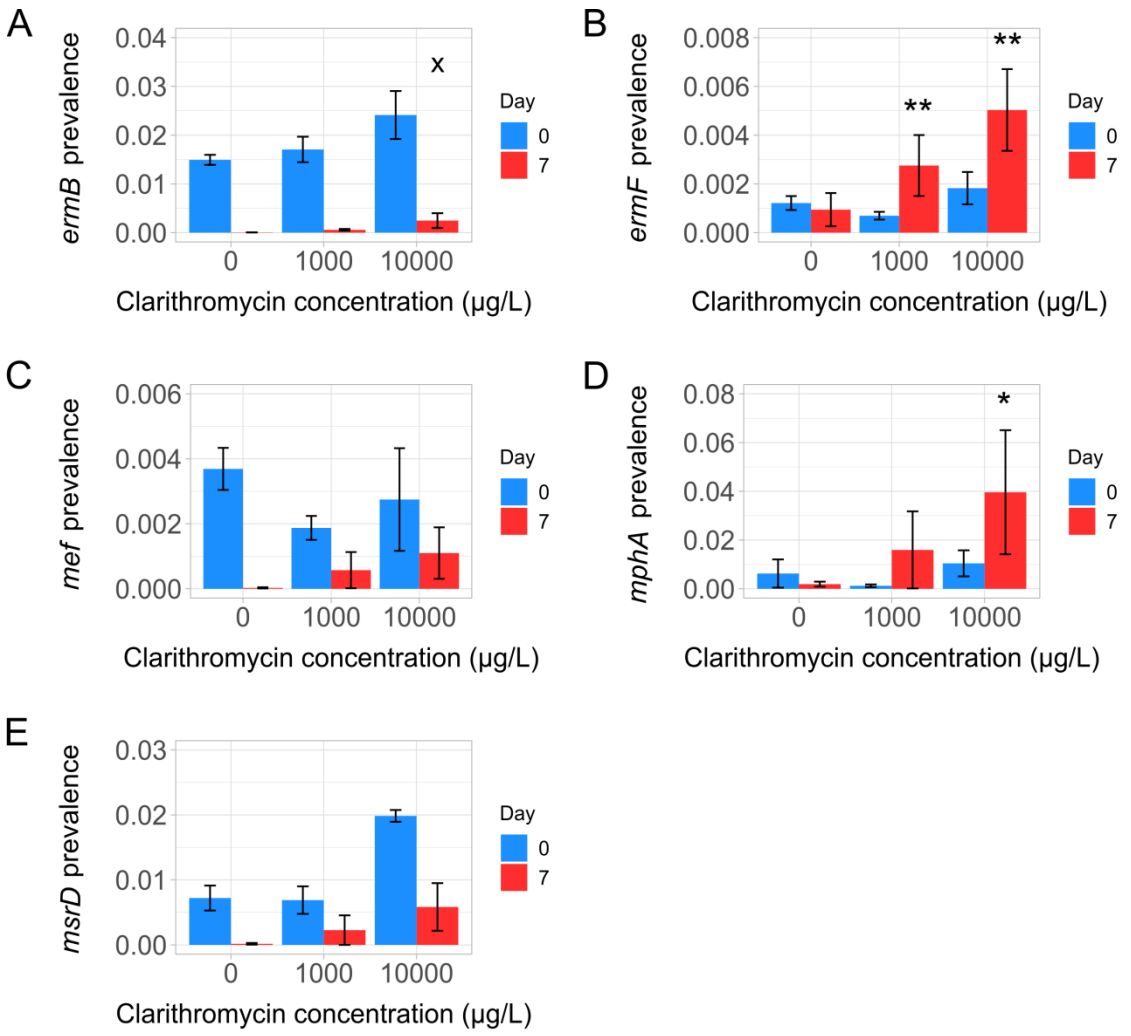


Figure 8A: e*rmB* as a function of high clarithromycin concentrations. 8B: e*rmF* as a function of high clarithromycin concentrations. 8C: *mef* family as a function of high clarithromycin concentrations. 8D: *mphA* as a function of high clarithromycin concentrations. 8E: *msrD* as a function of high clarithromycin concentrations. x = significant increase in comparison to the control to 90% confidence. * = significant positive selection to 90% confidence, ** = significant positive selection to 95% confidence. Standard error is represented by the error bars.

As with AZ and CLA, no significant positive selection was seen for *ermB, mef* family and *msrD* at any of the concentrations of ERY (Figure 9A, C and E, respectively). A significant increase in prevalence of *ermB*, in comparison to the no antibiotic control, was observed to 90% confidence at 1,000 (p = 0.0705, Dunn's test) and 10,000 µg/L (p = 0.0565, Dunn's test) of ERY and to 95% condfidence at 100,000 µg/L (p = 0.0157, Dunn's test). None of these were found to be higher than the prevalence found in the day 0 samples and, therefore, no positive selection was occurring.

For *ermF* (Figure 9B), significant selection was seen to 90% confidence at 1,000 µg/L (p = 0.0745, Dunn's test) and to 95% confidence at 10,000 (p = 0.0108, Dunn's test) and 100,000 µg/L (p = 0.0163, Dunn's test) of ERY.

Finally, significant selection for *mphA* (Figure 9D) was first observed to 90% confidence at 10,000 µg/L (p = 0.0606, Dunn's test) and to 95% confidence at 100,000 µg/L (p = 0.0023, Dunn's test) of ERY.

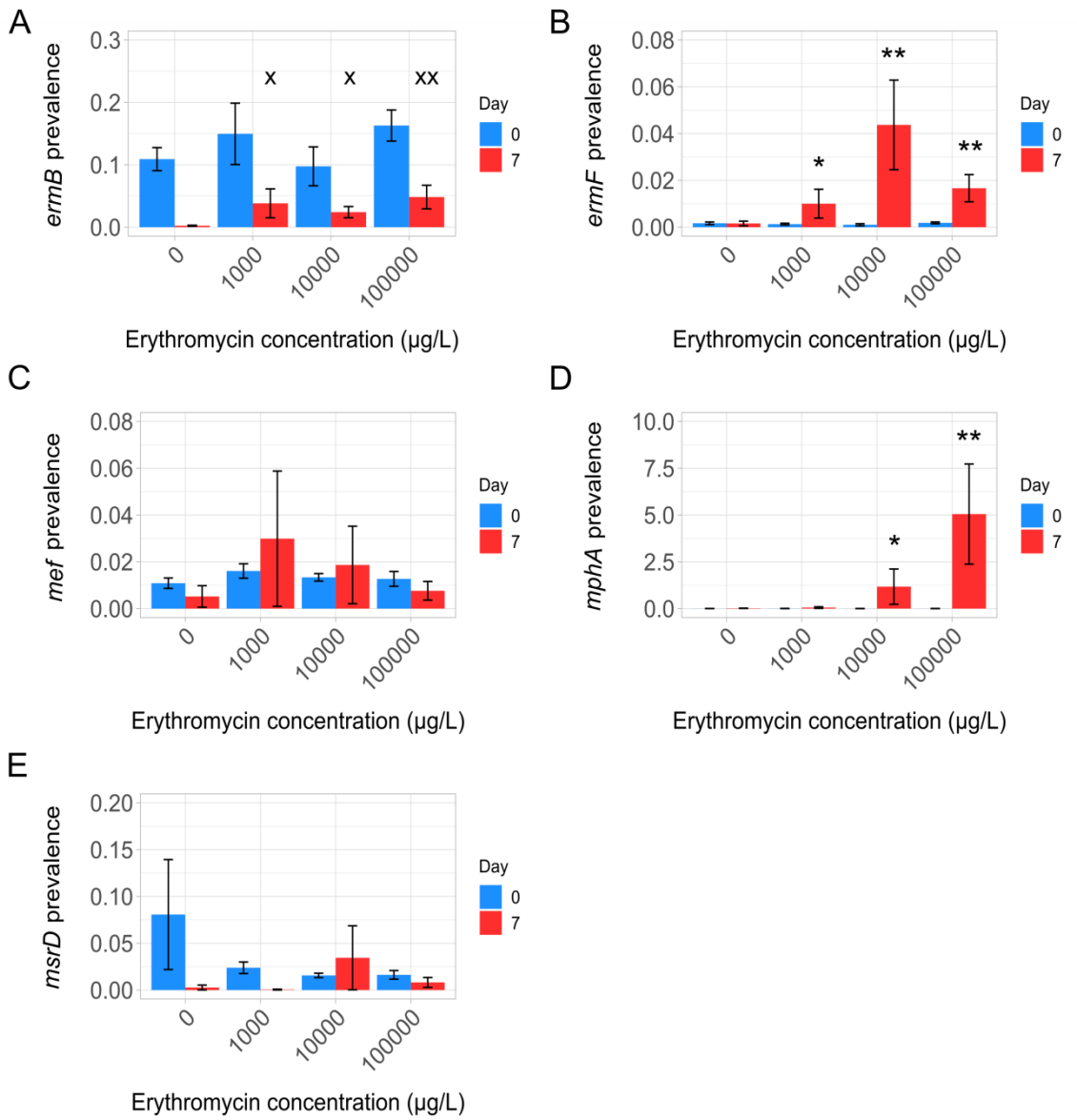


Figure 9A: e*rmB* as a function of high erythromycin concentrations. 9B: e*rmF* as a function of high erythromycin concentrations. 9C: *mef* family as a function of high erythromycin concentrations. 9D: *mphA* as a function of high erythromycin concentrations. 9E: *msrD* as a function of high erythromycin concentrations. x = significant increase in comparison to the control to 90% confidence. xx = significant increase in comparison to the control to 95% confidence. * = significant positive selection to 90% confidence, ** = significant positive selection to 95% confidence. Standard error is represented by the error bars.

3.4.4 Final selection experiments

The preliminary data for *ermF* showed no significant selection at 100 µg/L but significant selection at 1,000 µg/L for all three macrolides. It was decided, therefore, to target a concentration range between these two values to enable a more accurate MSC to be determined for the three macrolide compounds. This concentration range was 100, 250, 500, 750, 1,000, 10,000 and 100,000 µg/L. Based on preliminary data (as seen in Section 3.4.2 and 3.4.3), *mef* family, *ermB* and *msrD* were no longer tested. The integron gene *intI1,* however, was included in the analysis alongside *ermF* and *mphA*. The qPCR results can be seen in Section 3.4.4.1.

To determine whether spontaneous mutations or other macrolide resistant genes, not being quantified by qPCR, were selected for at concentrations lower than was seen with the current qPCR targets, culture dependent methods were used (see Section 3.4.4.3) and metagenome libraries were produced (see Section 3.4.4.4). Metagenome libraries were also analysed for the 16S rRNA community structure change with increasing concentration of macrolides, and for the co-selective ability of AZ, CLA and ERY to select for antibiotic resistance genes specific to other classes of antibiotics.

3.4.4.1 Real-time PCR analysis

Azithromycin

ermF

The gene that showed positive selection at the lowest AZ concentration was *ermF.* As can be seen in Figure 10, no significant selection was seen compared to the no antibiotic control at 100, 250 and 500 µg/L, but significant selection was seen to 90% confidence at 750 µg/L (p = 0.0616, Dunn's test) and to 95%

confidence at 1000 µg/L (p = 0.0015, Dunn's test) and 10,000 µg/L (p = 0.0242, Dunn's test). At 100,000 µg/L, no significant selection was seen. The LOEC was, therefore, determined to be 1,000 µg/L.

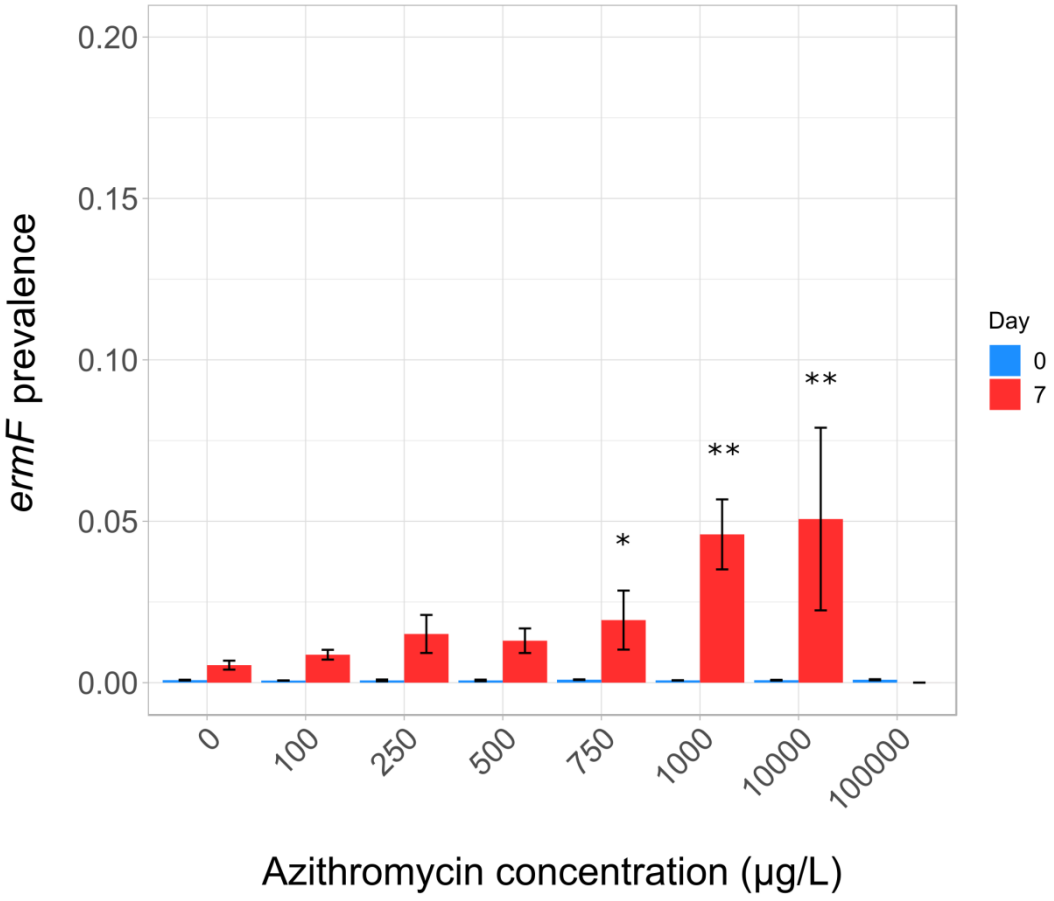


Figure 10: Selection for *ermF* by azithromycin. A LOEC of 1,000 µg/L is determined. Standard error is represented by the error bars. * = significant positive selection to 90% confidence, ** = significant positive selection to 95% confidence.

mphA

The graph showing the response of *mphA* to AZ can be seen in Figure 11. A strong biological effect can be seen at 1,000, 10,000 and 100,000 µg/L. When the Dunn's test was undertaken, however, only 10,000 µg/L showed a significant response (p = 0.0839, Dunn's test). This was, presumably, due to the

high variation between the biological replicates. This variation can be seen in Figure 80, Appendix. As a result, instead of using the Dunn's tests, a GLM was used to analyse this data. This produced more credible statistical significance.

Positive selection by AZ was, therefore, observed at 1,000 (p = 9.21E-5, GLM (Gamma, log)), 10,000 (p = 0.000413, GLM (Gamma, log)) and 100,000 µg/L (p = 0.003762, GLM (Gamma, log)). The LOEC was, therefore, determined to be 1,000 µg/L.

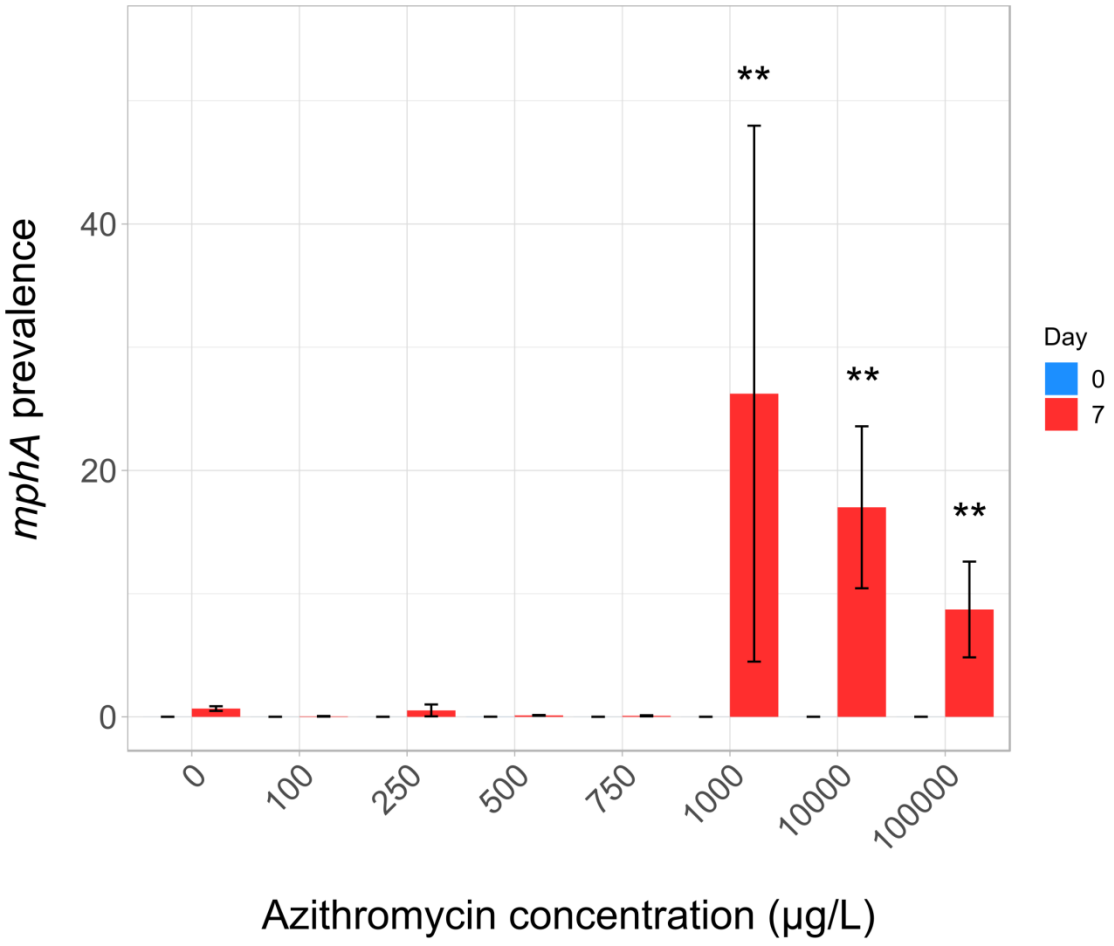


Figure 11: Selection for *mphA* by azithromycin. A LOEC of 1,000 µg/L was determined. Standard error is represented by the error bars. ** = significant positive selection to 95% confidence.

intI1

Similarly to *mphA,* the Dunnett's/Dunn's post hoc test did not align to the biological effect seen by AZ on *intI1*. GLM was, therefore, undertaken. No LOEC could be determined as significant selection was not observed to 95% confidence at any concentration. AZ positively selected for *intI1,* to 90% confidence*,* in comparison to the no antibiotic control, at 1,000 µg/L (p = 0.0886, GLM (Gamma, inverse)), 10,000 µg/L (p = 0.0886, GLM (Gamma, inverse)) and 100,000 µg/L (p = 0.0932, GLM (Gamma, inverse)), Figure 12. This was a more credible, and more protective, result than the Dunn's test which only determined significant selection at 10,000 (p = 0.0174, Dunn's test) when a clear biological effect was also seen at 1,000 and 100,000 µg/L.

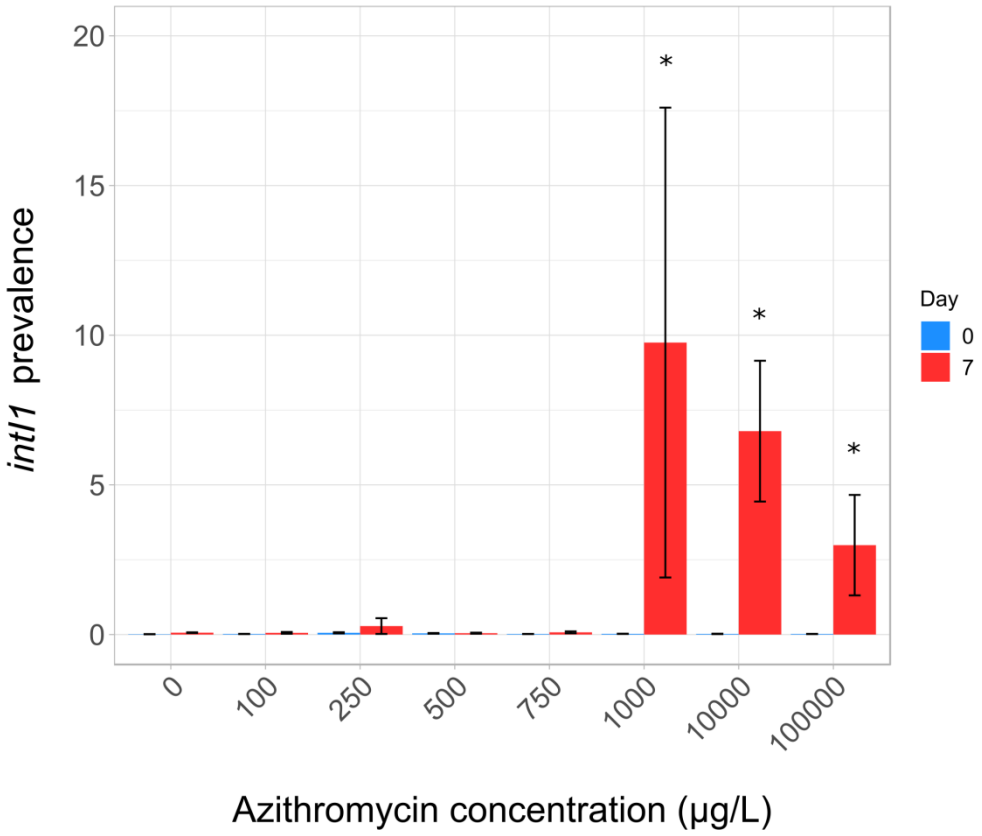


Figure 12: Selection for *intI1* by azithromycin. No LOEC could be defined. Significant positive selection was seen to 90% confidence at 1,000 µg/L. Standard error is represented by the error bars. * = significant positive selection to 90% confidence.

Clarithromycin

ermF

As with AZ, *ermF* showed a response to CLA at the lowest concentration (Figure 13). At 100, 250 and 500 µg/L no significant selection was seen compared to the no antibiotic control. Significant selection was seen to 95% confidence at 750 (p = 0.0336, Dunn's test), 1,000 (p = 0.0018, Dunn's test) and 10,000 µg/L (p = 0.0242, Dunn's test). Again, as with AZ, no significant selection was seen at 100,000 µg/L. A LOEC of 750 µg/L was, therefore, determined.

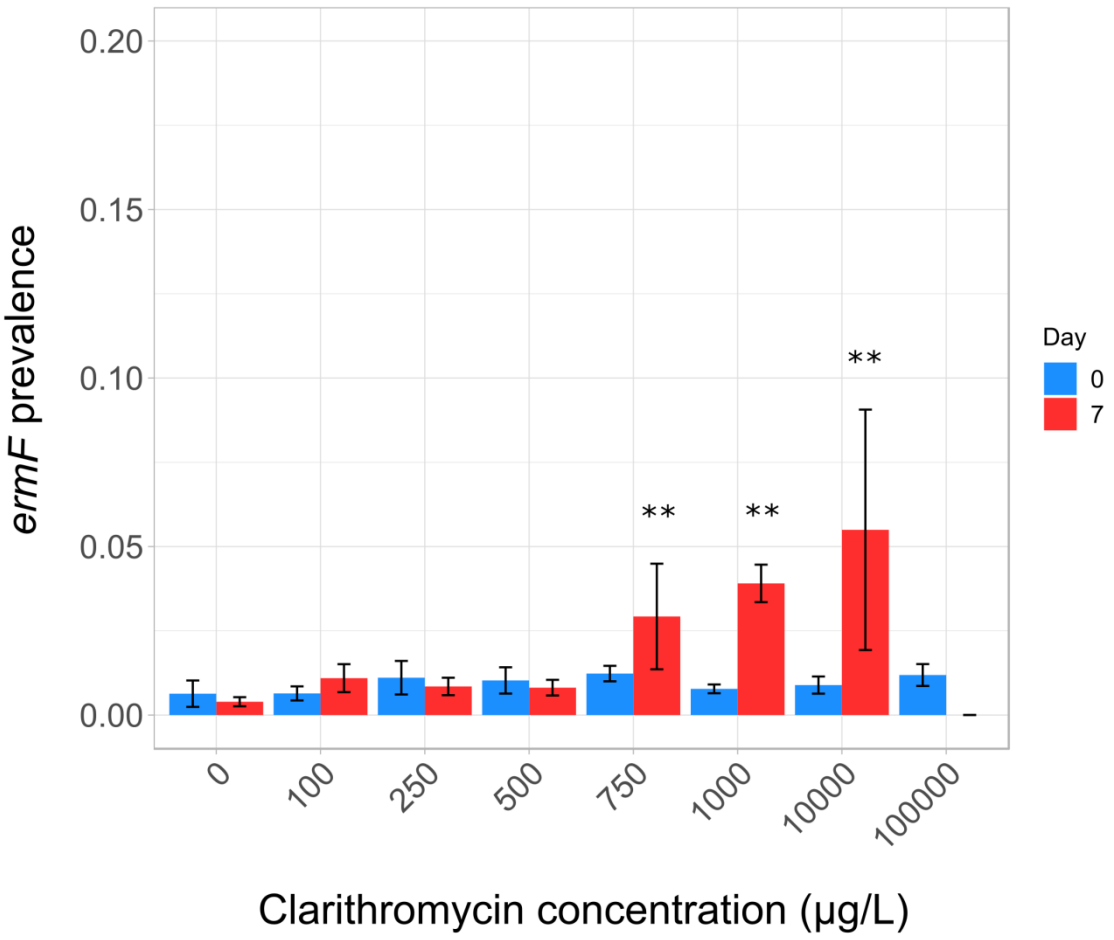


Figure 13: Selection for *ermF* by clarithromycin. A LOEC of 750 µg/L of CLA was determined. Standard error is represented by the error bars. ** = significant positive selection to 95% confidence.

mphA

Variation was observed in prevalence at day 0 (p = 0.001574, Kruskal Wallis). The difference in prevalence between day 0 and day 7 was, therefore, used for statistical analyses. A LOEC for *mphA* selection by CLA was 100,000 µg/L (p = 0.0446, Dunn's test (difference)), although a biological effect was seen at 10,000 µg/L, Figure 14. This was a significantly higher concentration of CLA in comparison to the concentration (750 µg/L) of CLA needed to positively select for *ermF.* A much stronger response was seen, however, in terms of prevalence

at day 7 and change in prevalence over the 7 day period of *mphA* by CLA in comparison to *ermF*.

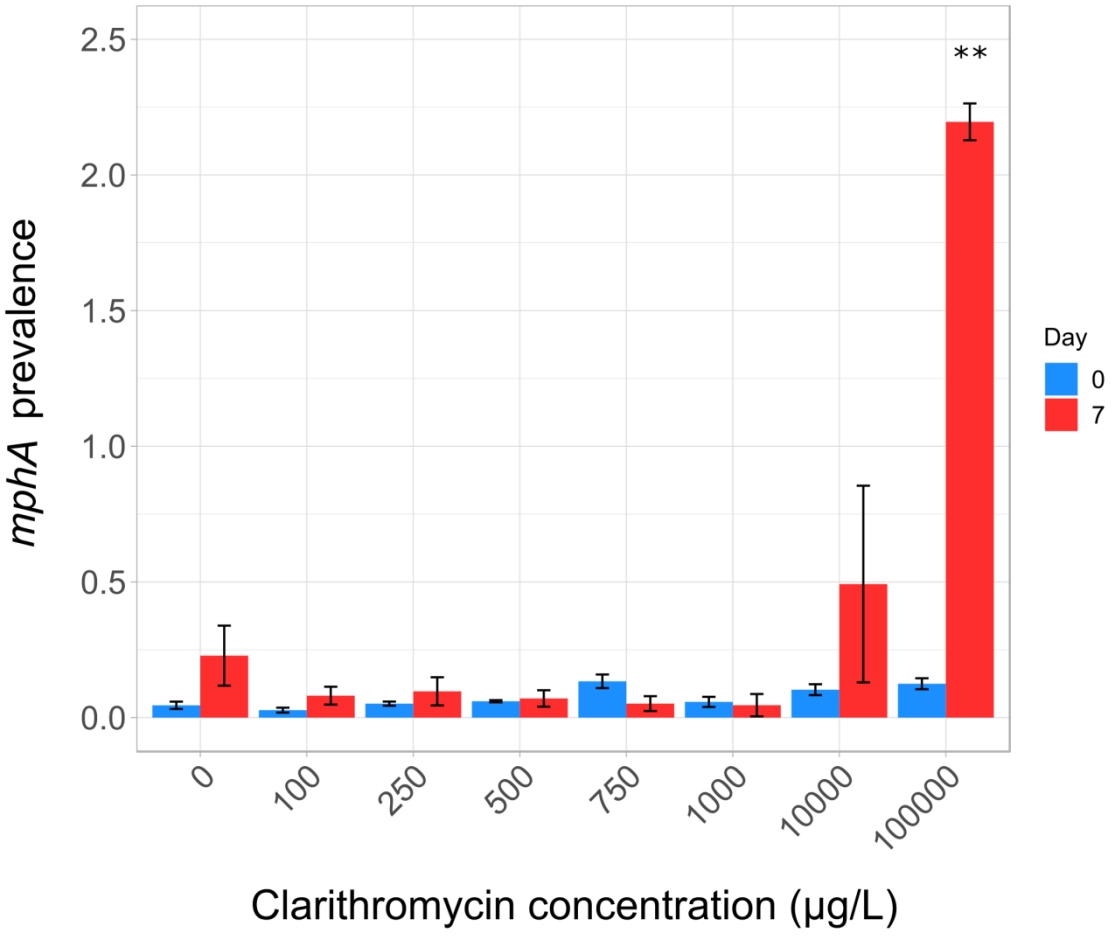


Figure 14: Selection for *mphA* by clarithromycin. A LOEC of 100,000 µg/L of CLA was determined. Standard error is represented by the error bars. ** = significant positive selection to 95% confidence.

intI1

As with the selection for *intI1* and *mphA* by AZ, the Dunnett's/Dunn's test did not align to the biological effect seen when *intI1* was selected by CLA. Variation was observed in the prevalence at day 0 (p = 0.0018, Kruskal Wallis) and therefore the change in prevalence was used for statistical analysis. Because, at certain concentrations prevalence decreases over time, the Gamma GLM

was not used as this model cannot fit to negative numbers. Significant positive selection was observed at 100,000 µg/L of CLA only for *intI1* (p = 1.46e-05, Gaussian GLM (difference)), Figure 15.

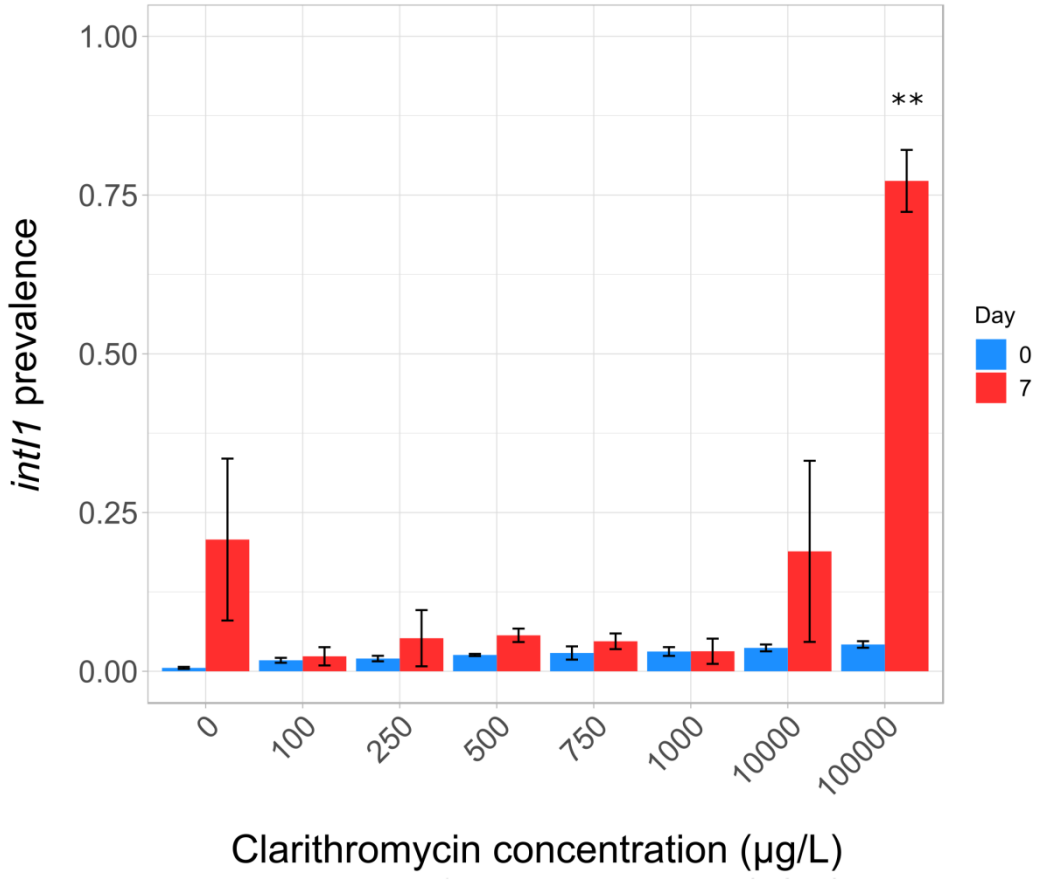


Figure 15: Selection for *intI1* by clarithromycin. A LOEC of 100,000 µg/L of CLA was determined. Standard error is represented by the error bars. ** = significant positive selection to 95% confidence.

Erythromycin

ermF

For ERY, the gene that illustrated significant selection at the lowest antibiotic concentration was, again, *ermF.* Again, no significant selection was seen at 100, 250 or 500 µg/L. Significant selection was seen to 90% confidence at 750 µg/L (p = 0.0663, Dunn's test) and to 95% confidence at 1000 (p = 0.0164,

Dunn's test) and 10,000 µg/L (p =0.0049, Dunn's test). As with AZ and CLA, no significant selection was seen at 100,000 µg/L. A LOEC was, therefore, determined to be 1,000 µg/L. This data can be seen in Figure 16.

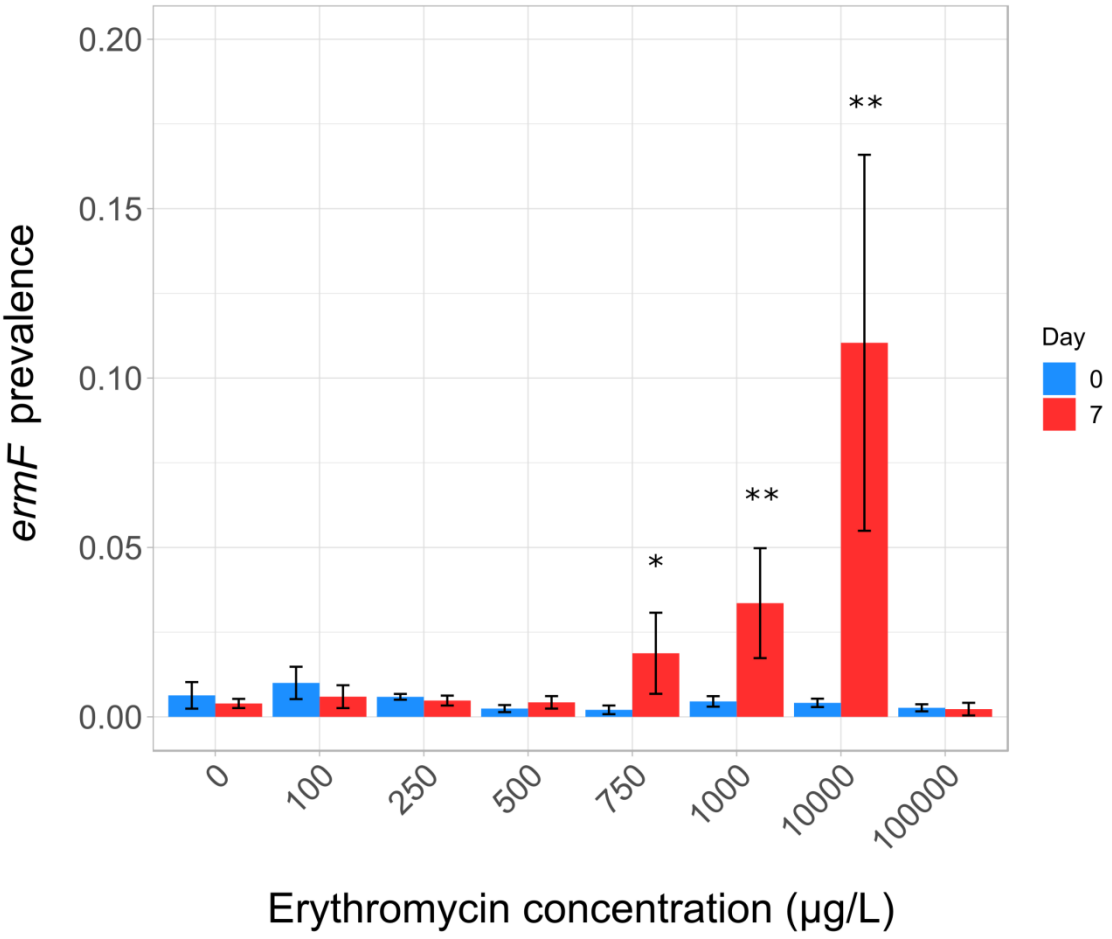


Figure 16: Selection for *ermF* by erythromycin. A LOEC of 1,000 µg/L was determined. Standard error is represented by the error bars. * = significant positive selection to 90% confidence, ** = significant positive selection to 95% confidence.

mphA

Similarly to CLA, significant positive selection for *mphA* by ERY was not observed until 100,000 µg/L (p = 0.0361, Dunn's test). A biological effect can be seen at 10,000 µg/L, although this was not statistically significant (Figure 17).

The effect ERY has on *mphA* was at a higher concentration, than what was observed with *ermF*, but the response of *mphA* was significantly stronger in regards to change in prevalence.

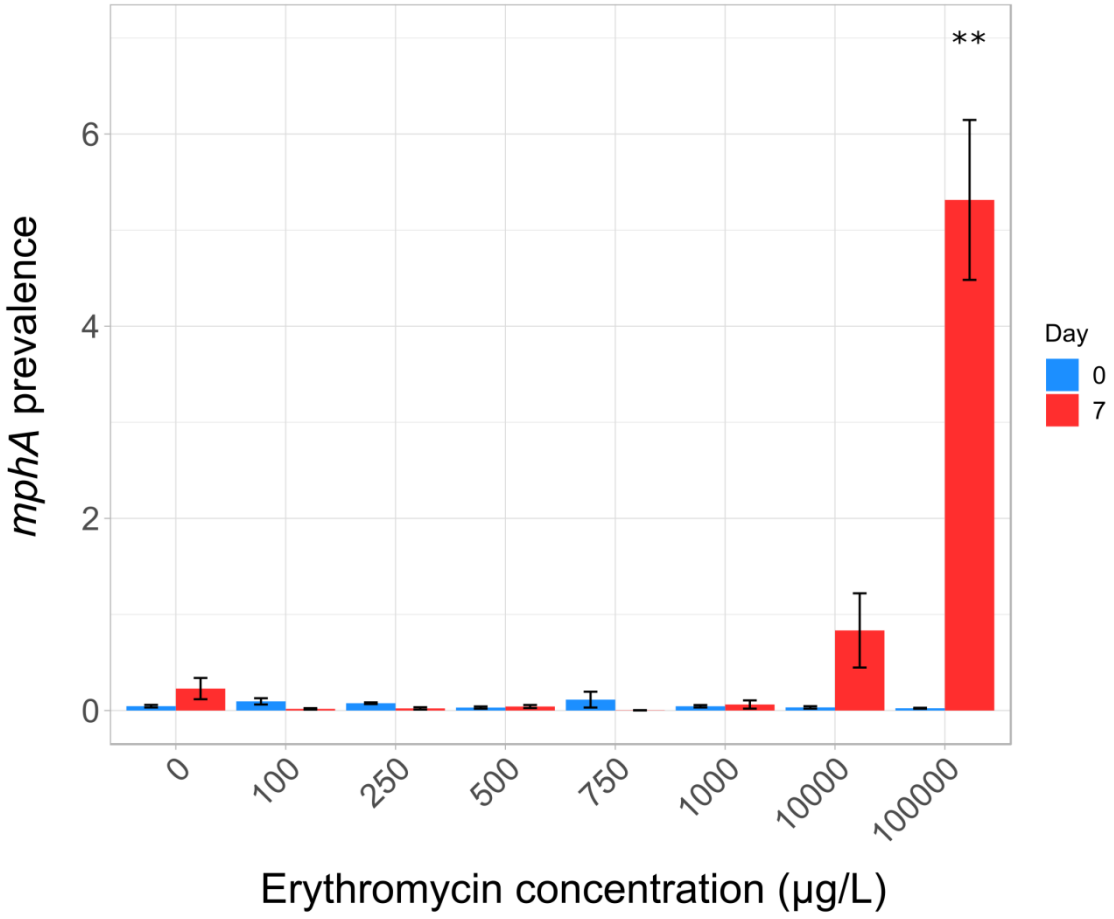


Figure 17: Selection for *mphA* by erythromycin. A LOEC of 100,000 µg/L of ERY was determined. Standard error is represented by the error bars. ** = significant positive selection to 95% confidence.

intI1

Here, statistical analysis was undertaken on the difference between day 0 and day 7 as there was a significant difference in prevalence between each of the day 0 samples (p = 0.0002008, Kruskal Wallis). Only 100,000 µg/L was

statistically significant, to 95% confidence, compared to the no antibiotic control (p = 0.0142, Dunn's test (difference)). A biological effect can be seen at 10,000 µg/L, but, as a result of variability between the biological replicates, this was not statistically significant (Figure 18). Although the response of *intI1* occurs at a higher concentration of ERY than *ermF*, the response shown by *intI1* was significantly stronger.

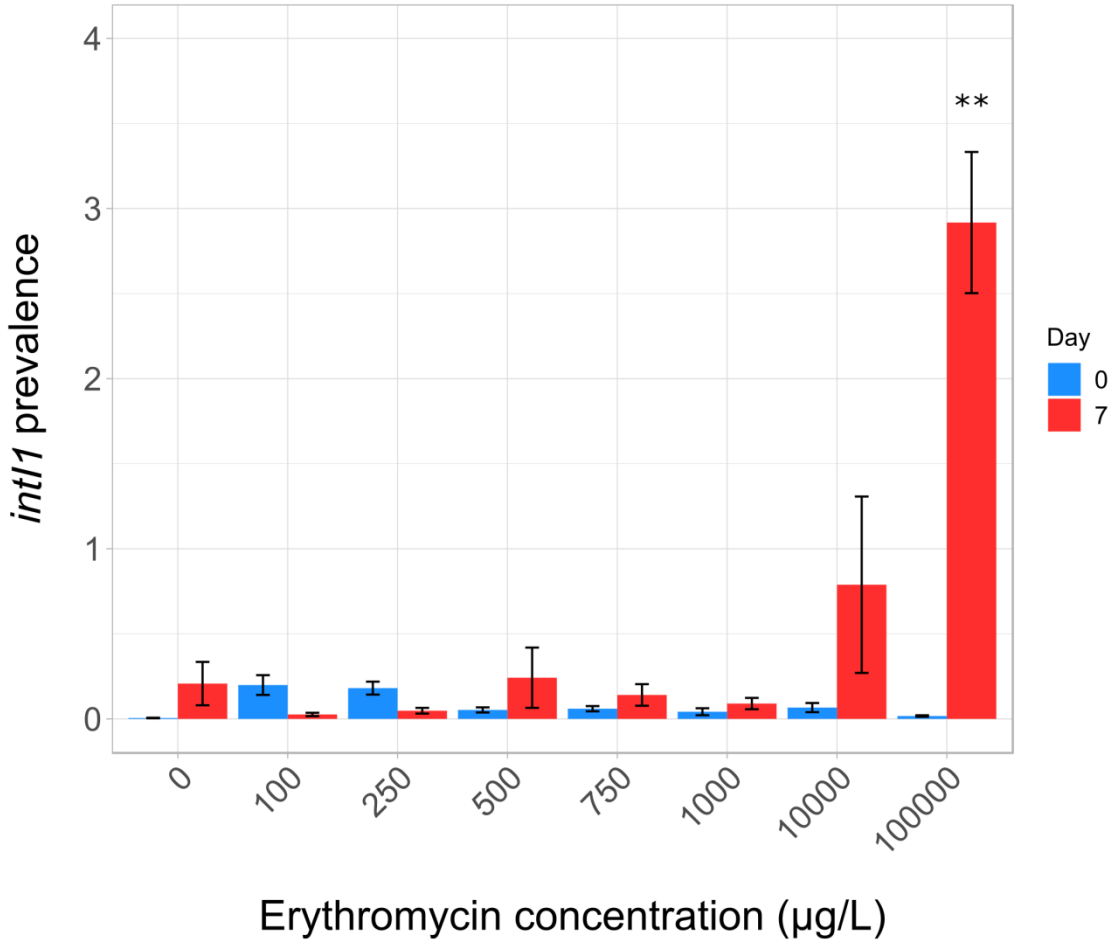


Figure 18: Selection for *intI1* by erythromycin. A LOEC of 100,000 µg/L was determined. Standard error is represented by the error bars. ** = significant positive selection to 95% confidence.

3.4.4.2 Selection coefficients

Using selection coefficient graphs can be a useful tool to evaluate the selective potential of antibiotics and other antimicrobial compounds. It may also offer a lower and, therefore, more protective value than the LOECs determined by statistical analysis. As significant selection was observed at the lowest concentration of AZ, CLA and ERY for the *ermF* gene, selection coefficients are only determined for this gene. Selection coefficients have their limitations, however. For example, it was only possible to determine a MSC (where the trendline crosses the x-axis) for *ermF* for ERY. For AZ and CLA, determining a MSC in this way was impossible as positive selection was observed in the no antibiotic control. This meant that the trendline for the selection coefficient graph for *ermF* by AZ was above the x axis at all concentrations. Not crossing the x axis at any point meant that no MSC value could be determined. Determining a selection coefficient for *ermF* by CLA had a similar problem. Again, the line of best fit was always above x axis although in some samples individual replicates were seen to be decreasing. The graphs for AZ and CLA can be seen in Figure 81 and 82 (Page 310 - Appendix), respectively. The LOECs were, therefore, used as the selective endpoint.

For ERY, however, a selection coefficient, and therefore MSC, was calculated (Figure 19). A polynomial order 4 curve was determined to be the line of best fit (R^2 = 0.1709, standard error = 0.3612). The equation of the line ($y = -2.544e^{-12}x^4 + 1.564e^{-09}x^3 + 2.59e^{-06}x^2 - 0.001432x + 0.01684$) was used to determine a MSC of approximately 514 µg/L for ERY (x intercept = 514.1). The natural variation between biological replicates meant that the MSC value could only be reported as an approximation.

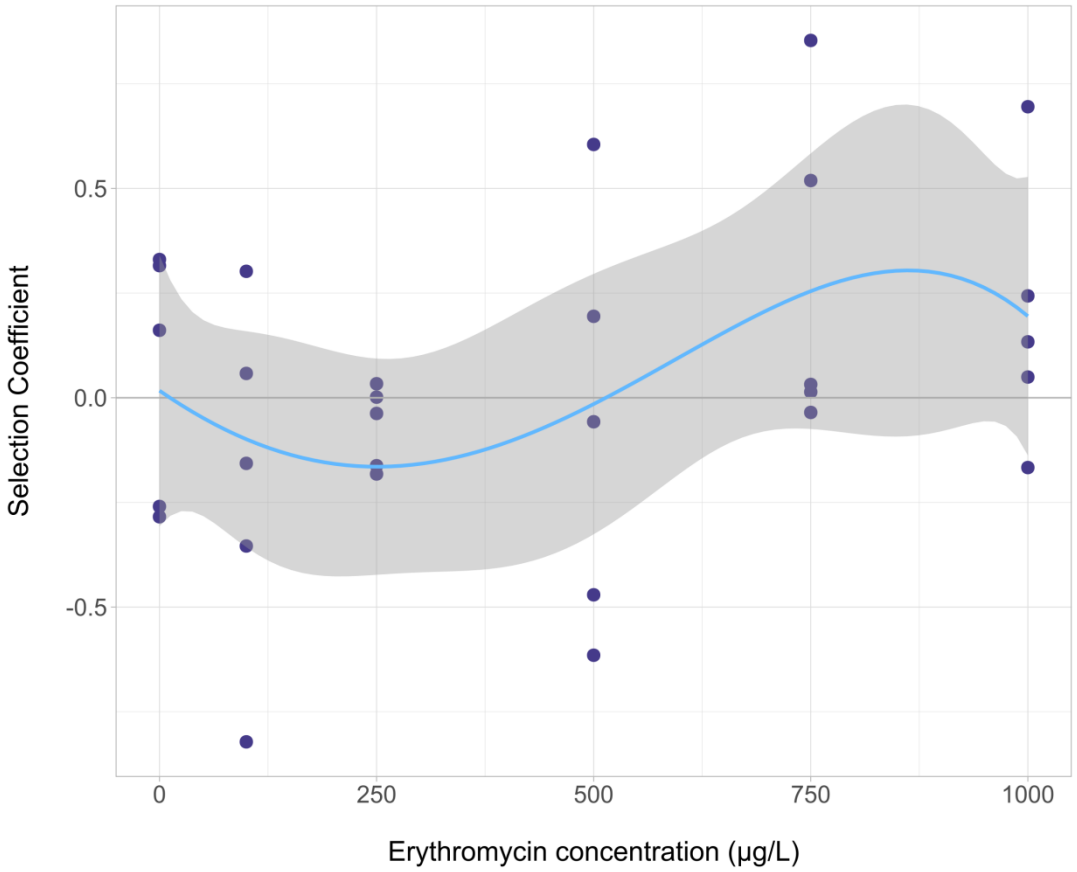


Figure 19: Selection coefficient of *ermF* in the presence of erythromycin. A MSC was determined to be approximately 514 µg/L. The blue line is the polynomial line of best fit (order 4), the grey shading represents the confidence intervals and the purple points represent the selection coefficients for each replicate.

3.4.4.3 Culture dependent analysis

A plating experiment was undertaken using the day 7 samples in the presence of AZ. This was to determine whether significant selection occurred below the LOEC determined by qPCR either by spontaneous mutations or by genes not targeted by qPCR analysis. Three types of agar were tested: Chromocult agar; Mannitol-salt agar and Muller-Hinton (Figure 20). No significant selection was seen at the concentration of 100 µg/L of AZ on any agar. For Mannitol-salt and Muller-Hinton agar, no significant selection was observed at any concentration of AZ. Bacteria plated on Chromocult agar saw a significant increase in

phenotypic resistance at 10,000 (p = 0.0493, Dunn's test) and 100,000 µg/L (p = 0.0493, Dunn's test).

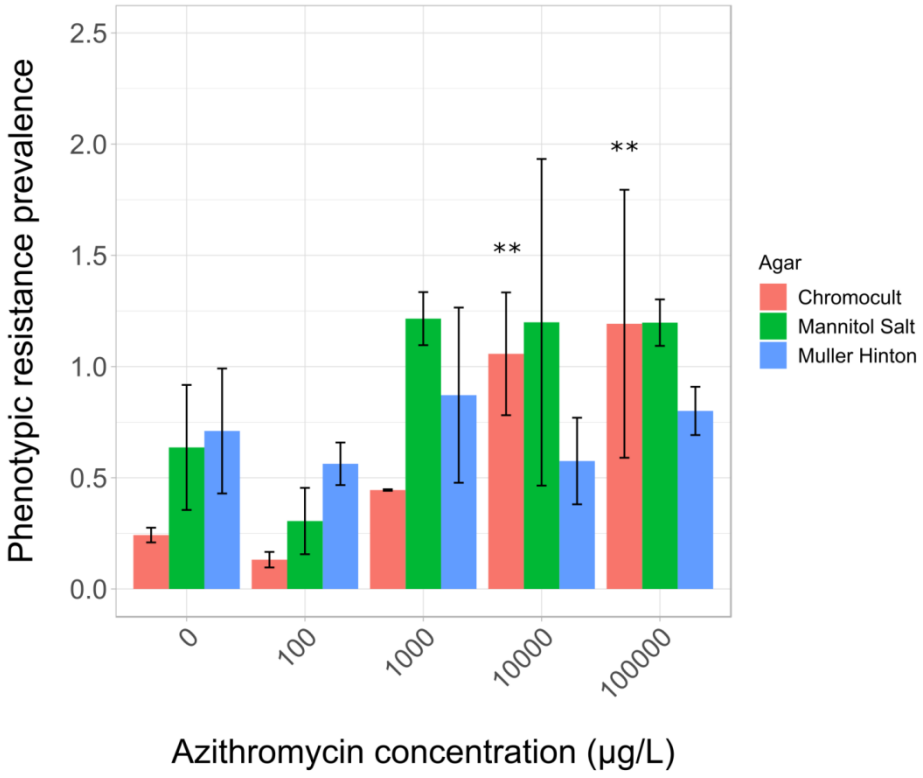


Figure 20: Selection for phenotypic resistance on three agars by azithromycin. Standard error is represented by the error bars. ** = significance to 95% confidence.

3.4.4.4 Metagenome analysis

Metagenome analysis was undertaken for a selection for samples taken from day 7 of the final selection experiment for AZ, CLA and ERY. As mentioned in Chapter 2, Aimee Murray undertook the raw sequence data analysis and produced spreadsheets that contained raw gene prevalence data per sample library (normalised to 16S rRNA copy number). All subsequent data analysis was undertaken by the author. By looking at the macrolide class as a whole, and by looking at individual macrolide resistance genes, the aim was to determine whether selection was occurring at a concentration lower than

determined by qPCR (LOECs of 1,000, 750 and 1,000 µg/L for AZ, CLA and ERY, respectively, and a MSC of approximately 514 µg/L for ERY). Metagenome analysis produced a large amount of data on relative abundance of all ARGs and, therefore, it was possible to examine the co-selective nature of AZ, CLA and ERY by investigating the change in other antibiotic class resistance genes and determine how increasing concentration of AZ, CLA and ERY affected bacterial community structure.

Investigating the change in prevalence of MLS genes using metagenomics

First, the effect of macrolide selection on MLS gene prevalence was investigated. The group MLS genes encode resistance to macrolide, lincosamide and streptogramin antibiotics. These antibiotics are often grouped together in the literature as, although they are chemically different compounds, they all have a similar mode of action inhibiting protein synthesis within the bacterial cell (Leclercq & Courvalin 1991). Here, the ARGs-OAP v2 pipeline groups them as one class.

Azithromycin

For AZ (Figure 21), a significant increase in prevalence, compared to the no antibiotic control, was seen to 95% confidence at 10,000 (p = 0.0280, Dunn's test) and 100,000 µg/L (p = 0.0047, Dunn's test) but at no AZ concentration lower than this. A biological effect was seen at 1,000 µg/L but, as a result of variation between replicates, statistical significance is not determined.

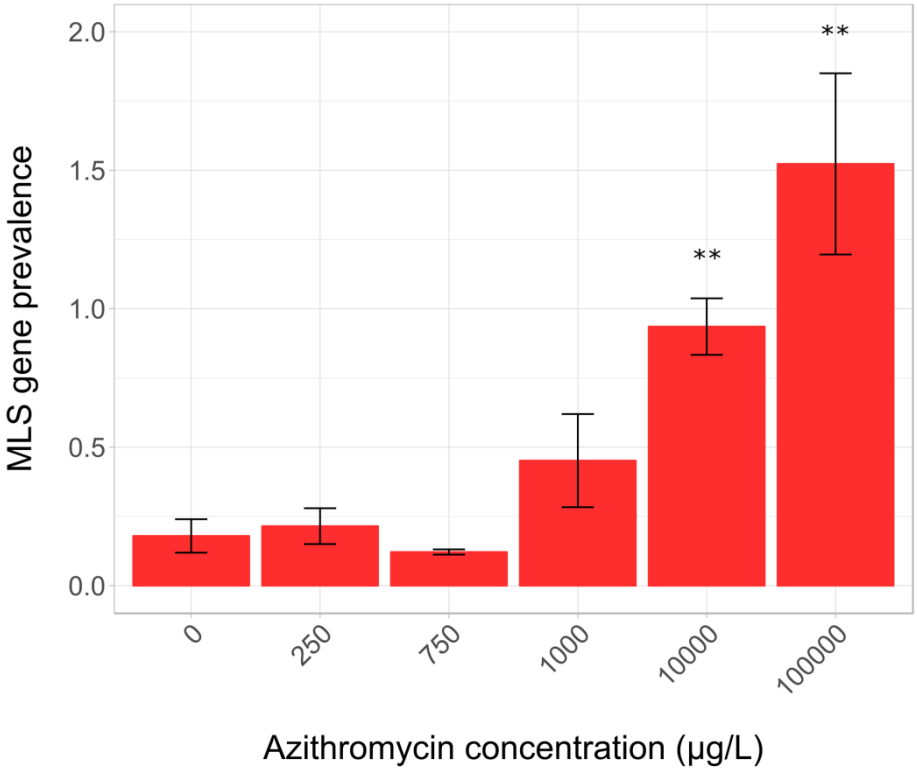


Figure 21: MLS gene prevalence as a function of azithromycin concentration. A LOEC of 10,000 µg/L of AZ was determined. Standard error is represented by the error bars. ** = significance to 95% confidence.

Clarithromycin

MLS genes only demonstrated a significant increase in prevalence, to 95% confidence, at 100,000 µg/L (p = 0.0089, Dunn's test) of CLA (Figure 22).

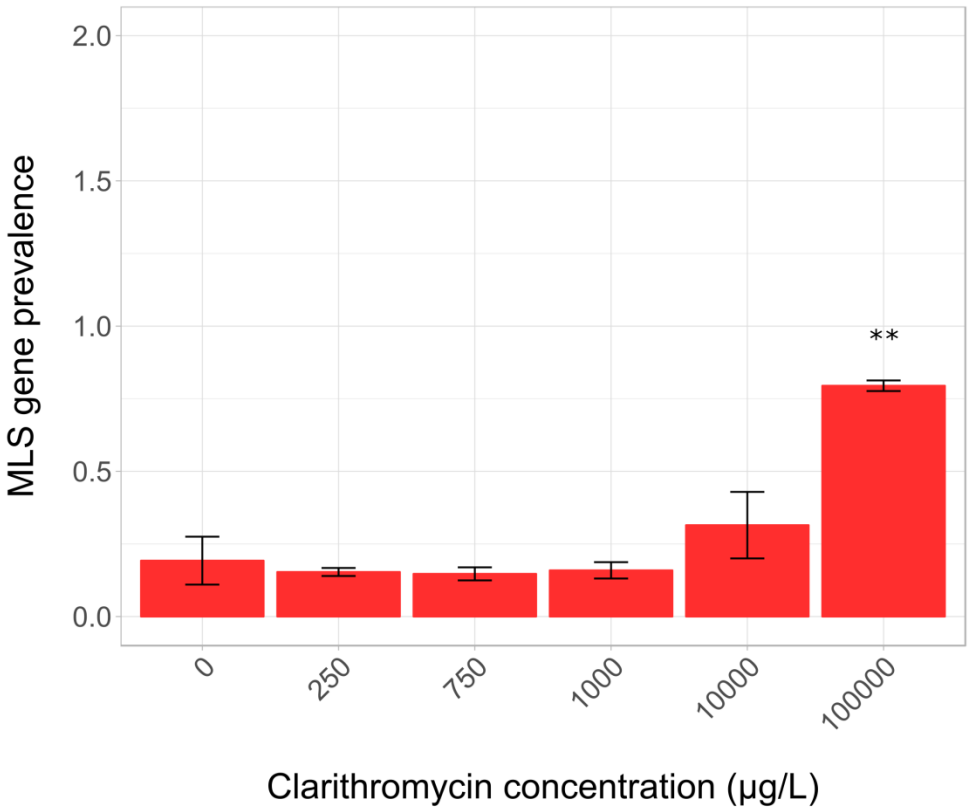


Figure 22: MLS gene prevalence as a function of clarithromycin concentration. A LOEC of 100,000 µg/L was determined. Standard error is represented by the error bars. ** = significance to 95% confidence.

Erythromycin

Finally, ERY only showed a significant increase in prevalence of MLS genes, compared to the no antibiotic control, to 90% confidence at 10,000 (p = 0.0843, Dunn's test) and, to 95% confidence, at 100,000 µg/L (p = 0.0109, Dunn's). This can be seen in Figure 23.

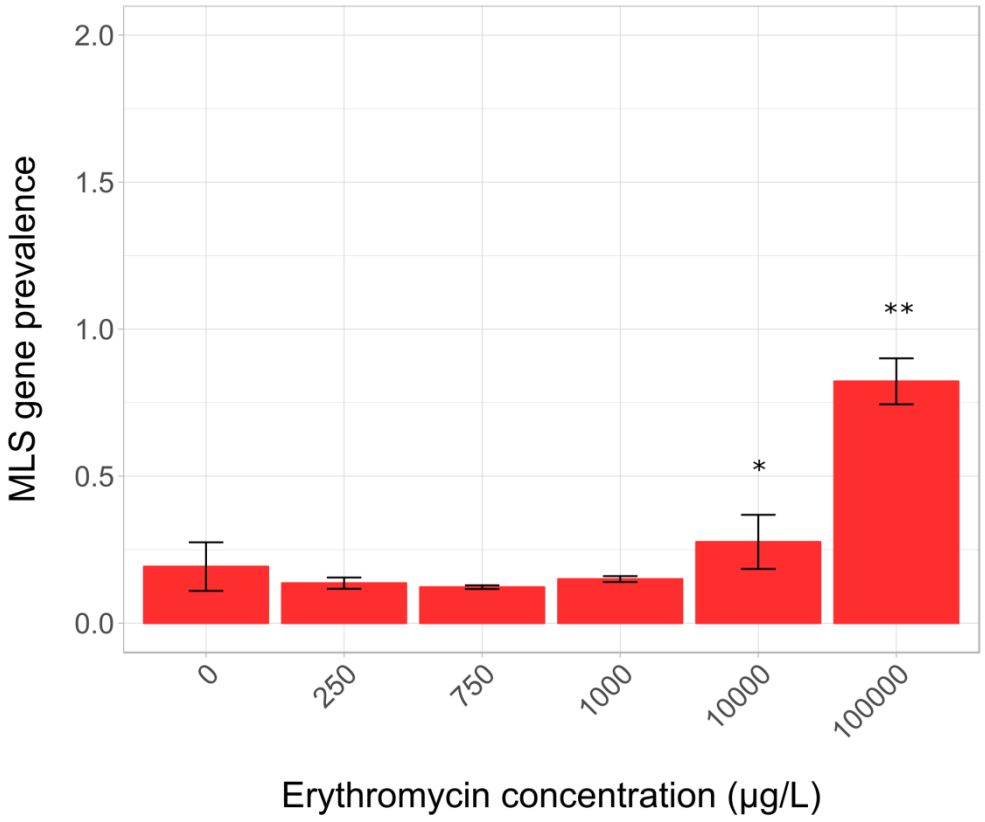


Figure 23: MLS gene prevalence as a function of erythromycin concentration. A LOEC of 100,000 µg/L was determined. Standard error is represented by the error bars. * = significance to 90% confidence, ** = significance to 95% confidence.

Investigating the change in prevalence of individual macrolide resistance genes

Heatmaps showing log(prevalence) of the individual macrolide resistance genes at various concentrations of AZ, CLA and ERY can be seen in Figure 24, 25 and 26, respectively. Genes that were below the detection limit or not present at every concentration of antibiotic were eliminated from heatmaps. The colour white on all three graphs represents where the genes were below the limit of detection in the samples taken for that concentration of macrolide. The raw prevalence data used to generate the heatmaps can be found in Tables 12, 13 and 14 (Page 312 – Appendix) for AZ, CLA and ERY, respectively.

To test whether there was any selection occurred below LOECs and MSC defined by qPCR data, the concentration 250 µg/L of AZ, CLA and ERY was investigated for all macrolide resistance genes. A secondary aim was to compare qPCR and the metagenome data as it has been stated that qPCR analysis is a more sensitive method to determine MSCs than metagenomics (Lundström et al. 2016). Investigation was, therefore, also undertaken into the response of *ermF* and *mphA* when analysed by metagenomics and how this compared to the qPCR data in Section 3.4.4.1.

Azithromycin

The metagenome analysis of individual macrolide resistance genes in the presence of AZ showed no significant increase in prevalence, to 95% confidence, of any of the genes in the ARG-OAP v2 database at 250 µg/L. A significant increase in prevalence was observed, to 90% confidence, at 250 µg/L for *ermX* (encoding a rRNA methylase enzyme) and *macA* (encoding a subunit of an efflux pump). There were no detectable *ermX* genes in the no

antibiotic control samples. The abundance of *ermX* in two of the three samples at 250 µg/L was extremely low. There was a correlation between the response of *ermF* and *mphA* in metagenome analysis and in the qPCR data presented in Section 3.4.4.1. From the metagenome data, a significant increase in prevalence of *ermF* was seen at 750 (p = 0.0131, Dunn's test), 1,000 (p = 0.0193, Dunn's test) and 10,000 µg/L (p = 0.0459, Dunn's test) and no detectable genes at 100,000 µg/L of AZ. From the qPCR results, a significant increase to 90% confidence was observed at 750 µg/L, the LOEC (significance to 95% confidence) was 1,000 µg/L for *ermF,* a significant increase to 95% confidence was observed at 10,000 µg/L and a complete reduction at 100,000 µg/L. The metagenome data was, therefore, relatively consistent with the qPCR results. In the metagenome data, a significant increase in *mphA* prevalence was first observed, to 95% confidence, at 10,000 µg/L (p = 0.0393, Dunn's test) and to 90% confidence at 100,000 µg/L (p = 0.0968, Dunn's test) in comparison to the no antibiotic control. QPCR determined a LOEC of 1,000 µg/L and significant positive selection at all subsequent concentrations, 10,000 and 100,000 µg/L.

Only a significant increase (** = to 95% confidence and * = to 90% confidence) is represented on the heatmap. Significant decreases, compared to the no antibiotic control, have not been represented. For example, the prevalence of *ereA* drops to undetectable in every sample with AZ present and significant decrease is, therefore, observed at every concentration of AZ (p = 0.04159, Dunn's test).

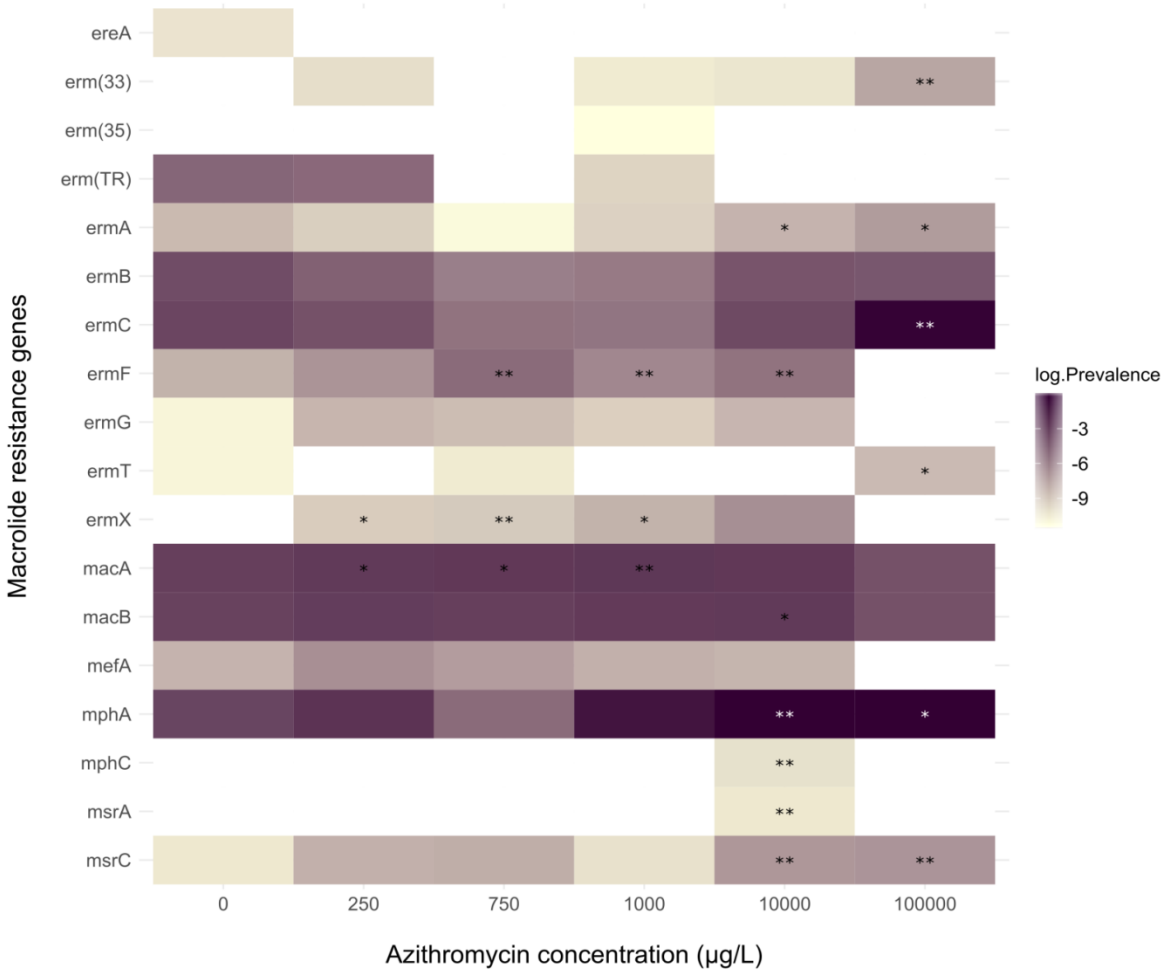


Figure 24: Heatmap showing the change in prevalence of macrolide resistance genes at various concentrations of azithromycin. The heatmap does not show the macrolide resistance genes that were below detection limit at every concentration of AZ. White represents where genes were below detection limit. The log(prevalence) is displayed here. Only a significant increase in prevalence is represented here (* = significance to 90% confidence, ** = significance to 95% confidence).

Clarithromycin

For CLA, significant selection, to 95% confidence, was seen at 250 µg/L for the genes *ermB* (p = 0.0037, Dunn's test), *ermC* (p = 0.0462, Dunn's test) and *mefA* (p = 0.0446, Dunn's test).

For *ermF* a significant increase in prevalence was seen, to 95% confidence, at 1,000 µg/L (p = 0.0273, Dunn's test) and to 90% confidence at 10,000 µg/L (p = 0.0669, Dunn's test). No significant increase in prevalence was observed at 750 µg/L, the LOEC determined by qPCR. The qPCR data gives, therefore, a lower estimation than the metagenome data. For *mphA,* no significant increase in prevalence compared to the no antibiotic control was observed until 100,000 µg/L (p = 0.0541, Dunn's test). This correlated with the qPCR data.

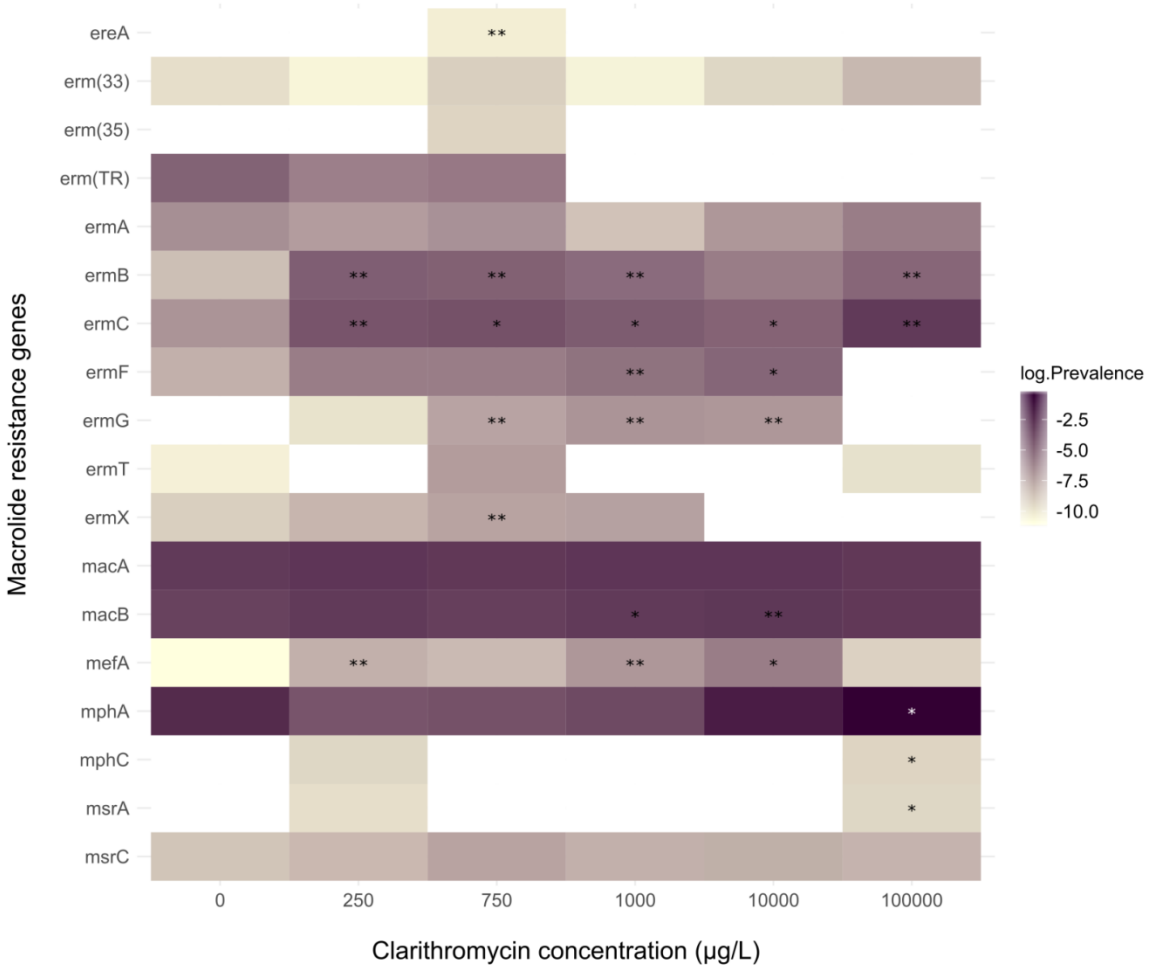


Figure 25: Heatmap showing the change in prevalence of macrolide resistance genes at various concentrations of clarithromycin. The heatmap does not show the macrolide resistance genes that were below detection limit at every concentration of CLA. White represents where genes were below detection limit. The log(prevalence) is displayed here . Only a significant increase in prevalence is represented here (* = significance to 90% confidence, ** = significance to 95% confidence).

Erythromycin

The metagenome results for ERY showed significant increase in prevalence compared to the no antibiotic control to 95% confidence at 250 µg/L for *ermC* (*p* = 0.0037, Dunn's test) and *macB* (*p* = 0.0109, Dunn's test). For *ermF,* no significant increase in prevalence was seen at 250 and 750 µg/L. A significant increase compared to the no antibiotic control was, however, seen to 95% confidence at 1,000 (*p* = 0.0233, Dunn's test) and 10,000 µg/L (*p* = 0.0462, Dunn's test). This was consistent with the qPCR data which showed a LOEC of 1,000 µg/L and a significant increase to 95% confidence at 10,000 µg/L. With the qPCR data significant selection was seen at 750 µg/L to 90% confidence but this was not replicated in the metagenome data. For *mphA,* no significant selection was seen at any concentration of ERY until 100,000 µg/L, where significant selection was seen to 90% confidence (*p* = 0.0541, Dunn's test), similarly to the qPCR results.

The statistical analysis for *ermB* did not match the biological effect seen. As can be observed in both the heatmap (Figure 26) and in the bar graph (Figure 83, Page 311 - Appendix) there was an increase in prevalence of *ermB* at all concentrations of 250 µg/L in comparison to the no antibiotic control. This was similar to the response seen when *ermB* was in the presence of CLA, although with CLA it was determined as statistically significant.

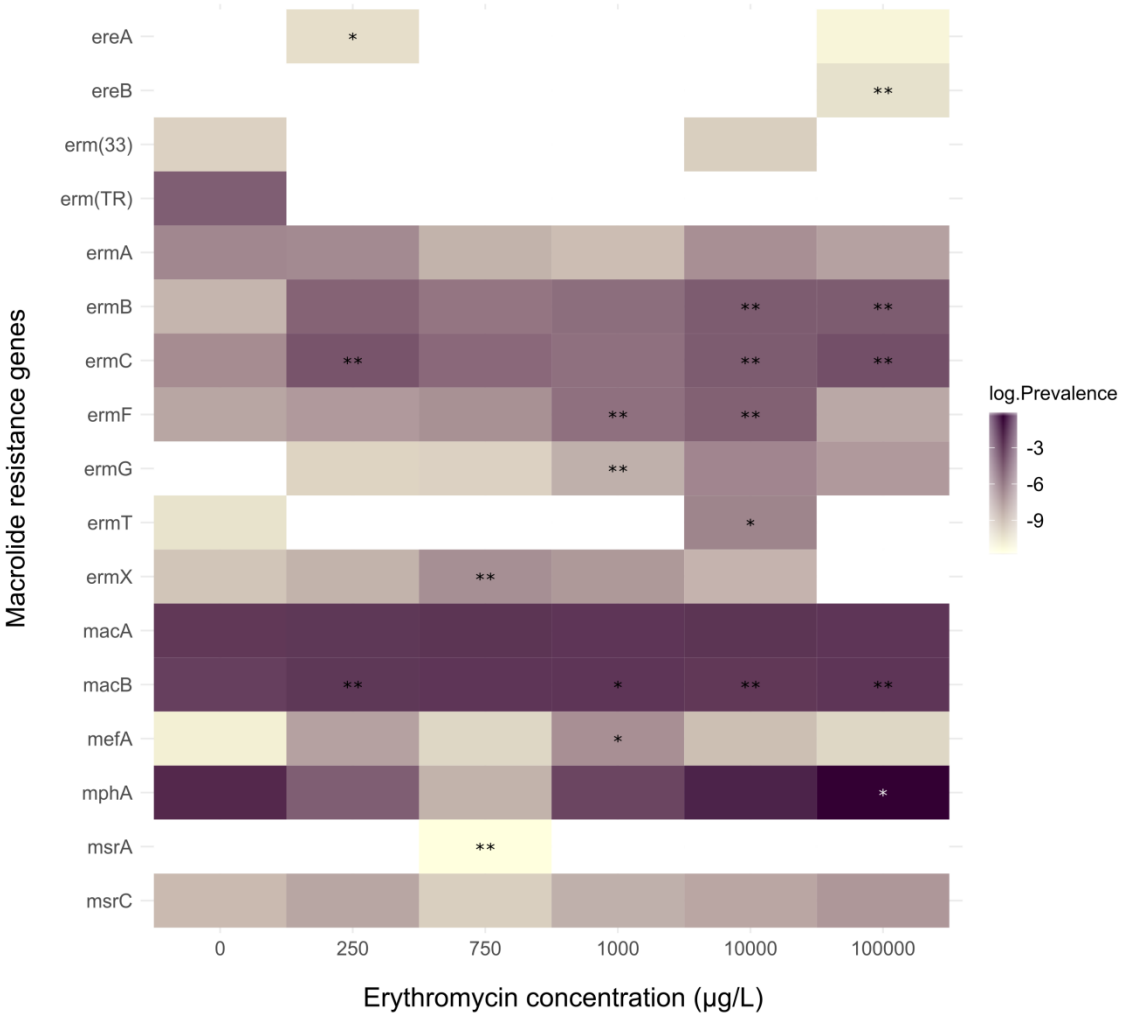


Figure 26: Heatmap showing the change in prevalence of macrolide resistance genes at various concentrations of erythromycin. The heatmap does not show the macrolide resistance genes that were below detection limit at every concentration of ERY. White represents where genes were below detection limit. The log(prevalence) is displayed here. Only a significant increase in prevalence is represented here (* = significance to 90% confidence, ** = significance to 95% confidence).

As sequencing was not undertaken on the day 0 samples and because qPCR has been suggested to be more sensitive than metagenome analysis (Lundström et al. 2016), it was decided to use qPCR to target *macB* and *ermB.* The purpose was to investigate whether there was a positive selective effect on these genes or if these significant differences may be due to differential rates of negative selection. Also, as has been seen from some of the metagenome data, such as *ermF* in the presence of CLA, qPCR is a more sensitive method than metagenome analysis.

The *macA* gene was not targeted as *macA* and *macB* encode 2 subunits (MacA and MacB, respectively) of the ABC-type efflux pump, MacAB (Rouquette-Loughlin et al. 2005; Bogomolnaya et al. 2013) and are, therefore, always found in combination. The *macB* primers can be found in Table 2 in Section 2.3.4.1. The gene is not well conserved and four subtypes of the gene were identified from sequences obtained on GenBank and aligned in MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/). Four sets of primers were, therefore, designed. Rather than testing the whole range of antibiotic concentrations for all 3 macrolides, ERY was tested as an example, as the macrolides have behaved in a similar way for *ermF* which has been used to define the LOEC. It was also decided to only test the ERY concentration at 1,000 µg/L as if no significant positive selection was seen at this concentration, then there was no need to test lower concentrations.

For two of the *macB* subtypes (subtypes 3 and 4) no genes were detected in these samples. For subtype 1 and 2, however, the results can be seen in Figure 27A and B, respectively. For *macB* subtype 1, positive selection was observed in both the no antibiotic control and at 1,000 µg/L but no significant difference was observed between the two. For *macB* subtype 2, no positive selection was observed.

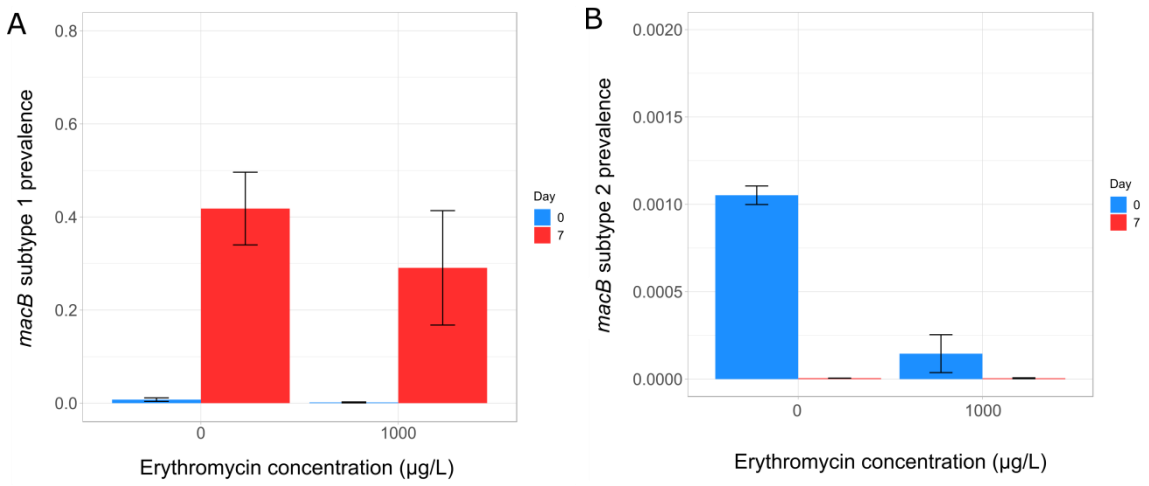


Figure 27A: *macB* subtype 1 as a function of erythromycin concentration. No significant positive selection was observed in comparison to the no antibiotic control. **Figure 27B: *macB* subtype 2 as a function of erythromycin concentration.** No significant positive selection was observed in comparison to the no antibiotic control Standard error is represented by the error bars.

The *ermB* gene was targeted as it showed a similar response to *ermC*. It is also one of the genes targeted in the preliminary experiments undertaken in Section 3.4.2 and 3.4.3. The qPCR, as with *macB*, was run on samples selected for by 1000 µg/L of ERY and the no antibiotic control. The results of the qPCR can be seen in Figure 28. Similarly to *macB,* and the response to *ermB* seen in the preliminary range finding experiments in Section 3.4.2 and 3.4.3, no positive

selection was observed for *ermB* at 1000 µg/L. Therefore, none of the genes tested using qPCR, that showed an increase in relative abundance in the metagenome data, proved to be under positive selection.

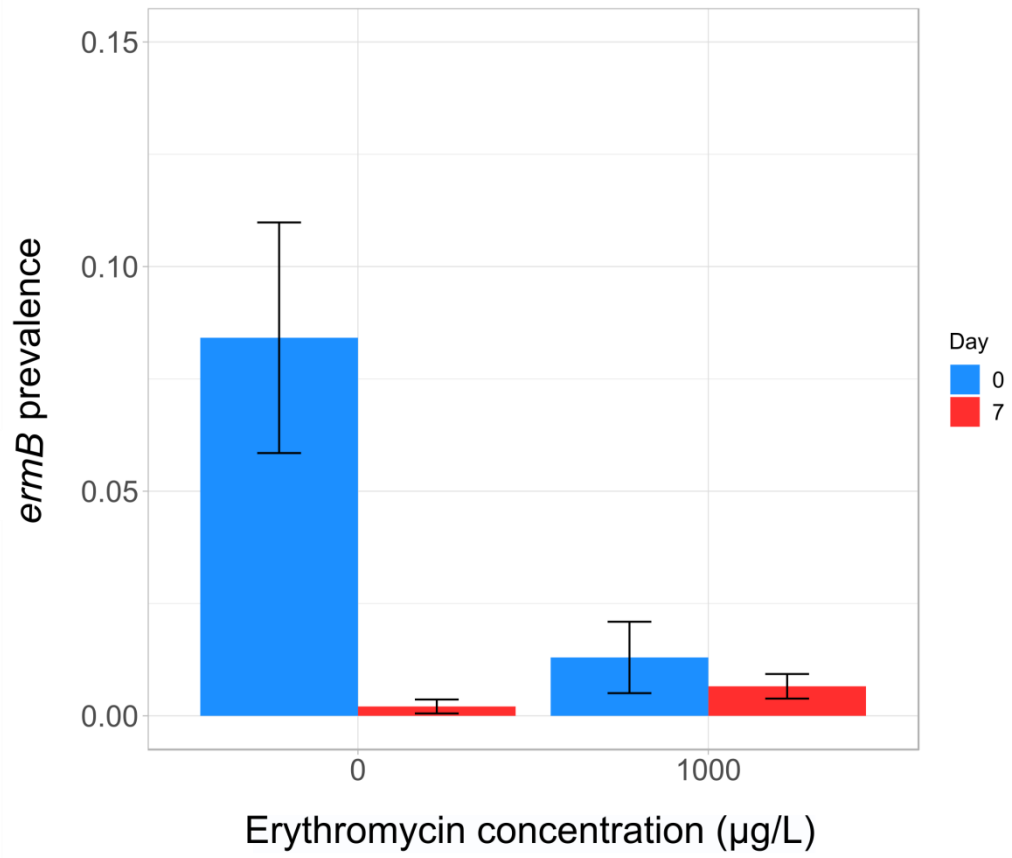


Figure 28: *ErmB* as a function of erythromycin concentration. No significant positive selection was observed in comparison to the no antibiotic control Standard error is represented by the error bars.

Investigating the co-selective potential of macrolides

Heatmaps showing the change in prevalence of resistance genes belonging to different classes of antibiotic can be seen in Figures 29, 30 and 31 in the presence of increasing concentrations of AZ, CLA and ERY, respectively. Significant increase in prevalence compared to the no antibiotic control has been displayed on the graphs to 95% confidence = ** and to 90% confidence =

*. Significant decreases have not been represented. The raw prevalence data used to make the heatmaps can be found in Tables 15, 16 and 17 (Page 313 – Appendix) for AZ, CLA and ERY, respectively.

Certain gene classes have been removed from these heatmaps, specifically the MLS gene class (as this was previously described in depth earlier in this section) and gene classes where prevalence was either below the limit of detection or not present at all concentrations. The white areas on all three graphs represent a lack of detectable genes.

Azithromycin

No genes were detected at any concentration of AZ for carbomycin, fusidic-acid, puromycin, rifamycin, spectinomycin and tetracenomycin C resistance. No significant co-selective effect was seen at any concentration of AZ for the resistance gene classes of bleomycin or fosfomycin compared to the no antibiotic control.

No significant increase in prevalence, compared to the no antibiotic control, was seen until 1,000 µg/L for any antibiotic resistance gene class. The class kasugamycin saw a significant increase in prevalence to 95% confidence at 1,000 (p = 0.0462, Dunn's test) and 10,000 µg/L (p = 0.0280, Dunn's test). Polymyxin (p = 0.0631, Dunn's test), sulfonamide (p = 0.0631, Dunn's test) and unclassified (p = 0.0938, Dunnett's test) resistance genes demonstrated a significant increase to 90% confidence at 1,000 µg/L. Polymyxin (p = 0.0393, Dunn's test) and unclassified (p = 0.0309, Dunnett's test) classes both increased significantly, to 95% confidence, at 10,000 µg/L but no significant increase in the presence of 100,000 µg/L of AZ was observed. Sulfonamide resistance genes saw a significant increase, compared to the no antibiotic

control, to 10,000 (p = 0.0058, Dunn's test) and 100,000 µg/L (p = 0.0234, Dunn's test).

Seven gene classes saw an initial increase in the prevalence of resistance genes at 10,000 µg/L. These classes consisted of aminoglycosides (p = 0.0133, Dunn's test), beta-lactams (p = 0.0332, Dunn's test), chloramphenicol (p = 0.012, Dunnett's test), quinolones (p = 0.0385, Dunn's test), tetracycline (p = 0.0195, Dunn's test), trimethoprim (p = 0.0133, Dunn's test) and vancomycin (p = 0.0233, Dunn's test). Only the classes bacitracin and fusaric-acid saw no significant increase until 100,000 µg/L. This data can be seen in the heatmap - Figure 29.

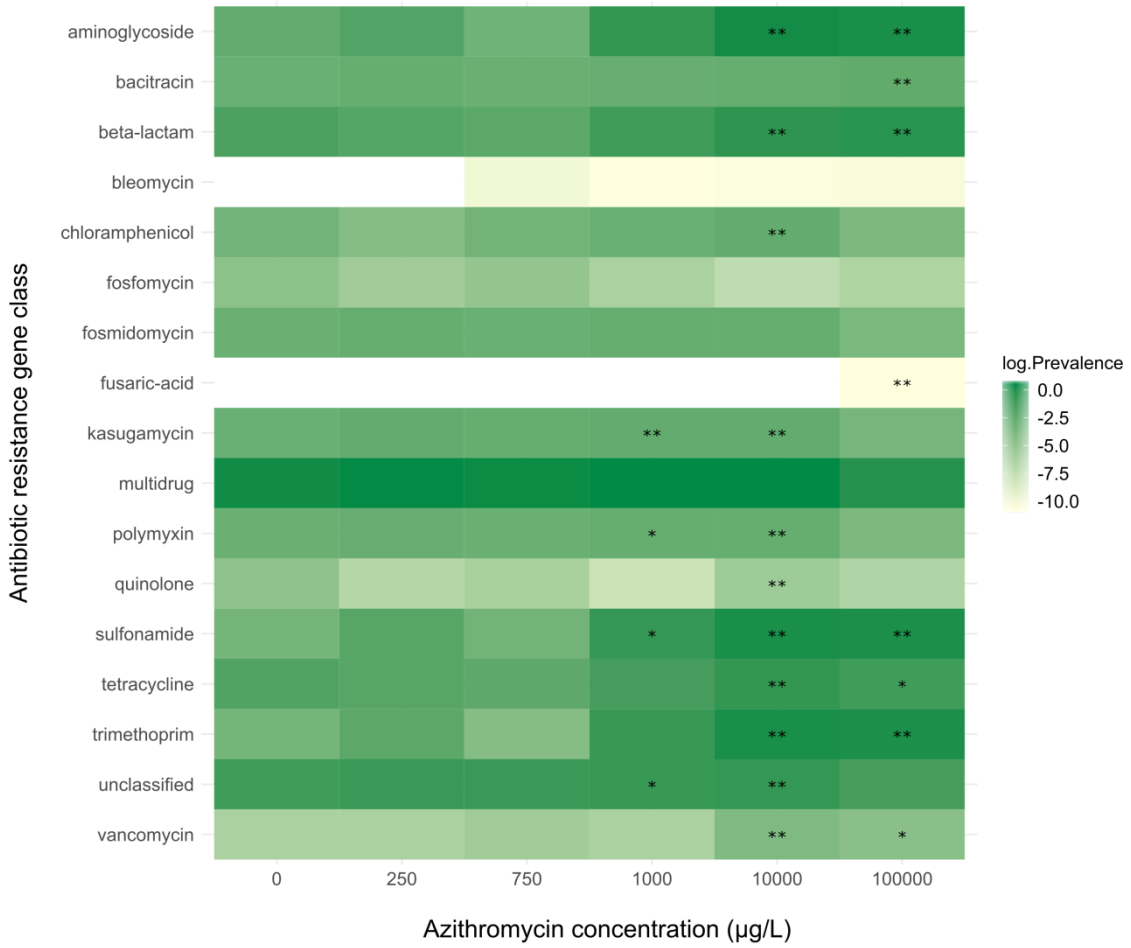


Figure 29: Heatmap showing the co-selection for antibiotic resistance gene classes at various concentrations of azithromycin. The heatmap does not show the classes that were below detection limit at every concentration of AZ. White represents where genes were below detection limit. The log(prevalence) is displayed here. Only a significant increase in prevalence is represented here (* = significance to 90% confidence, ** = significance to 95% confidence).

Clarithromycin

As with the metagenome results from the selection experiment with AZ, no resistance genes were detected at any concentration of CLA for the antibiotic classes carbomycin, fusidic-acid, puromycin, rifamycin, spectinomycin and tetracenomycin C. No significant increase in prevalence of resistance genes

was determined at any of the CLA concentrations tested for the antibiotic classes bleomycin, fosfomycin, quinolone or vancomycin. Significance was seen at 10,000 and 100,000 µg/L for fosmidomycin (p = 0.0388 and 0.0436, respectively, Dunnett's test), kasugamycin (p = 0.0192 and 0.0304, respectively, Dunnett's test), polymyxin (p = 0.0427 and 0.0390, respectively, Dunnett's test) and unclassified genes (p = 0.0418 and 0.0208, respectively, Dunnett's test).

Bacitracin saw a significant increase, to 90% confidence, at 10,000 µg/L (p = 0.06320, Dunnett's test) and to 95% confidence at 100,000 µg/L (p = 0.00302, Dunn's test) compared to the no antibiotic control.

Each of the seven other gene classes first saw a significant increase at 100,000 µg/L. These included aminoglycoside (p = 0.0161, Dunn's test), fusaric-acid (p = 0.0416, Dunn's test), sulfonamide (p = 0.0332, Dunn's test), tetracycline (p = 0.0393, Dunn's test) and trimethoprim (p = 0.0393, Dunn's test) to 95% confidence. Resistance to beta-lactams (p = 0.0541, Dunn's test) and chloramphenicol (p = 0.0968, Dunn's test) saw an increase at 100,000 µg/L to 90% confidence.

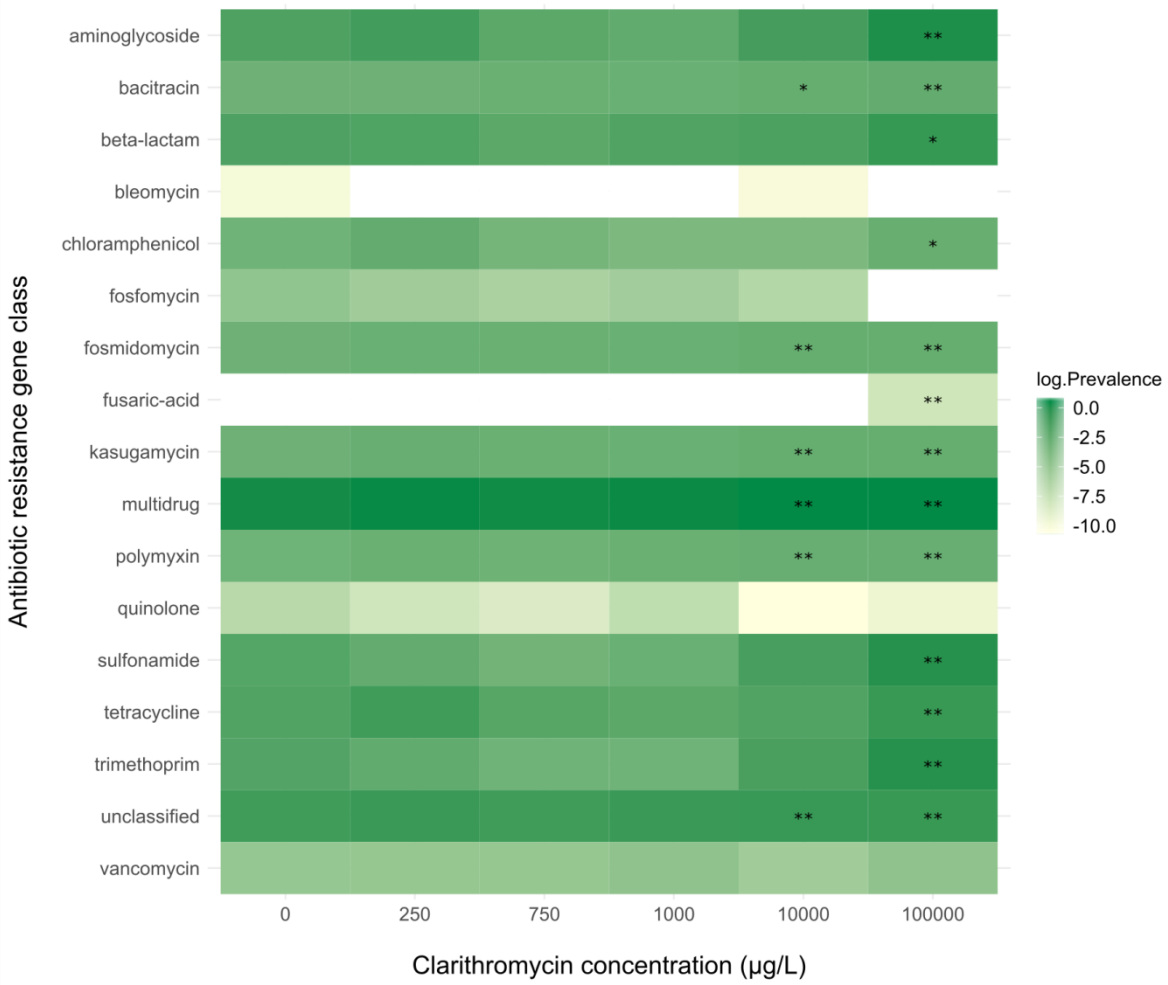


Figure 30: Heatmap showing the co-selection for antibiotic resistance gene classes at various concentrations of clarithromycin. The heatmap does not represent the classes that were below detection limit at every concentration of CLA. White represents where genes were below detection limit. The log(prevalence) is displayed here. Only a significant increase in prevalence is represented here (* = significance to 90% confidence, ** = significance to 95% confidence).

Erythromycin

For all concentrations of ERY, and similar to the results seen for AZ and CLA, there were no detectable resistance genes for the classes of carbomycin, fusaric-acid, fusidic-acid, puromycin, rifamycin, spectinomycin and tetracenomycin C. These classes were, therefore, eliminated from the heatmap

(Figure 31). No significant selection was seen at any concentration of ERY for resistance genes from the classes' fosfomycin, quinolone and vancomycin. Neither was any significant selection seen for multidrug resistance.

Significant selection to 95% confidence was seen at 250 µg/L for bacitracin and fosmidomycin, although if the individual bar graphs are considered (Figure 32 and Figure 33), this does not appear to be correct, particularly as no significant selection was observed in some higher concentrations of ERY. This looks, therefore, to be more like natural variation in the biological samples and also appears to be true for the significance seen at 750 µg/L for the classes kasugamycin, polymyxin and unclassified. An example (bacitracin) of an individual bar graph for these can be seen in the (Figures 32). It is possible that there is a difference in the prevalence of these class specific resistance genes in the starting inoculum that accounts for the variation observed at day 7. As day 0 samples were not sequenced, however, it cannot be established for certain. If other classes, such as aminoglycoside, sulfonamide, tetracycline and trimethoprim, are viewed as bar graphs a true dose response is evident. An example of one of these graphs (aminoglycosides) can be seen in Figure 33. This is in contrast to the data presented in Figure 32.

The beta-lactam class of antibiotics saw a significant increase in gene prevalence to 90% confidence at 100,000 µg/L (p = 0.0843, Dunn's test). The remaining classes saw a significant increase in prevalence, to 95% confidence, in the presence of 100,000 µg/L of ERY. These classes were aminoglycoside (p = 0.0089, Dunn's test), sulfonamide (p = 0.0109, Dunn's test), tetracycline (p = 0.0616, Dunn's test) and trimethoprim (p = 0.0234, Dunn's test).

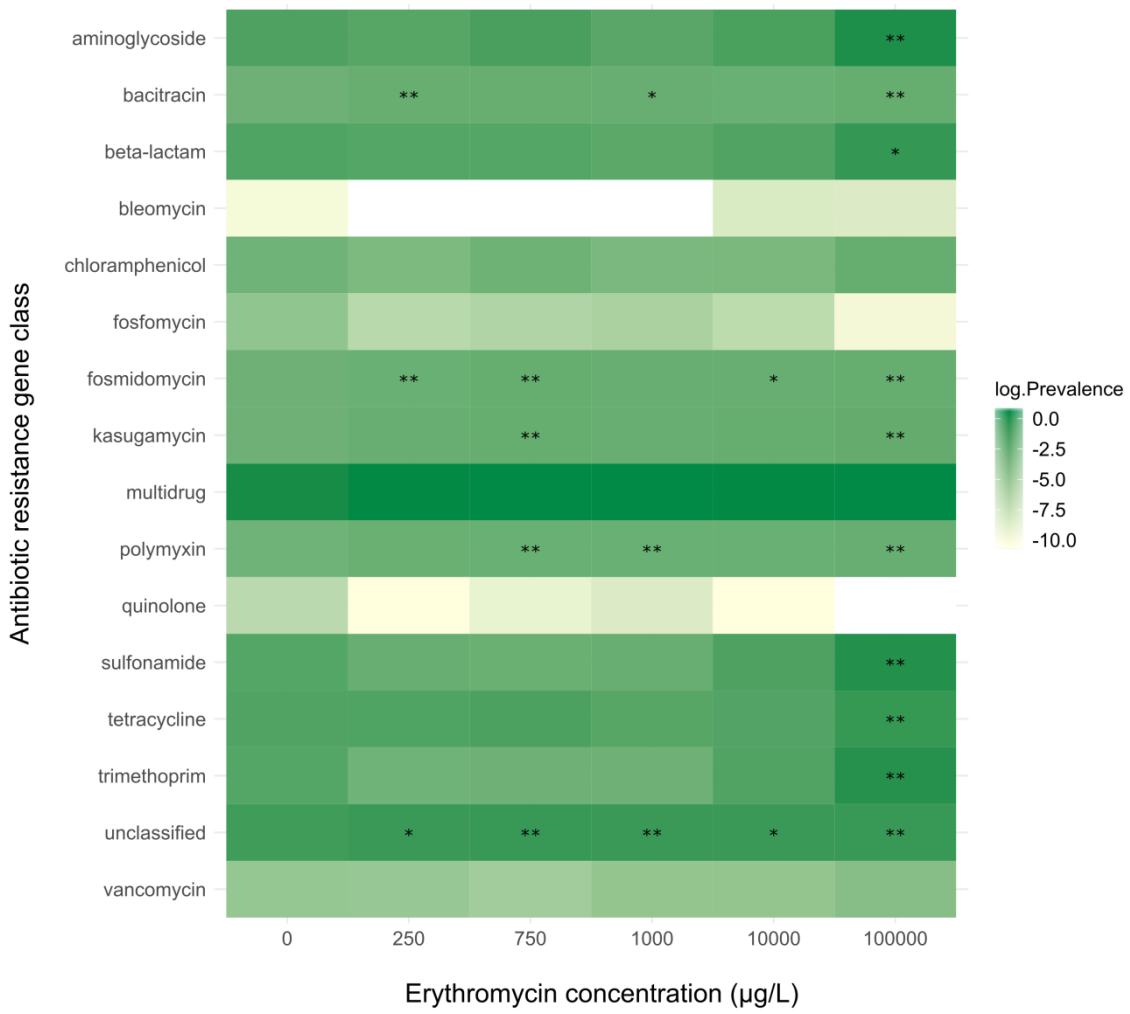


Figure 31: Heatmap showing the co-selection for antibiotic resistance gene classes at various concentrations of erythromycin. The heatmap does not represent the classes that were below detection limit at every concentration of ERY. White represents where genes were below detection limit. The log(prevalence) is displayed here. Only a significant increase in prevalence is represented here (* = significance to 90% confidence, ** = significance to 95% confidence).

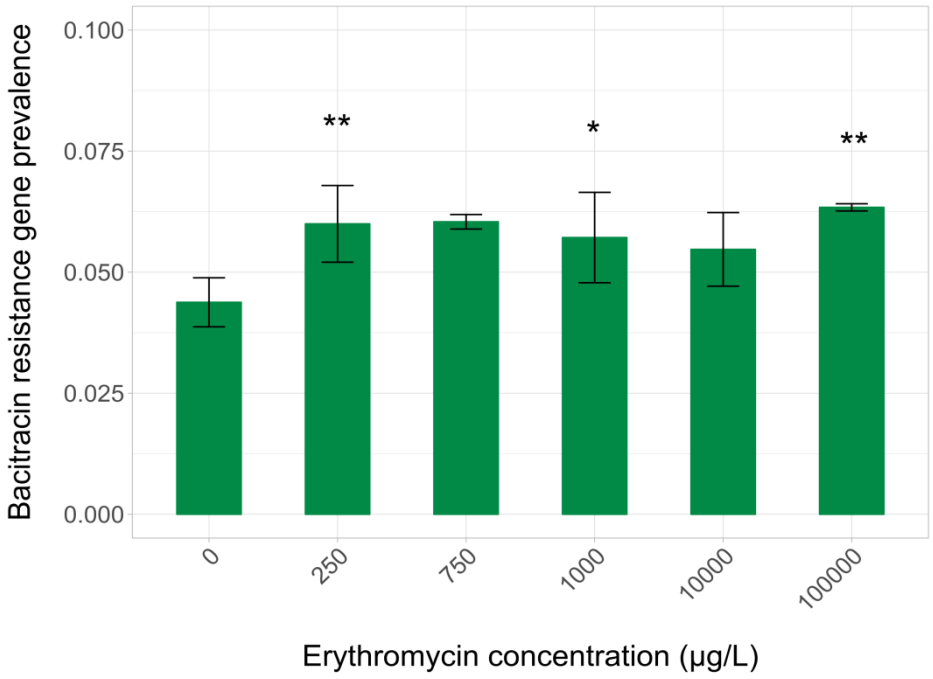


Figure 32: Bacitracin resistance gene prevalence as a function of ERY concentration. * = significance to 90% confidence, ** = significance to 95% confidence. Standard error is represented by the error bars.

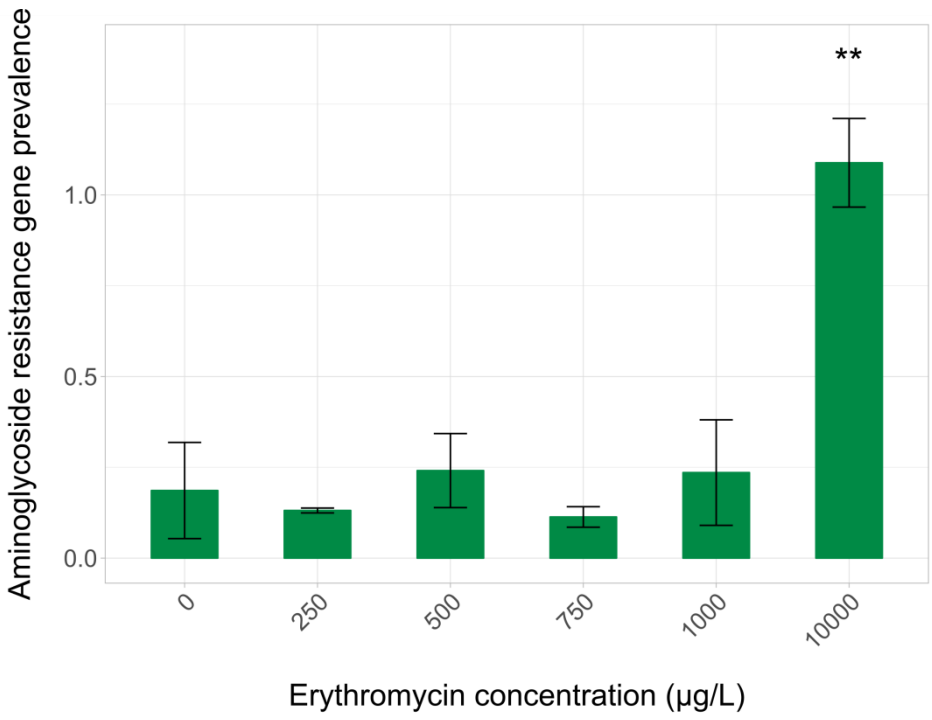


Figure 33: Aminoglycoside resistance gene prevalence as a function of ERY concentration. ** = significance to 95% confidence. Standard error is represented by the error bars.

Investigating the effect of concentration of AZ, CLA and ERY on 16S community structure

The analysis of community structure was run by Aimee Murray as part of the metagenome analysis pipeline. Heatmaps showing the abundance (colour scale) of the top 25 most abundant species grouped by relatedness (y axis) found in each experiment can be seen in Figures 34, 35 and 36 for AZ, CLA and ERY (x axis), respectively. Heatmaps showing all species detected in these samples can be seen in Figures 84, 85 and 86 (Page 316, 319 and 322, respectively - Appendix), for AZ, CLA and ERY, respectively, and tables showing the raw data that corresponds for the top 25 most abundant species heat maps can be found in Table 18, 19 and 20 (Page 317, 320 and 323, respectively - Appendix) for AZ, CLA and ERY, respectively.

Azithromycin

By far the most abundant species in these libraries were *Escherichia* (unclassified species), *Escherichia coli* and *Proteus mirabilis.* For both of the *Escherichia* groups, abundance was high in all of the libraries produced, from no antibiotic control samples to all samples treated with all concentrations of AZ tested. For *Proteus mirabilis,* abundance of species was high in the no antibiotic control samples and all AZ treated samples except 100,000 µg/L where AZ becomes toxic and no *P. mirabilis* is detected.

Similarly to *Proteus mirabilis*, other species have similar abundances in the AZ treated samples to the no antibiotic treated control except at 100,000 µg/L, where AZ becomes toxic. These species were *Morganella morganii* and *Bacteroides uniformis*, neither of which were detected at 100,000 µg/L of AZ. *Klebsiella pneumoniae* saw an increase in abundance when in the presence of

AZ compared to the no antibiotic control at all antibiotic concentrations except 10,000 and 100,000 µg/L where this species was detected infrequently.

Other species proved to be more sensitive to AZ, being present in libraries from samples not treated with AZ and becoming less abundant as the concentration of AZ increased. These species included *Bacteroides taiotaomicron, Bacteroides fragilis, Eubacterium limosum, Bacteroides dorei* and *Fusobacterium ulcerans.* The species *Streptococcus infantarius, Peptoniphilus harei, Staphylococcus aureus, Veillonella ratti* and *Acinetobacter baumannii* were even more sensitive and were all present in the no antibiotic control samples. Except for the occasional sample, these species were completely lost when samples were treated with AZ.

The species *Enterobacter cloacae, Enterococcus faecium and Enterococcus avium* were seen in low abundance in the no antibiotic control samples but increased slightly in abundance in certain samples that had been treated with AZ.

The remaining species *Enterococcus faecalis, Providencia alcalifaciens, Cronobacter phage CR5* and *Staphylococcus haemolyticus* saw no real pattern with the application of AZ. Certain samples showed the presence of the species, and in the case of *Cronobacter phage CR5* and *S. haemolyticus* at very high abundance, but in the vast majority of samples these species were below the limit of detection.

Overall, with the application of AZ the samples get less diverse compared to the samples that have not been treated with AZ. A correlation can also be seen between AZ concentration and samples becoming less diverse as concentration increases.

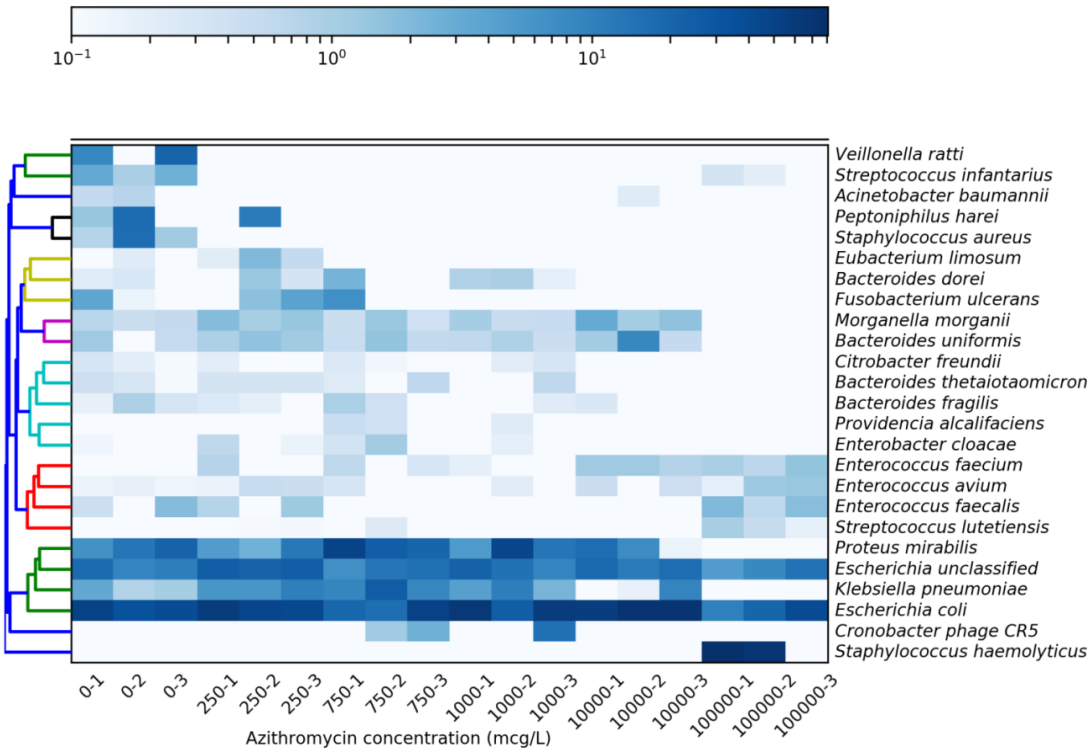


Figure 34: Heatmap showing the change in community structure at increasing concentrations of azithromycin. Heatmap shows the top 25 most abundant species (y axis) in the samples. Scale represents relative abundance in the community. Community structure is shown for individual replicates. Individual replicates are represented by 1, 2 or 3. For example, 0 – 1 is the first replicate of the not antibiotic control.

Clarithromycin

Similarly to AZ, the most abundant species found in this set of libraries was *Escherichia* unclassified and *Escherichia coli.* Not only were these the most abundant species, but they were found in high numbers throughout, at every concentration of CLA. No dose dependent response to CLA was observed here for these two species.

Proteus mirabilis is also seen with similarly high abundance to *Escherichia coli* and *Escherichia* unclassified. The abundance of this species started to

decrease, however, at 1,000 µg/L and higher. By 100,000 µg/L, none of this species was detected in any of the samples.

Other species also saw a dose dependent response to CLA. These included *Morganella morganii, Bacteriodes uniformis* and *Klebsiella pneumoniae.* Both *B. uniformis* and *K. pneumoniae* saw a small increase in abundance at 250, 750 and 1,000 µg/L compared to the abundance seen in the no antibiotic control. A decrease in abundance was then seen at 10,000 µg/L compared to the no antibiotic control and both species were completely absent from 100,000 µg/L of CLA. *M. morganii* was seen at similar levels of abundance to the no antibiotic control at concentrations up to 1,000 µg/L of CLA. At 10,000 and 100,000 µg/L, this species was not detected.

Other bacterial species were more sensitive to the presence of CLA. Species such as *Staphylococcus aureus* and *Streptococcus infantarius* were detected in most of the no antibiotic control samples but were rarely detected in any sample where CLA had been used.

One species, *Bifidobacterium pseudolongum*, was below detection limit in any of the no antibiotic control samples but low abundance of this species was detected at low concentrations of CLA. This bacterial species then disappears in samples at higher CLA concentrations.

The remaining species shown in Figure 35 showed no pattern. These were grouped into three categories. The first group of bacterial species were detected in every no antibiotic control sample but were detected sporadically throughout the rest of the samples when CLA was present. These species included *Bacteroides fragilis* and *Bacteroides dorei*. The second group consisted of bacterial species that were found sporadically throughout the libraries no matter

the treatment. These species were *Peptoniphilus harei, Bacteroides ovatus, Enterococcus raffinosus, Enterococcus asini, Enterococcus faecium, Klebsiella* unclassified, *Enterobacter cloacae, Citrobacter freundii, Bacteroides thetaiotaomicron, Staphylococcus simulans, Staphylococcus haemolyticus* and *Enterococcus faecalis.* The final category consisted of just *Enterococcus avium* and had low abundance throughout treatments but with no pattern able to be determined.

Overall, a dose response relationship between CLA and diversity of species can be seen. There is a substantial reduction (and often complete loss) in abundance in a number of species at 100,000 µg/L. In addition, a reduction in diversity can clearly be seen at 10,000 µg/L.

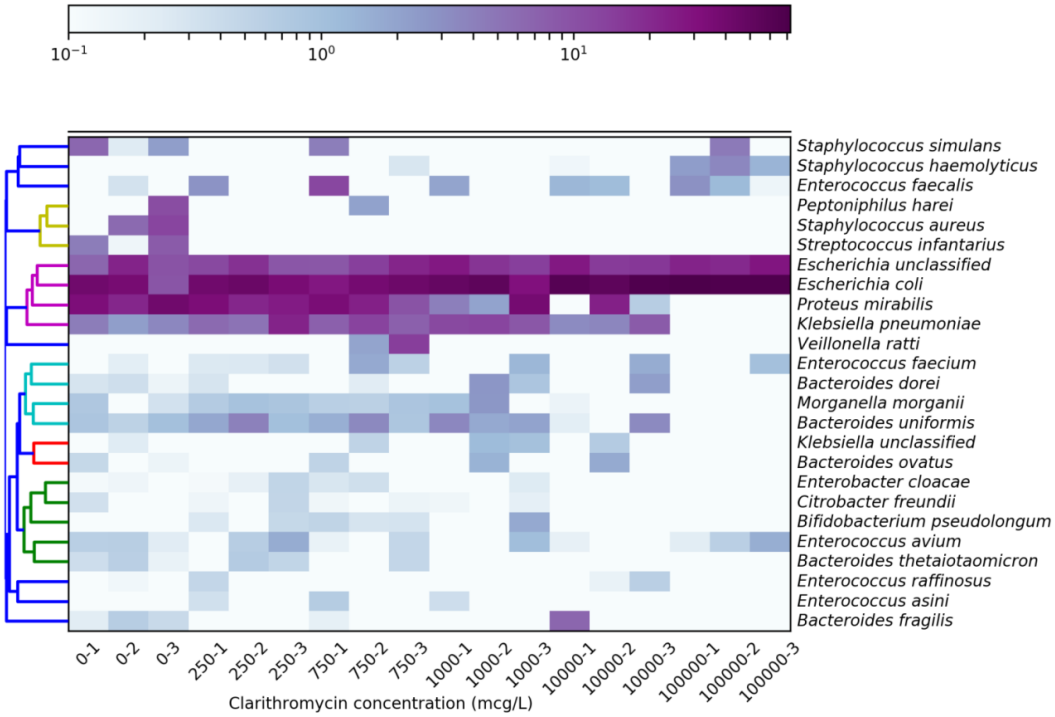


Figure 35: Heatmap showing the change in community structure at increasing concentrations of clarithromycin. Heatmap shows the top 25 most abundant species (y axis) in the samples. Scale represents relative abundance in the community. Community structure is shown for individual replicates. Individual replicates are represented by 1, 2 or 3. For example, 0 – 1 is the first replicate of the not antibiotic control.

Erythromycin

Similarly to AZ and CLA, *Escherichia* unclassified and *Escherichia coli* were found at high abundances throughout all libraries independent of treatment with ERY. As with AZ and CLA, *Proteus mirabilis* was also found at high abundance in the no antibiotic control samples. However, unlike the community structure with AZ and CLA treatment, when *P. mirabilis* was in the presence of ERY a decrease in abundance was seen at 250 µg/L and subsequent higher concentrations and was below the detection limit at 100,000 µg/L.

Other species that saw a dose dependent reaction to ERY were *Bacteroides uniformis* and *Klebsiella pneumoniae*. *B. uniformis* had similar abundance in the samples treated with ERY and without, except at the higher concentrations of 10,000 and 100,000 µg/L where one and two samples, respectively, had undetectable levels of this species. *K. pneumoniae* had similar abundance in all treatments and the no antibiotic control except at 100,000 µg/L where no bacteria were detected.

Species that were more sensitive to ERY include *Staphylococcus simulans* and *Streptococcus infantarius*. These species were seen in the no antibiotic control treatment but were seen only sporadically or not at all in any of the samples treated with ERY.

The vast majority of species detected displayed no association between concentration of ERY and abundance. The species that were found sporadically throughout samples with no pattern included *Veillonella ratti, Enterobacter clocae, Bifidobacterium longum, Bifidobacterium pseudolongum, Enterococcus faecium, Enterococcus faecalis, Klebsiella* unclassified, *Enterococcus gallinarum, Bifidobacterium pseudolongum, Streptococcus gallolyticus,*

Streptococcus pastuerianus. Other species, including *Bacteroides thetaoitaomicron, Bacteroides ovatus* and *Morganella morganii* were found to be present in the no antibiotic control samples but only sporadically in the presence of ERY. Finally, *Enterococcus avium* was found in most of the samples with no distinct pattern.

Overall, unlike with AZ and CLA, there was no clear pattern in terms of reduction in diversity with increasing concentrations of ERY. More species were seen at higher abundance at the highest concentration of ERY and there was a mixed effect at lower concentrations. In other words, there was no obvious dose response with increasing application of ERY although samples in the presence of ERY have decreased diversity in comparison to the no antibiotic control.

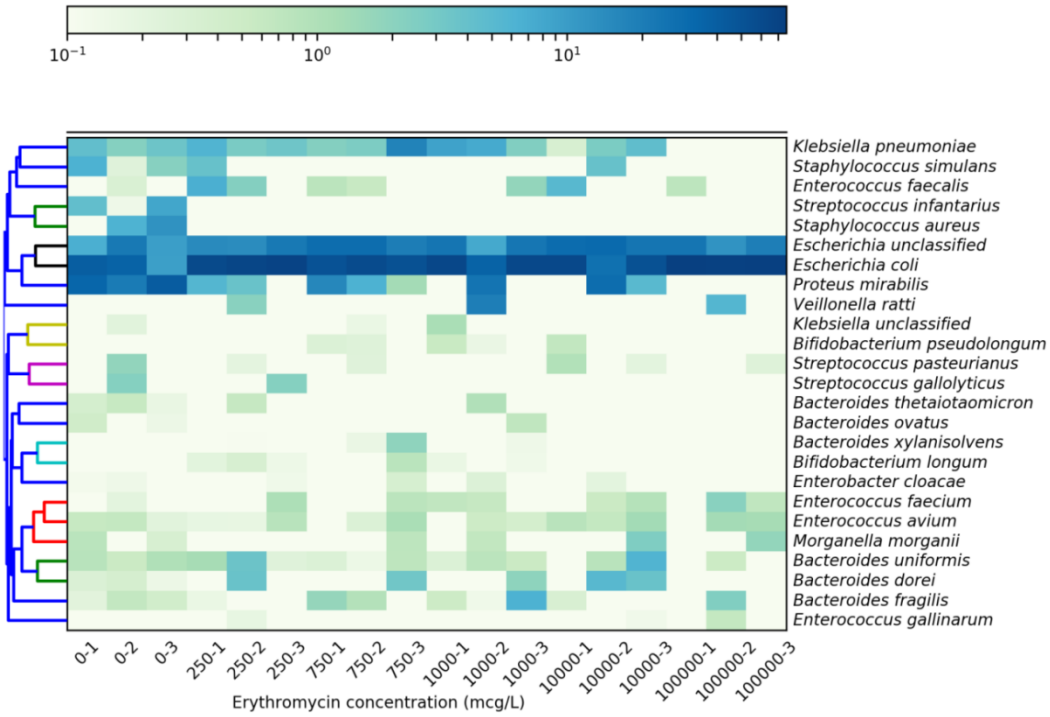


Figure 36: Heatmap showing the change in community structure at increasing concentrations of erythromycin. Heatmap shows the top 25 most abundant species (y axis) in the samples. Scale represents relative abundance in the community. Community structure is shown for individual replicates. Individual replicates are represented by 1, 2 or 3. For example, 0 – 1 is the first replicate of the not antibiotic control.

3.5 Discussion

3.5.1 Comparison to current measured environmental concentrations

The data presented in this chapter suggests that, using this experimental system, no selection for macrolide resistance is likely to occur at the measured environmental concentrations (MEC) gleaned from the results of the literature search reported in Section 3.4.1.

A LOEC was determined, by investigating selection for *ermF,* as 750 µg/L for CLA (with a NOEC of 500 µg/L) and a LOEC of 1,000 µg/L for both AZ and ERY (with a NOEC of 750 µg/L for both). The LOEC of AZ was 666.6 times and the NOEC 500 times higher the maximum MECs (MEC_{max}) of AZ (1.5µg/L (Bartelt-Hunt et al. 2009)) calculated through the analysis of the literature review of MECs (Section 3.4.1). According to that analysis, the MEC_{max} of CLA was 1 µg/L (Morasch et al. 2010). The LOEC was, therefore, 750 times higher than the MEC_{max} and the NOEC was 500 times higher. Finally, the MEC_{max} for ERY, according to the literature review analysis, was 2.42 µg/L (Yao et al. 2015) and 4 µg/L for ERY-H_2O (Tong et al. 2014). The LOEC for ERY was, therefore, approximately 446.4 times higher and the NOEC was 334.8 times higher than the MEC_{max} ERY and was 250 times (LOEC) and 187.5 times (NOEC) higher the MEC_{max} of ERY-H_2O. The MSC of 514 µg/L was, therefore, 229.5 times higher than the MEC_{max} of ERY and 128.5 times higher than that of ERY-H_2O. After the literature search was conducted (2015), the Umwelt Bundesamt (UBA) released a database of measured environmental concentrations from around the world (Umweltbundesamt 2016). Maximum, mean and median values were determined for AZ, CLA and ERY by searching for surface waters, ground waters, WWTP influent and effluent and hospital, urban and industrial sewage.

Values that were below the detection limit were eliminated when calculating mean and medians although for there were many undetectable hits for all three compounds. A maximum value of 9.7 µg/L (WWTP influent) was observed for AZ. Mean and median values were determined from this table to be 0.36 and 0.098, respectively. For CLA, the maximum concentration on this database was 62.241 µg/L. This is an unusually high concentration found in untreated hospital wastewater. The mean of CLA was 1.47 µg/L and a median of 0.057 µg/L was determined. For ERY, a MEC_{max} of 7.84 µg/L was determined from industrial sewage in China. MEC_{mean} and MEC_{median} were determined to be 0.3336 µg/L and 0.058 µg/L, respectively, for ERY. Finally, for ERY-H_2O a maximum concentration of 10.025 µg/L was measured in untreated wastewater in Wales. MEC_{mean} and MEC_{median} values for ERY-H_2O were determined to be 1.335 µg/L and 0.038 µg/L, respectively. Similar to the literature search undertaken in 2015, except for the one unusually high CLA concentration from hospital wastewater, the concentrations determined from this database were significantly lower than the LOECs and MSC for the macrolide compounds determined.

If a 10-fold assessment factor is applied to the NOEC for each macrolide antibiotic, a PNEC can be determined. This allows for a conservative safe release limit to be determined. The PNEC for AZ and CLA was, therefore, 75 and 50 µg/L, respectively. To be the most protective of selection in the environment, the assessment factor was applied to the MSC (514 µg/L) of ERY, as it was lower than the NOEC. A PNEC of 51.4 µg/L of ERY was, therefore, determined. All of these PNECs are approximately an order of magnitude higher than current MEC_{max} seen for typical environmental concentrations.

3.5.2 Comparison to previously published endpoints

If the LOECs and the MSC of the individual macrolide compounds presented here are compared to previously published selective endpoints, similar results were determined to the single species assays by Gullberg et al. 2014 but were significantly higher than those in Bengtsson-Palme & Larsson 2016 which are estimated by applying a 10-fold assessment factor to MICs from the EUCAST database. In Gullberg et al. 2014, MSCs for both plasmid and chromosomal based resistance against ERY were determined. MSCs were 200 µg/L and 3000 µg/L for a chromosomal resistance element and the same resistance element on a plasmid, respectively, and, therefore, span the LOECs, NOECs and the MSC determined here. However, the endpoints determined here were significantly higher than the $PNEC_R$s that were calculated by a mathematical approach by Bengtsson-Palme & Larsson 2016 as 0.25 µg/L (AZ), 0.25 µg/L (CLA) and 1 µg/L (ERY).

The method used to produce the LOECs and MSC presented in this chapter uses a complex, environmental microbial community. For this reason, this methodology is the most representative of what may be occurring in the environment in comparison to the endpoints presented in Gullberg et al. 2014 and Bengtsson-Palme & Larsson 2016. The method used in Gullberg et al. 2014 used single species competition experiments and the method used in Bengtsson-Palme & Larsson 2016 applied a mathematical model to clinical MIC values and these, therefore, may not produce representative results that are indicative of the selective effect of antibiotics in environmental microbial populations.

3.5.3 *intI1* and *mphA*

The LOECs determined by *intI1* and *mphA* were significantly higher than those determined by *ermF* for AZ, CLA and ERY. The total prevalence observed at day 7 for these two genes was, however, significantly higher than the prevalence of *ermF* determined. In addition, a stronger response in regards to the increase in prevalence over the 7 day period, at concentrations where positive selection was observed, was seen in both of these genes compared to *ermF.* This was the case for AZ, CLA and ERY for both *intI1* and *mphA*. It was concluded, therefore, that these antibiotics, whilst having a selective effect on *ermF* at lower concentrations, have a significantly increased selective effect on *intI1* and *mphA* at higher concentrations. The reason this was seen for *intI1* is presumably because it is often associated with many different ARGs and MGEs. The class specific macrolide resistance gene, *ereA*, for example, is often found linked to class 1 integrons (Roberts 2008). Selection for many genes that have not been targeted by qPCR may be represented by selection for *intI1.* The number of resistance genes that are associated with *intI1* may, therefore, explain the much greater prevalence of *intI1* compared to *ermF.* The *mphA* gene has been found linked to class 1 integrons (Roberts 2008) and this could explain the similar pattern to where selection was first seen and the high prevalence of both genes.

3.5.4 *ermB, msrD* and *mef* family

Of the 5 macrolide class specific genes tested by qPCR, three did not undergo any positive selection at any concentration tested of any of the three macrolides tested, including higher, clinically relevant, concentrations. These genes were *ermB, msrD* and *mef* family. In a majority of the experiments for *ermB* and for one of experiments for the *mef* family, a significant increase in comparison to

the no antibiotic control was observed of concentrations typical of the environment but this increase was never higher than the starting prevalence of the gene. There are a variety of reasons why positive selection may not occur.

One explanation, for example, could be there was low prevalence of these genes in the original inoculum used for this experiment. This was not the case, however, as the average prevalence at day 0 for *ermF* was lower than all of the macrolide resistance genes tested (except for *msrD,* which was only 0.002 lower).

Another explanation might be that the genes that did not undergo selection were more clinically relevant and exert a high fitness cost so are not maintained in complex microbial populations as opposed to single species infections. *MsrD* has, for example, been detected in the clinical strain *Ureaplasma urealyticum* isolates in Guanzhou, China (Lu et al. 2010). In Japan, *ermB, mefA* and *mefE* have all been found in clinical isolates of macrolide resistant *Streptococcus pneumoniae* (Isozumi et al. 2007; Hotomi et al. 2005). However, as previously mentioned, *ermB* (along with *ermF*) was suggested to be a genetic indicator for macrolide resistance in the environment (Berendonk et al. 2015). This was one of the reasons it was chosen to study as it was expected to be maintained in environmental bacterial populations. Alternatively, the genes that are not positively selected for might be harboured in bacterial species that do not survive well in the experimental conditions used: they may be outcompeted by bacterial species that thrive in those conditions and reduced, therefore, over the experimental period.

A final plausible explanation may be that these genes are maintained in bacterial populations by other antibiotics or co-selective compounds. *MsrD*, for

example, provides resistance to both macrolide and streptogramin antibiotics (Lu et al. 2010). A streptogramin antibiotic may, therefore, exert a selective pressure on *msrD.*

MefA and *msrD* are often found in association with each other (Roberts 2008), this may, therefore, explain the similarities observed in regards to the lack of positive selection seen between *msrD* and the *mef* family.

3.5.5 Culture based and metagenome analysis

The culture based assay and metagenome data were used to verify the qPCR data. Although the class specific genes were chosen for the reasons outlined in the methods section, there are many other macrolide resistance genes and potential point mutations that convey phenotypic macrolide resistance that were not targeted by qPCR. For this reason, a culture based assay was used to capture any phenotypic resistance (including resistance genes and point mutations), and metagenome analysis was used to identify any other macrolide resistance genes that were being selected, occurring below the LOECs and MSC determined by qPCR.

The metagenome and culture based data were able to verify the qPCR data, as previously found in Murray et al. 2018. Overall MLS resistance, determined by metagenome analysis, and the change in phenotypic resistance on Chromocult agar (the only agar where a significant difference in phenotypic resistance was observed) aligned well. Both MLS resistance and phenotypic resistance saw a biological increase at 1,000 µg/L, but this was not significant, and significantly increased at 10,000 and 100,000 µg/L. Both of these changes were observed at higher concentrations than that of LOECs/MSC determined by the qPCR assay for *ermF* and aligned better with the *intI1* and *mphA* data. Significant selection

for *ermF* was seen (compared to the no antibiotic control) at a lower concentration of all three antibiotics than selection for *intI1* and *mphA.* The latter two genes increased to a higher prevalence than *ermF* over the 7 day period. *MphA* and *intI1* could, therefore, be having a stronger effect on the overall MLS resistance and phenotypic resistance, as their prevalence at the end of the 7 day experiment is significantly higher than *ermF,* and this could, therefore, account for the results observed in the metagenome and plating analysis.

There were a number of macrolide resistance genes found in the libraries. There appeared to be no trend between which type of resistance gene (for example, efflux pump or enzyme) was selected. Some genes were seen to be increasing, compared to the no antibiotic control, at concentrations below the LOECs defined by qPCR. However, in some instances, this was because the gene was undetectable in the no antibiotic control but present in one replicate run at 250 µg/L. The statistical analysis then deemed this to be significant selection, although the gene is never detected at any other concentration or any other replicate at that concentration. This was not, therefore, determined to be positive selection.

In other cases, such as *macB* and *ermB,* genes were tested by qPCR to determine whether positive selection was taking place. Based on the preliminary range finding experiments, it was expected that the increase seen in *ermB* was would not be positive selection. Preliminary range finding experiments found that a significant increase was observed in comparison to the no antibiotic control but that *ermB* did not increase over the starting inoculum. This concept is discussed further in Chapter 6. QPCR was used to verify this. It was also used to verify that no positive selection for *macB* was

occurring. This shows the benefits of quantifying the prevalence of the target gene at the beginning of the experiment as only then can positive selection over time be determined (this is discussed further in Chapter 6).

Metagenome analysis also found that macrolides have the potential to co-select for resistance genes of other classes of antibiotics at high concentrations. AZ appeared to be the most co-selective, in comparison to CLA and ERY. The dose response aligns well with the qPCR data investigating the selection for *intI1* as integrons are known to harbour various resistance genes from multiple antibiotic resistance gene classes. Common classes that are often found in association with class 1 integrons are sulfonamides and trimethoprim (Moura et al. 2009). Genes resistant to these classes are enriched at concentrations similar to those where significant selection for the *intI1* gene is seen. Co-selection for other classes is observed only at high concentrations of the three macrolide antibiotics tested. Previous data by Murray et al. 2019 showed the co-selective potential of ciprofloxacin and trimethoprim at 500 and 2,000 µg/L, respectively, although these were the only concentrations of these antibiotics investigated by the authors. These concentrations are similar to where the co-selective potential of AZ is first observed for certain classes of antibiotic resistance genes (1,000 µg/L) but are significantly lower than that of CLA and ERY (predominantly at 100,000 µg/L) (Murray et al. 2019).

In all samples, the community was dominated by *E. coli* and *Escherichia* (unclassified species), irrespective of macrolide concentration. This was unsurprising as the bacterial sample used was raw wastewater and *E. coli* is a faecal coliform bacteria that is regularly used by environment agencies/drinking

water companies as an indicator for contamination by faeces (Edberg et al. 2000).

A decrease in species diversity was seen in the presence of all three macrolides, in comparison to samples grown without antibiotic, particularly for samples passaged at 100,000 µg/L of AZ and CLA. This could explain the significant decrease in the *ermF* gene at 100,000 µg/L. The bacterial species harbouring this gene were, presumably, the bacteria that are significantly affected at this concentration and this is why a complete loss of *ermF* is observed at this concentration. For example, *ermF* has been found in clinical isolates of a *Bacteroides* species (McArthur et al. 2013; Kangaba et al. 2015). A number of different *Bacteroides* species are found in the samples from the AZ, CLA and ERY experiments. These species, with the exception of two instances, are never found in the replicates passaged at 100,000 µg/L of antibiotic supporting the above hypothesis.

One interesting discovery was the presence of *Cronobacter* phage 5 (CR5) in the top 25 most abundant species heatmap in the presence of AZ. CR5 is a phage that infects the bacterial species *Cronobacter sakazakii* (also known as *Enterobacter sakazakii*) (Lee et al. 2016; Healy et al. 2010). This phage is not found in the top 25 most abundant species of CLA or ERY and *C. sakazakii,* the species it infects, is not found in the top 25 most abundant species for any of the 3 macrolide compounds. If all of the species present in the day 7 samples are looked at, however, (Figures 84, 85 and 86, Page 316, 319 and 322 - Appendix) instead of only the top 25 most abundance species, CR5 is found in samples from both the AZ and CLA experiments and *C. sakazakii* is found in the experiments passaged in the presence of CLA and ERY. As the

experiments are run with pseudo replicates of the same wastewater influent inoculum, these results suggest that both CR5 phage and *C. sakazakii* were present in the wastewater when the inoculum sample was taken. Although *C. sakazakii* is commonly associated with food products such as formula milk (Zuber et al. 2008), it has been previously isolated from wastewater (Thulasinathan et al. 2019).

3.5.6 Difference in response to AZ, CLA and ERY

For some of the work presented here, it appears that CLA and ERY correlate well with each other, whereas AZ was often selecting for resistance at a lower concentration. This appears to be particularly true for the genes *intI1* and *mphA*. AZ was seen to positively select at 1,000 µg/L for both genes (for *mphA* to 95% confidence and for *intI1* to 90% confidence), whereas significant selection by CLA and ERY was not observed until 100,000 µg/L (both to 95% confidence).

A similar correlation was seen for MLS resistance data from the metagenome analysis. A significant increase was seen in comparison to the no antibiotic control (to 95% confidence) at 10,000 µg/L of AZ. A clear biological effect was also seen at 1,000 µg/L, although this was not statistically significant. For CLA, a significant increase was seen (to 95% confidence) at 100,000 µg/L and for ERY, a significant increase was observed at 100,000 µg/L (95% confidence) although a significant increase (to 90% confidence) was also seen at 10,000 µg/L. No biological effect was observed at 1,000 µg/L for CLA or ERY in contrast to what was seen for AZ.

One possible explanation for why this could be is the different chemical makeup of the three antibiotics. CLA and ERY both have 14 member lactone rings, whereas AZ has a 15 member ring (Leclercq & Courvalin 2002). This similarity

in the chemistry of CLA and ERY may be the reason for similar patterns in selection compared to AZ. In addition, AZ has been shown to be a significantly more potent drug than ERY (Jelić & Antolović 2016). One study showed that increased potency was seen in a number of different bacterial species including many of the *Enterobacteriacae* family. Furthermore, MIC concentrations of AZ were also shown to be lower than those of ERY (Retsema et al. 1987). A lower MIC of AZ in comparison to the MIC of ERY seen in Retsema et al. 1987 mimics the pattern observed in the selective endpoint data of *mphA, intI1* and the MLS resistance data presented in this chapter when comparing AZ and ERY.

3.5.7 Future work

This study has not considered the effects of mixtures of antibiotics on LOECs/MSCs/PNECs/sub-MSC effects. The many studies investigated to produce typical MEC values (Table 5, Section 3.4.1) show that these antibiotics are never found in isolation, whether that be, for example, in WWTPs or in surface waters. In addition, macrolide antibiotics all target bacteria by deploying the same mechanism, preventing protein synthesis by binding to 23S rRNA region of the large ribosomal subunit (Kannan & Mankin 2011; Mazzei et al. 1993). Furthermore, the semi-synthetic derivatives of ERY were developed for better tolerability and pharmacokinetics in patients, not to overcome the development of ERY resistance. Resistance mechanisms are, therefore, able to resist all three compounds (Leclercq 2002; Leclercq & Courvalin 2002). As a result, it can be assumed that the combination of these 3 compounds would produce an additive effect. It might, therefore, be more sensible to determine the safe release level PNEC as 51.41 µg/L as a conservative estimate of the total concentration of macrolide antibiotic until more information is determined to

ensure the most stringent protection of the development of selection in the environment. If the maximum environmental concentrations determined in Table 5 are combined, a total maximum concentration of 8.92 µg/L is determined, which is only 5.8 times the PNEC suggested for total macrolide concentration. The selective potential of mixtures of macrolides was determined in Chapter 4.

3.6 Conclusions

The LOECs, MSCs and PNECs determined for the three macrolide antibiotics, AZ, CLA and ERY, in the assay using a complex microbial community, were similar to those previously reported in a single species assays and significantly higher than estimated $PNEC_R$s and MECs for these antibiotics.

Basing a recommendation exclusively on the selective endpoint, this data would appear to suggest that these compounds could safely be removed from the watch list produced by the European Commission (European Commission 2015b) if only selective potential was a concern. The recommendation for removal should, however, consider ecological endpoints, as well as the potential to develop resistance. PNECs for ecological endpoints were determined by the European Commission in 2015 when considering whether to include these compounds on their watch list. PNECs were derived for freshwater to be 0.09, 0.13 and 0.2 µg/L for AZ, CLA and ERY, respectively. These PNECs were derived from ecotoxicology data with an assessment factor of 50-fold applied. Without the assessment factor, NOECs of these compounds are 4.5 (AZ), 6.5 (CLA) and 10 µg/L (ERY). Both the PNECs and NOECs determined by the European Commission were of a similar order of magnitude as current environmental concentrations (European Commission 2015b) and were significantly lower than LOEC/NOEC/MSCs determined for selection here.

Similarly, a meta-analysis by Le Page et al. (2017) determined PNECs for surface water ($PNEC_{SW}$) as 0.019 µg/L (AZ), 0.084 µg/L (CLA) and 0.2 µg/L (ERY). Here they determined the $PNEC_{SW}$ values by using the lowest NOEC found for all three by investigating data from many different studies and applied a 10-fold assessment factor. The NOECs determined were, therefore, 0.19 µg/L (AZ), 0.84 µg/L (CLA) and 2 µg/L (ERY). Again, as with data from the European Commission 2015, both these NOEC and PNEC values are typical of current environmental concentrations and significantly lower than the values that have been determined here for selection. Taking ecotoxicology endpoints into account, these compounds should remain on the watch list for better monitoring as the PNECs from European Commission 2015 and Le Page et al. 2017 are more protective than the work presented here and similar to concentrations found in the environment.

It is not the case, however, that all antibiotics have LOECs, NOECs and MSCs higher than environmental concentrations. The study by Murray et al. 2018 determined a MSC for cefotaxime of 0.4 µg/L. This is significantly different than the LOECs/MSCs determined here for the macrolides. The MSC of cefotaxime, unlike the selective endpoints determined for the macrolides, is similar to MECs. For example, according to the UBA database the MEC_{max} of cefotaxime is 1.1 µg/L in wastewater influent (in typical environmental concentrations and eliminating pharmaceutical wastewater from the search criteria) (Umweltbundesamt 2016). Similarly, selective endpoints for ciprofloxacin have been determined by Aimee Murray and will be published alongside the macrolide antibiotics in Stanton *et al.*, 2019. Data from this experiment can be seen in Figure 87 and 88 (Page 325 - Appendix). A MSC of approximately 11 µg/L was found. This is, again, significantly lower than the concentration of

macrolide needed to select for resistance and similar to the range of ciprofloxacin concentrations seen in the environment (Umweltbundesamt 2016). This demonstrates the need for compound specific testing of the selective potential of antimicrobial agents at a range of concentrations.

Determining the selective concentration of new and existing antibiotics, and potential co-selective agents is highly important. Assessment of the risk these compounds pose to the environment should be used in combination with other ecological endpoints as one may not always be protective of the other. This allows for a more informed assessment of the risk that compounds, such as the macrolides, pose to the environment as a whole.

Chapter 4: The effect of combinations of antibiotic compounds on selective endpoints

Author contribution

The selection experiment and DNA extraction for TRMP experiment 1 was undertaken by the author and Jessica Wright (placement student). The qPCR analysis on this experiment was undertaken by Aimee Murray. The author undertook the statistical analyses on the results from this experiment.

All aspects of SMX experiment 1 was undertaken by placement student, Jasmin Rauseo, under the supervision and direction of the author.

All other experiments (macrolide mixing, macrolide verification, TRMP-SMX mixing, TRMP experiment 2 and SMX experiment 2) were undertaken by the author under the supervision of names supervisors.

4.1 Abstract

Antibiotics are rarely found in isolation in the environment. They are often observed in complex mixtures with their degradation products, other antibiotics, pharmaceutical compounds and co-selecting agents such as heavy metals or biocides. Antibiotics are reported as acting synergistically when found in combination. This assertion is based on their clinical bacteriostatic or bactericidal effects and not on their ability to select for resistance. However, mixtures could have additive or potentially antagonistic effects. The aim of the work presented in this chapter was, therefore, to investigate simple combinations of antibiotics to determine if selective endpoints are affected when mixtures are used in comparison to endpoints of individual compounds.

Selection experiments were used to investigate two combinations. These were azithromycin, clarithromycin and erythromycin used in a 1:1:1 ratio and trimethoprim and sulfamethoxazole used in a 1:4 ratio. QPCR was used to quantify macrolide specific resistance genes and *intI1* in the macrolide experiment. Additionally, *intI1* was targeted by qPCR for the trimethoprim and sulfamethoxazole experiment. Change in prevalence over a 7 day period was observed to determine the difference in selective endpoints when compounds were used in isolation and when they were combined.

It was not possible to determine with certainty whether a synergistic effect occurred when investigating the selection for resistance genes by the macrolides but an additive response was clear. An additive and synergistic effect was observed, however, when investigating the difference in *ermF* prevalence response when comparing average prevalence for the individual compounds to the prevalence for the mixed compounds at each concentration tested. The selective endpoints of both trimethoprim and sulfamethoxazole were observed to behave synergistically when combined in a 1:4 combination. Further, a varied response was observed when investigating the sum of *intI1* prevalence for individual compounds in comparison to prevalence for the mixture.

Work on selective endpoints for the macrolide antibiotics using *ermF* (presented in Chapter 3) suggests that no selection is likely at current environmental concentrations. An additive effect created by combining these antibiotics would considerably reduce the lowest observable effect concentrations (LOECs) and, subsequently, predicted no effect concentration (PNEC) values so that

mitigation strategies may need to be implemented if these antibiotics are found in combination at current environmental concentrations.

When trimethoprim was used in isolation, selection for resistance was observed at current environmental concentrations. Combining trimethoprim with sulfamethoxazole reduced its LOEC by 2-fold. Sulfamethoxazole, however, was not selective at current environmental concentrations when in isolation. When combining sulfamethoxazole with trimethoprim, however, both the LOEC and minimal selective concentration (MSC) decreased to concentrations similar to those found in the environment.

It is clear that risk assessment should take mixtures of antibiotics into account when determining the threat posed to the environment. Based on these two datasets, combining antibiotics can allow for selection of resistance at environmental concentrations even where it is not observed for individual compounds.

4.2 Introduction

Chemicals from anthropogenic pollution are rarely found in isolation in environmental settings (Brandt et al. 2015). Complex mixtures of chemical pollutants tend to be the "*rule rather than the exception*" (Altenburger et al. 2015). A number of these anthropogenic pollutants, such as pharmaceuticals (including, antibiotics), cleaning products and personal care products, are found in combinations along with their degradation products in wastewater treatment plants (WWTPs) and receiving waters (Backhaus & Faust 2012; Marx et al. 2015).

A considerable number of surveillance studies have determined that complex chemical mixtures are found in many environmental settings (Mitosch &

Bollenbach 2014; Backhaus & Faust 2012). For example, one study undertook an investigation into pharmaceuticals in a Spanish river and found the presence of 57 different compounds, 17 of which were antibiotics belonging to 5 different classes (Proia et al. 2013). Another study determined the occurrence of pharmaceuticals in 4 European sewage treatment plant effluents. They found an average of 18 out of 26 of the chemicals they were testing for (69.23% detection rate). Of these 26 compounds, 7 were antibiotics from 3 different classes. Except for sulfamethoxazole in one of four sewage treatment plants, the concentrations of all 7 antibiotics tested were above detection limits in every effluent tested. Detectable concentrations ranged from 0.01 to 0.58 µg/L of antibiotic (Andreozzi et al. 2003). Finally, a further study investigated the presence of 56 pharmaceuticals (of which 7 were antibiotics) in the effluent of 50 wastewater treatment plants. The average detection rate for all antibiotics in the 50 WWTPs tested was 56%. This ranged from two antibiotics that were undetected in all WWTPs to sulfamethoxazole which had a detection rate of 89.8%. These antibiotics were found in combination with each other and with many other pharmaceuticals (Kostich et al. 2014). Although only three surveillance studies have been presented here, the presence of mixtures of pharmaceuticals in hotspots such as WWTPs and receiving waters is the norm rather than the exception. Many other similar surveillance studies show complex mixtures of chemical compounds are introduced to the environment.

In 2006, a group of stakeholders, including industry, academic and government organisations gave their views on how to manage pharmaceuticals in environmental settings. They stated that the potential detrimental effect that mixtures posed was unknown and that research into the effects of mixed pharmaceuticals is important (Doerr-MacEwen & Haight 2006). Further, the

Council of the European Union has been aware, for almost a decade, of the dangers posed to human health and the environment by mixtures of chemical compounds as stated in their Council conclusions document from a meeting in 2009 (Council of the European Union 2009; Altenburger et al. 2015). Documents produced by the European Commission have also stated that there is an "*almost infinite number of possible combinations of chemicals*" (European Commission 2012), and that a systematic approach to evaluating the risk of these chemicals must, therefore, be developed (SCHER et al. 2012; Altenburger et al. 2015). Although the dangers that mixtures pose to the environment and to human health have been in discussion in Europe for over a decade, environmental risk assessments still only require the investigation of the dangers posed by anthropogenic contaminants on an individual basis (Backhaus & Faust 2012).

There are three possible outcomes when chemicals interact in combination: the effect may be additive (a linear relationship is observed), synergistic (a stronger effect is observed than when the chemicals are in isolation) or antagonistic (a weaker effect is observed than when the chemicals are in isolation) (Mitosch & Bollenbach 2014; Marx et al. 2015). The effect of individual chemicals must first be characterised to determine whether additive, synergistic or antagonistic effects are seen when chemicals are in combination (Marx et al. 2015). Data suggests that antibiotics tend to display a synergistic effect when found in combination (Marx et al. 2015).

To date, no studies have investigated the effect that combining antibiotics has on their selective endpoints in a mixed microbial community. A number of studies have focused on the effects that antibiotic combinations have on non-

target organisms such as zooplankton (Freitas et al. 2018), algae (Hagenbuch & Pinckney 2012) and zebrafish (Zhang et al. 2016). Some studies have also looked at the effect on bacteria, although data has so far focused on change in community structure (Brosche & Backhaus 2010) or toxicity to a specific bacterial species (Backhaus et al. 2000; Long et al. 2016). A study that has determined selective endpoints for combinations of antibiotics is a mathematical study by Bengtsson-Palme and Larsson, 2016. In this study the EUCAST database of clinical breakpoints was used to calculate a Predictive No Effect Concentration for selection of resistance ($PNEC_R$). As EUCAST is a clinical database, it also has breakpoints for combinations of antibiotics that are frequently used in the clinic, for example trimethoprim (TRMP) with sulfamethoxazole (SMX). The same mathematical approach was, therefore, able to be applied to MIC values for combinations to calculate $PNEC_R$s (Bengtsson-Palme & Larsson 2016). Gullberg *et al.*, 2014 also investigated how combining two antibiotics affected MSCs in a single species assays. Here, they investigated a mixture of erythromycin (ERY) and TRMP and found a synergistic effect on selection and combining the two reduced MSC (Gullberg et al. 2014).

The aim of work presented in this chapter was to explore the effect combining antibiotics has on experimentally defined minimal selective concentrations (MSCs) and lowest observable effect concentrations (LOECs) in a complex microbial community. The effect of two different mixtures of antibiotics was explored. These were the three macrolide antibiotics in combination and a combination of TRMP and SMX.

The macrolide antibiotics were chosen as their LOECs (defined by *ermF*), the response of other macrolide specific resistance genes and of *intI1* to the individual compounds were defined in Chapter 3. The macrolides are a class of antibiotics that target protein synthesis by binding to the large subunit of the ribosome and preventing synthesis of new peptides (Kannan & Mankin 2011; Mazzei et al. 1993). New generations of macrolides have been developed for improved pharmacokinetics and tolerability in patients, rather than to overcome pre-existing resistance mechanisms developed to ERY (Leclercq 2002; Leclercq & Courvalin 2002). As all three compounds tested in Chapter 3 have the same mode of action and bacteria deploy the same resistance mechanisms to combat them, it follows that when the macrolide antibiotics are mixed together they would produce at least a combined additive effect on any macrolide specific resistance genes.

Results generated in Chapter 3 demonstrate that current measured environmental concentrations of macrolide antibiotics may not select for resistance. The LOECs of azithromycin (AZ), clarithromycin (CLA) and ERY associated with increased *ermF* prevalence were 1,000, 750, 1,000 µg/L, respectively. It would be of interest, therefore, to investigate whether the LOEC of the total concentration of those combined macrolide antibiotics (termed AZ-CLA-ERY) would be similar to the LOECs determined in Chapter 3. There is no available data to determine how macrolide antibiotics influence each other. Studies tend to focus on whether synergism occurs between a macrolide and another antibiotic from a clinical perspective (Saiman et al. 2002; Drago et al. 2011; Descours et al. 2011; van der Paardt et al. 2015).

TRMP and SMX were chosen for investigation as they are commonly used in combination in the clinic. This combination (TRMP-SMX) was first introduced in the United States in 1973 (Foltzer & Reese 1987). TRMP-SMX works as a broad spectrum antibiotic and is effective against a range of infections (Woormser et al. 1982). Both of these antibiotics work by disrupting the synthesis of folic acid, but use different methods to do so (Eliopoulos & Huovinen 2001). SMX's mode of action is to inhibit the penultimate step in the formation of dihydrofolate. It achieves this by binding the dihydropteroate synthase enzyme and thereby preventing the formation of dihydropteroic acid from PABA and DHPPP (Sköld 2000). TRMP works by inhibiting a different enzyme: the dihydrofolate reductase. This enzyme converts dihydrofolate into tetrahydrofolate (Eliopoulos & Huovinen 2001) which is extremely important in a number of biological processes, such as the formation of purines (Bermingham & Derrick 2002).

TRMP and SMX have both mutation based resistance and transferable genes that confer resistance to them (Eliopoulos & Huovinen 2001). Transferable resistance to TRMP is conferred by dihydrofolate reductases (*dfr* genes) (Domínguez et al. 2019). There are in excess of 20 different *dfr* genes (Eliopoulos & Huovinen 2001) and these are commonly found associated with integrons which means they spread and integrate into many bacterial species (Domínguez et al. 2019; Brolund et al. 2010). In comparison to the substantial number of TRMP resistance genes, SMX has only 3 resistance genes. These are *sul1, sul2* and *sul3* which all encode dihydropteroate synthases. The *sul1* and *sul3* genes are often found in association with the class 1 integron as part of its genetic cassette, whereas the *sul2* gene is more commonly associated with plasmids (Domínguez et al. 2019).

TRMP-SMX is used in combination in the clinic as the two antibiotics act synergistically to treat infections. Clinical administration occurs in a 1:5 ratio of TRMP:SMX as this leads to the optimal serum ratio in regards to best synergistic effect of the drug to allow for the most effective treatment (1:19 and 1:20, TRMP:SMX, dependent on the reporting study) (Woormser et al. 1982; Masters et al. 2003).

A previous study has tested whether TRMP-SMX produced a synergistic effect on the minimum inhibitory concentration (MIC) of a number of bacterial strains when using these antibiotics in the serum ratio. Of every bacterial strain tested, a synergistic effect was produced and a reduction in MICs observed (Bushby 1975). It is hypothesised, therefore, that their interaction could also produce a synergistic effect on MSCs/LOECs and thereby reduce the concentration needed for the selection of resistance genes.

The objectives of the work presented in this chapter were, therefore, to determine the combined effect on the MSCs of the individual antibiotics both of the 3 macrolide antibiotics in a 1:1:1 ratio and of TRMP-SMX in a 1:4 ratio.

4.3 Methods

4.3.1 Growth rate experiment

The growth rate assay used for this work is the one developed in a study by Murray *et al.*, unpublished. This assay is able to determine a LOEC of an antibiotic or combination of antibiotics. The study found that as growth of bacteria enters the exponential phase, a dose dependent response is observed in regard to growth rate that is comparable to LOECs determined by selection experiments and qPCR. This assay was used during this work to predict the concentration range that should be targeted for selection experiments.

Iso-sensitest broth was inoculated with 10% v/v of washed wastewater (as in the set up for the week long selection experiments described in Section 2.2). 200 µl was used per well in a 96-well plate. Antibiotics were diluted in a 2-fold serial dilution with 6 replicates per concentration, alongside a no antibiotic control and a broth only control. The BioTek Synergy 2 or Varioskan Flash plate reader was used to determine optical density (OD) at 600 nm. Plates were left shaking at “medium” speed or 120 rpm, respectively, for 24 hours at 37 °C. Readings were taken every hour.

Pearson’s correlation/Spearman’s rank correlation (for parametric and non-parametric data, respectively) was used to determine where the dose response was most evident and subsequently, Dunnett’s/Dunn’s tests (for parametric and non-parametric data, respectively) were used to compare the OD of those in the presence of antibiotics to the no antibiotic control. The lowest concentration at which a significant difference was observed to the no antibiotic control was defined as the MSC.

4.3.2 Antibiotic and antibiotic concentrations

4.3.2.1 Macrolides

Macrolides used were AZ (Sigma-aldrich), CLA (Molekula) and ERY (Acros Organics). AZ and ERY were dissolved in ethanol absolute (Fisher) and CLA in acetone (Acros Organics).

This experiment was run at a concentration range where it was expected that significant selection would be observed, based on previous results. The total concentration of macrolide of 500, 750, 1,000 and 10,000 µg/L was again run in a 1:1:1 ratio of AZ:CLA:ERY and was compared to a no antibiotic control. As this was a new batch of sewage in comparison to the work undertaken and

presented in Chapter 3, individual compounds were run alongside the mixture experiment to ensure direct comparison.

A verification experiment was undertaken using ERY at a concentration range of 100, 250, 500, 750, 1,000, 10,000 and 100,000 µg/L.

4.3.2.2 Trimethoprim and Sulfamethoxazole

TRMP (Sigma-Aldrich) was dissolved in DMSO (Sigma-Aldrich) and SMX (Molekula) in methanol (Fisher).

TRMP experiment 1

The initial TRMP selection experiment, performed by the author together with a placement student, Jessica Wright, used concentrations of TRMP from the EUCAST breakpoint for *Enterobacteriaceae* and used a 2-fold dilution down to environmental concentrations of TRMP. These concentrations were 4,000, 2,000, 1,000, 500, 250, 125, 62.5, 31.25, 15.625 µg/L of TRMP.

SMX experiment 1

The initial SMX growth rate experiment, performed by the author and Jasmin Rauseo, used the same approach as TRMP experiment 1. The top concentration of SMX used was the EUCAST breakpoint for *Enterobacteriaceae*. A 2-fold dilution was performed and the concentrations used here were: 76,000, 38,000, 19,000, 9,500, 4,750, 2,375, 1,187.5, 593.75, 296.8, 148.4, 74.21, 37.1, 18.55 and 9.27 µg/L.

The results from these growth rate experiments then influenced the concentrations used in the SMX selection experiment 1. These were 2, 20, 200, 2,000, 20,000 µg/L alongside a no antibiotic control.

TRMP-SMX mixture experiment

The results from both of these preliminary selection experiments (TRMP experiment 1 and SMX experiment 1) were used to inform the concentrations used for both the mixture growth rate and mixture selection experiments. The concentrations used were: 1,000, 500, 250, 125, 62.5, 31.25 and 15.625 µg/L of TRMP and 4,000, 2,000, 1,000, 500, 250, 125 and 62.5 µg/L of SMX. These concentrations were combined in a 1:4 ratio of TRMP-SMX for the mixture experiments leading to total antibiotic concentrations of 78.125, 156.25, 312.5, 625, 1,250, 2,500 and 5,000 µg/L. The ratio of these antibiotics was based on their median surface water concentrations in Europe from the studies Straub, 2013 and Straub, 2015 (0.012 and 0.049 µg/L for TRMP and SMX, respectively).

In addition to the mixture selection experiment (TRMP-SMX experiment) the antibiotics were also run in isolation as a different inoculum was used to the preliminary experiments (TRMP experiment 1 and SMX experiment 1). These isolation experiments will be referred to as TRMP experiment 2 and SMX experiment 2 and will be reported alongside the TRMP-SMX experiment.

4.3.3 Resistance genes for qPCR quantitation

4.3.3.1 Macrolides

Based on the results from Chapter 3, the only class specific resistance genes tested were *ermF* and *mphA.* This was alongside the *intI1* gene and the 16S rRNA gene as a proxy for the presence of AMR genes and for bacterial cell count, respectively.

4.3.3.2 Trimethoprim and Sulfamethoxazole

For both of these compounds for all experiments (TRMP experiment 1, SMX experiment 1, TRMP experiment 2, SMX experiment 2 and TRMP-SMX mixing experiment), no class specific resistance genes were tested. QPCR was used to target *intI1* and 16S rRNA genes. For both SMX and TRMP, most of the class specific resistance genes associated with resistance to these drugs are commonly associated with the *intI1* gene (Moura et al. 2009).

4.3.4 Data analysis

Shapiro-Wilk test was used to find if data was parametric or non-parametric. A 2 – tailed *t* test was used on parametric data, and a Mann Whitney *U* test on non-parametric data, to compare gene prevalence in the individual and mixture experiments. This was undertaken to establish whether the effect on gene prevalence was synergistic, additive or antagonistic. Average gene prevalence in the two experiments (individual or mixed) were determined to be different from each other if $p < 0.05$.

4.4 Results

4.4.1 Macrolides mixing experiment

A limited range of antibiotic concentrations were used to determine LOECs for AZ, CLA, ERY and all three in a 1:1:1 mixture. Concentrations targeted ranged from the lowest NOEC for *ermF* from Chapter 3 (500 µg/L of CLA) to 10,000 µg/L for AZ, CLA, ERY and all three mixed in a 1:1:1 ratio.

For this experiment, as a positive increase was seen in the no antibiotic control for every gene tested, MSCs were not defined. Graphs produced from the

qPCR data and the LOECs derived from statistical analysis are presented below.

ermF

Figure 37A, B, C and D show the selection experiment data for *ermF* prevalence when in the presence of AZ, CLA, ERY and AZ-CLA-ERY, respectively. Here, significant positive selection was determined for all antibiotics and the combination of antibiotics at 500 µg/L (p = 0.0323, 0.0023, 0.0056 and 0.0034, Dunn's test, for AZ, CLA, ERY and AZ-CLA-ERY, respectively) and every subsequent concentration for all 3 antibiotics and the mixture. No concentration was, however, tested below 500 µg/L (no observable effect concentration (NOEC) in Chapter 3) and it was, therefore, impossible to determine if this was a LOEC or if selection was occurring below this concentration.

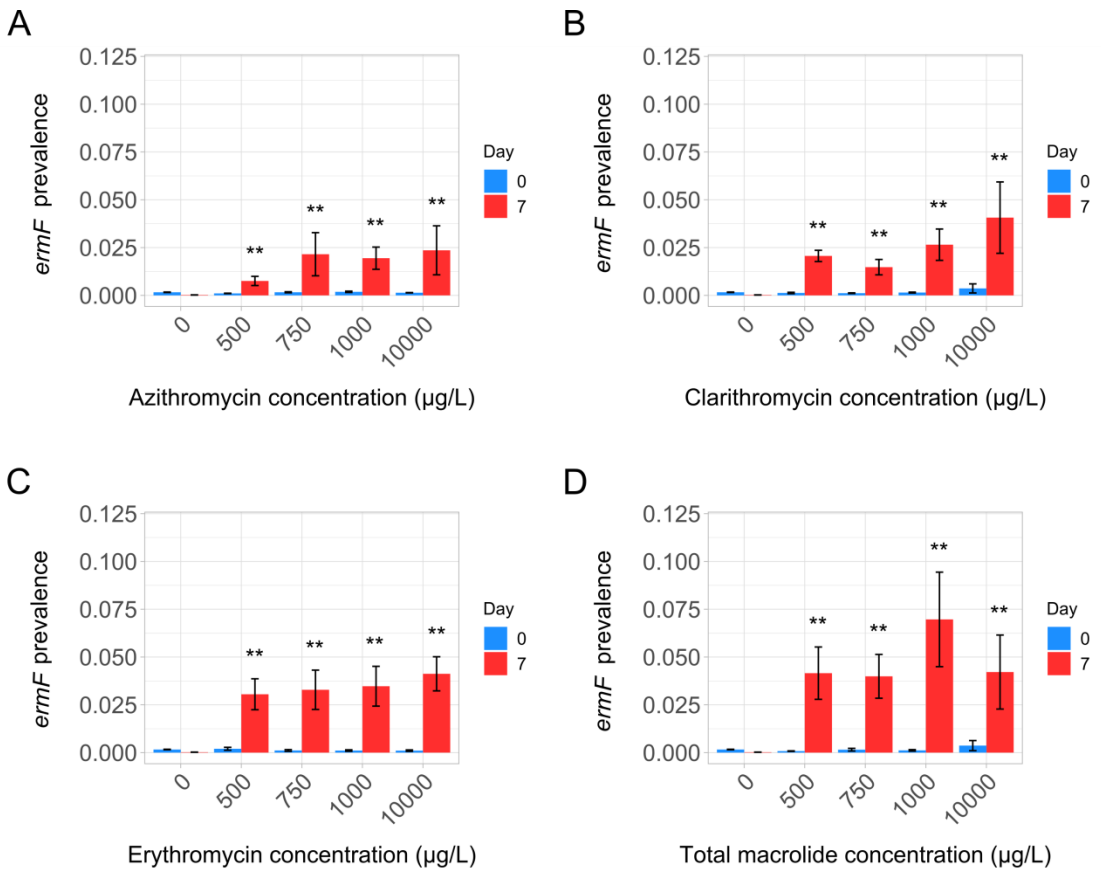


Figure 37: A comparison of the selection for *ermF* by (A) AZ, (B) CLA, (C) ERY and (D) AZ-CLA-ERY in a 1:1:1 ratio. Standard error is represented by the error bars ** = significant positive selection to 95% confidence.

Therefore, a verification experiment was undertaken for a more extensive range of concentrations using ERY, which illustrated the greatest response at 500 µg/L, Figure 38. As no concentration was tested below 500 µg/L, this experiment was undertaken to see if *ermF* significantly increased at any concentrations below this. A LOEC of 500 µg/L (p = 0.0291, Dunn's test) was determined, although a biological effect was seen at lower concentrations.

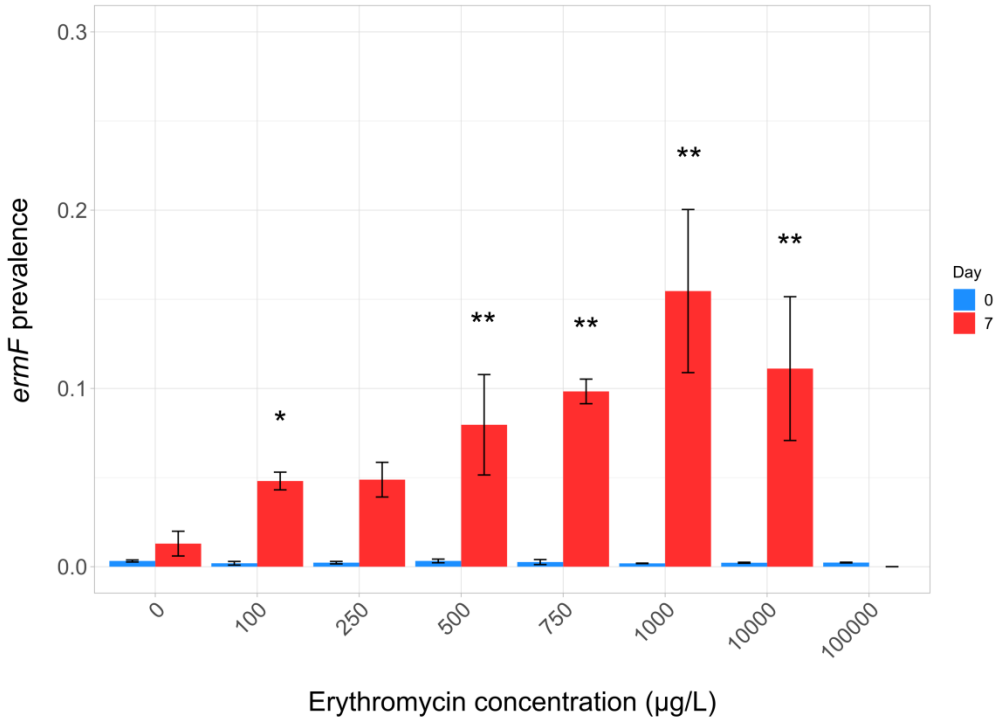


Figure 38: Mixing verification experiment graph showing *ermF* in the presence of ERY using Falmouth/Penryn 2017 wastewater as the inoculum. A LOEC of 500 µg/L was determined (p = 0.0291, Dunn's test). Standard error is represented by the error bars. * = significant positive selection to 90% confidence. ** = significant positive selection to 95% confidence

The average prevalence in the individual compound experiment was compared to the prevalence in the mixed experiment (Figure 39) and a significant difference between prevalence was observed at 500 (p = 0.0418, Mann-Whitney U test) and 1,000 µg/L (p = 0.03277, Mann-Whitney U test) indicating a synergistic response may occur. No significant difference was observed between the two experiments at 750 and 10,000 µg/L, suggesting an additive response.

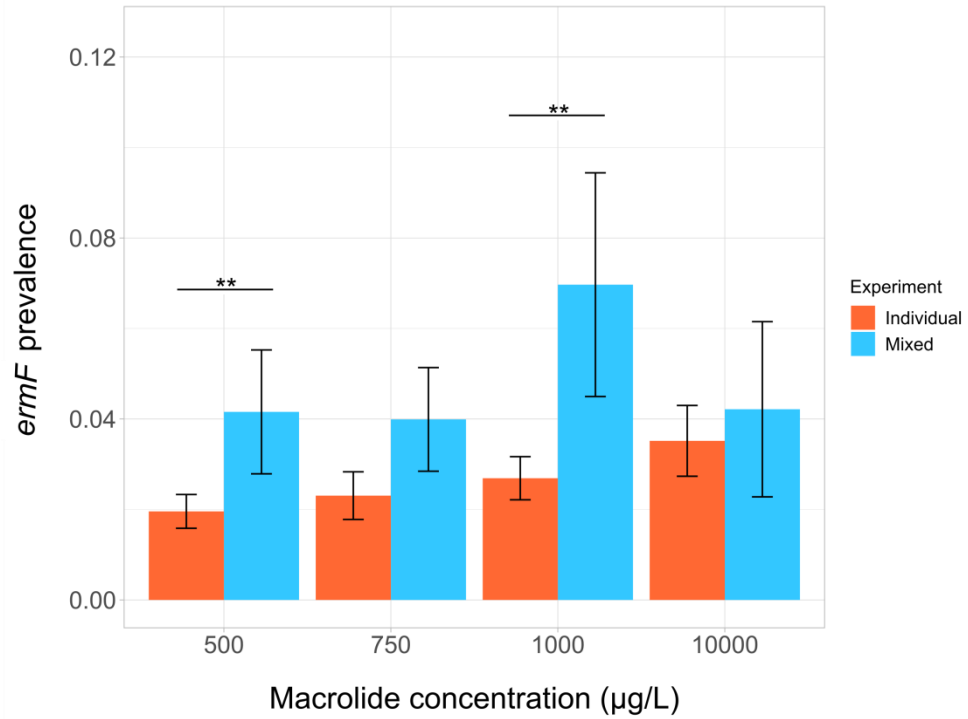


Figure 39: A comparison of the average *ermF* prevalence from the individual compound experiments to the prevalence from the mixed experiment. ** = a significant difference to 95% confidence.

mphA

Data on the change in prevalence of *mphA* over 7 days is shown in Figure 40A, B, C and D for AZ, CLA, ERY and AZ-CLA-ERY, respectively. For AZ, no LOEC was determined as positive selection to 95% confidence occurred at 500 µg/L (p = 0.0391, Dunn's test) and no concentration was tested lower than list. Subsequent higher concentrations of 750, 1,000 and 10,000 also demonstrated significant selection (p = 0.004, 0.0127, <0.0001, respectively, Dunn's test).

For CLA, a significant difference was observed in the prevalence of *mphA* at day 0 (p = 0.001834, Kruskal Wallis). The post-hoc analysis was, therefore, undertaken on the difference in prevalence between day 0 and day 7. A LOEC was determined to be 10,000 µg/L of CLA for *mphA* (p = 0.007, Dunn's test (difference)) although significant selection was observed to 90% confidence at

500 µg/L (p = 0.0914, Dunn's test (difference)). A LOEC of 10,000 µg/L was also determined for ERY (*p* = 0.0391, Dunn's test). Finally, the combination of AZ-CLA-ERY determined a LOEC of 750 µg/L (*p* = 0.0217, Dunn's test) and significant selection was seen at all subsequent higher concentrations (1,000 and 10,000 µg/L (p = 0.0391 and 0.0001, respectively, Dunn's test)).

No statistical significant difference was observed between the average *mphA* prevalence in the individual antibiotics experiments compared to the mixture experiment. This suggested an additive effect.

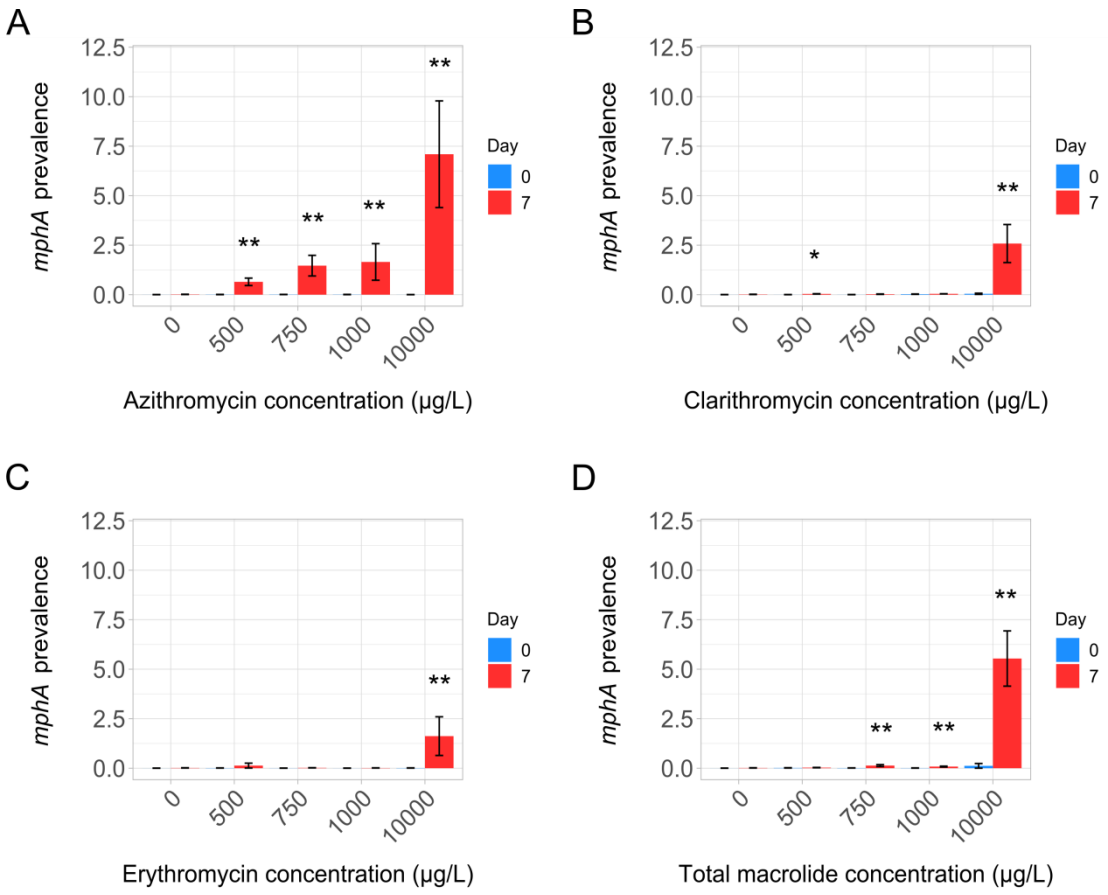


Figure 40: A comparison of the selection for *mphA* by (A) AZ, (B) CLA, (C) ERY and (D) AZ-CLA-ERY in a 1:1:1 ratio. * = significant positive selection to 90% confidence. ** = significant positive selection to 95% confidence.

intI1

Graphs showing *intI1* as a function of AZ, CLA, ERY and AZ-CLA-ERY concentration can be seen in Figure 41A, B, C and D, respectively. A LOEC was determined for *intI1* selection to be 10,000 µg/L of AZ to 95% confidence (p = 0.0081, Dunn's test) although a biological effect was observed at all concentrations tested. A significant difference between prevalence was observed for *intI1* at day 0 for both CLA and ERY (p = 0.01312 and 0.003518, respectively, Kruskal Wallis). The post-hoc tests were, therefore, undertaken on difference between the prevalence of *intI1* between day 0 and day 7. For CLA, a LOEC of 1,000 µg/L was determined (p = 0.0196, Dunn's test (difference)) although no significant difference was observed between the control and 10,000 µg/L for the difference between day 0 and day 7 and significance was observed to 90% confidence at 500 µg/L (p = 0.0663, Dunn's test (difference)). For ERY, no significant selection was observed at any concentration tested. No LOEC was able to be determined for the combination of AZ-CLA-ERY. Significant selection to 90% confidence was observed, however at 10,000 µg/L (p = 0.0846, Dunn's test).

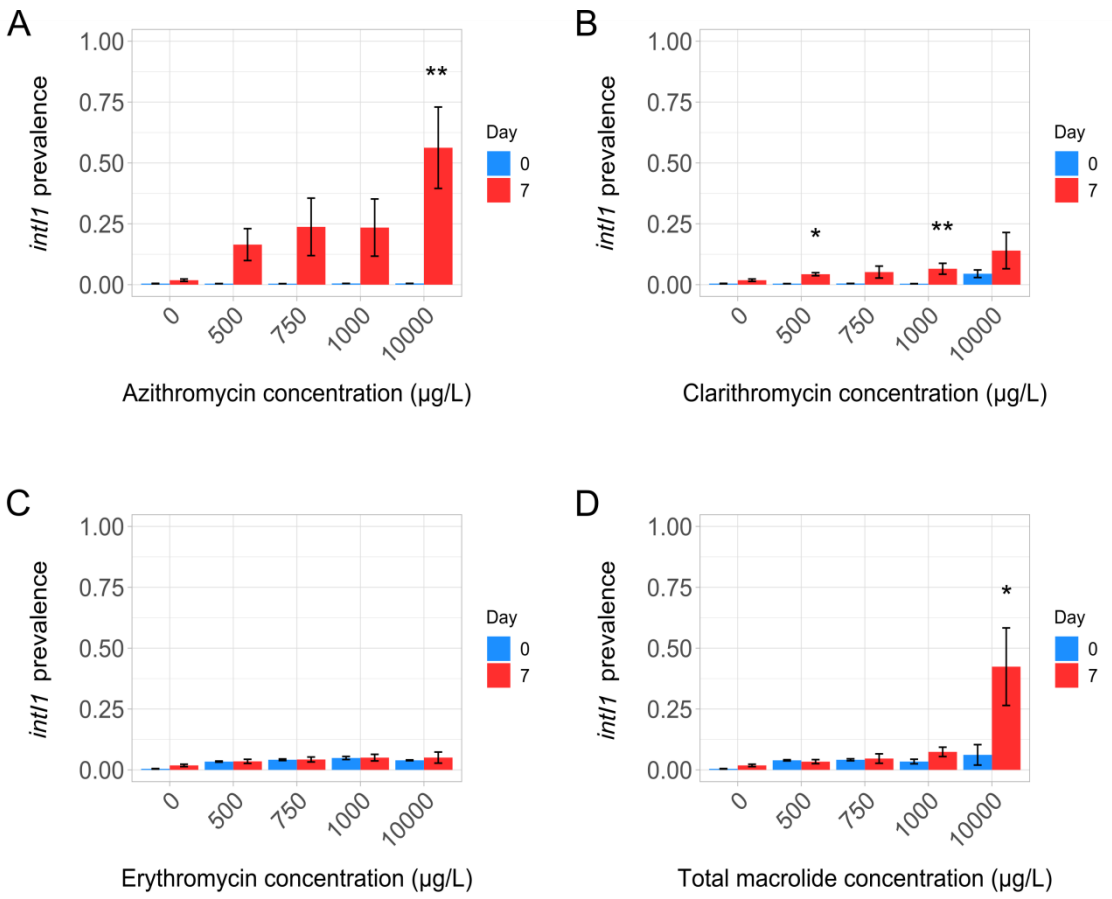


Figure 41: A comparison of the selection for *intI1* by (A) AZ, (B) CLA, (C) ERY and (D) AZ-CLA-ERY in a 1:1:1 ratio. Standard error is represented by the error bars. * = significant positive selection to 90% confidence. ** = significant positive selection to 95% confidence.

No statistical significant difference was observed between the average *intI1* prevalence for individual compounds compared to the antibiotic mixture, suggesting an additive effect.

4.4.2 Trimethoprim and Sulfamethoxazole

4.4.2.1 TRMP experiment 1

The selection experiment and DNA extractions for this work was undertaken by the author and a Nuffield bursary school student, Jessica Wright. The qPCR analysis was undertaken by Aimee Murray.

QPCR was used to investigate selection for *intI1* by TRMP. This can be seen in Figure 42. The prevalence of *intI1* at day 0 showed a significant difference (p = 0.005621, Kruskal Wallis), therefore the post-hoc analysis was undertaken on the difference in prevalence between day 0 and day 7. No significant increase in prevalence was determined at 15.625 and 31.25 µg/L of TRMP compared to the no antibiotic control. Significant selection to 90% confidence was observed at 62.5 (p = 0.0951, Dunn's test) and 125 µg/L (p = 0.0774, Dunn's test (difference)) compared to the no antibiotic control. Significant selection to 95% confidence was first observed at 250 (p = 0.0074, Dunn's test) and this was, therefore, defined as the LOEC.

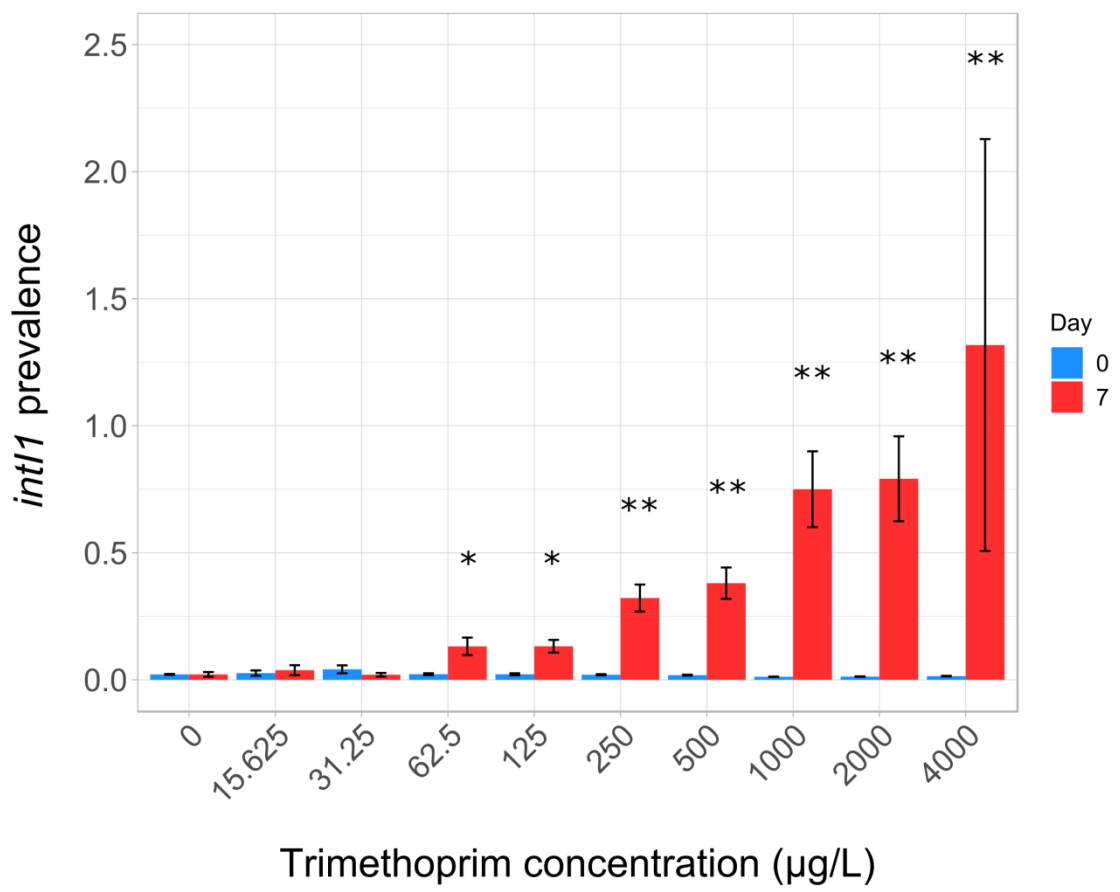


Figure 42: Selection of *intI1* by trimethoprim – TRMP experiment 1. Standard error is represented by the error bars. * = significant positive selection to 90% confidence. ** = significant positive selection to 95% confidence.

An MSC was determined as approximately 26 µg/L by plotting a selection coefficient with polynomial order 2 of best fit ($y = -0.11 + 0.0045x - 1e^{-05}x^2$, $R^2 = 0.5227$, standard error = 0.5227, x intercept = 25.9). This graph can be seen in Figure 43. As a result of the natural variation between the biological replicates, only an approximate MSC can be calculated.

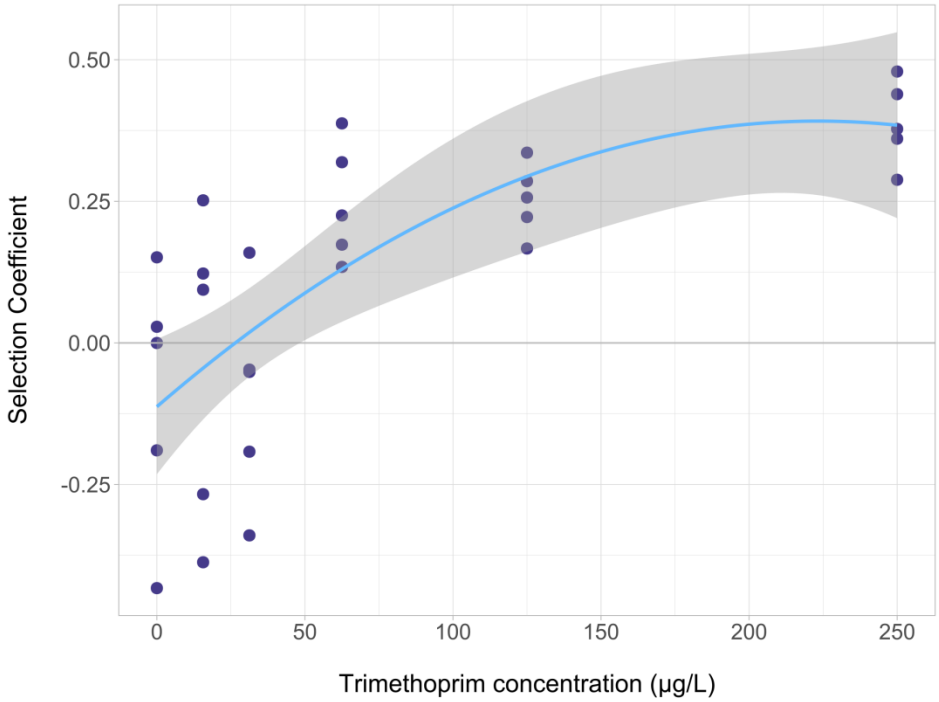


Figure 43: Selection coefficient graph of *intI1* in the presence trimethoprim – TRMP experiment 1. The blue line represents the line of best fit, the grey shading is the confidence intervals and the selection coefficient for each replicate is represented by the purple dots.

4.4.2.2 SMX experiment 1

This experiment was undertaken by Jasmin Rauseo, a visiting PhD student from the Water Research Institute – National Research Council in Italy. During her time at the University of Exeter, her project was planned by and she was supervised by the author who instructed her on the methodology of the experiment and assisted her with the analysis of her work.

4.4.2.2.1 Preliminary growth rate experiment

An initial growth plate experiment was run to determine the best concentration range to target. The greatest variation in regards to dose response was observed at hour 7 (determined by Spearman's rank correlation). A significant difference, in comparison to the antibiotic control was observed to 90% confidence at 1,187.5 µg/L (p = 0.0664, Dunn's test) and to 95% confidence at 2,375 µg/L (p = 0.0179, Dunn's test) and every subsequent concentration. A graph showing the exponential growth phase of this can be seen in Figure 44. The full growth rate can be seen in Figure 89 (Page 327 - Appendix).

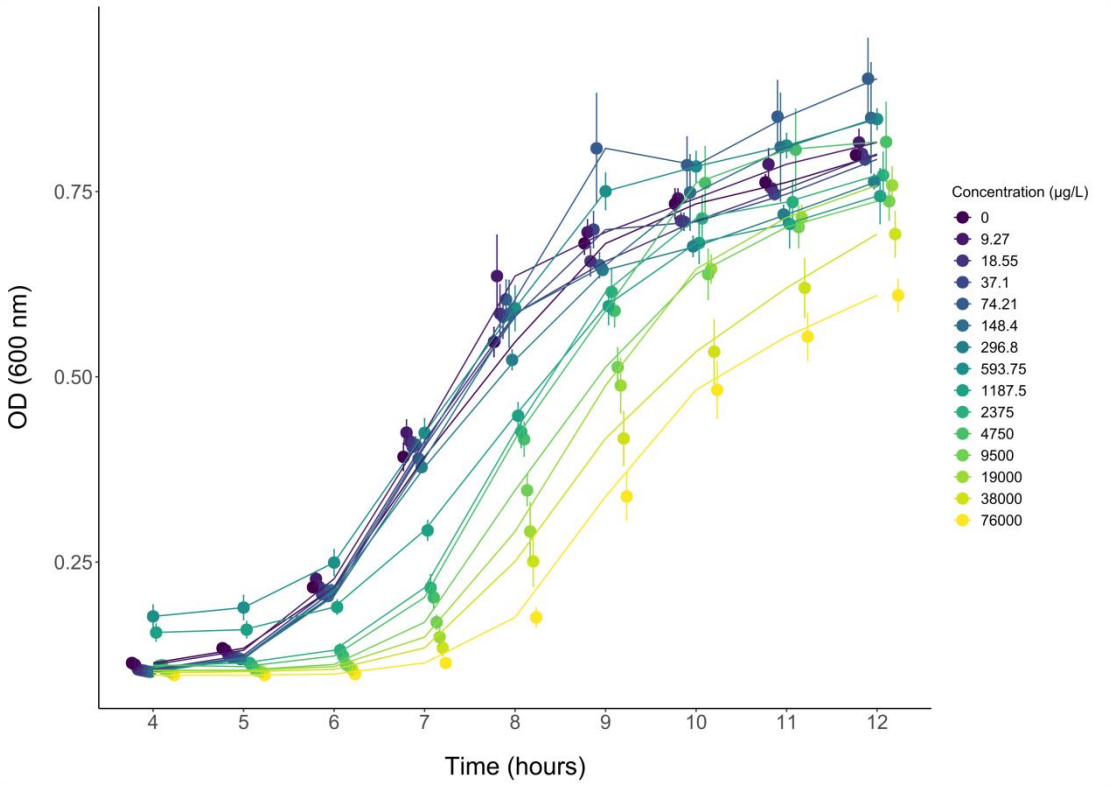


Figure 44: Growth rate as a function of SMX concentration. Graph shows OD over a limited time frame to best show exponential phase. Standard error is represented by the error bars.

4.4.2.2.2 Selection experiment

Based on this growth rate data, a week long evolution experiment was conducted, targeting the concentrations 2, 20, 200, 2,000 and 20,000 µg/L of sulfamethoxazole. QPCR showed significant positive selection for *intI1* to 95% confidence for the concentrations of SMX of 2,000 (p = 0.0006, Dunn's test) and 20,000 µg/L (p = 0.0001, Dunn's test). A LOEC of 2,000 µg/L and a NOEC of 200 µg/L was, therefore, determined. The results can be seen in Figure 45. A significant increase in *intI1* prevalence was seen at 200 µg/L to 90% confidence (p = 0.0806, Dunn's test) in comparison to the no antibiotic control although this did not increase above the starting prevalence of *intI1*.

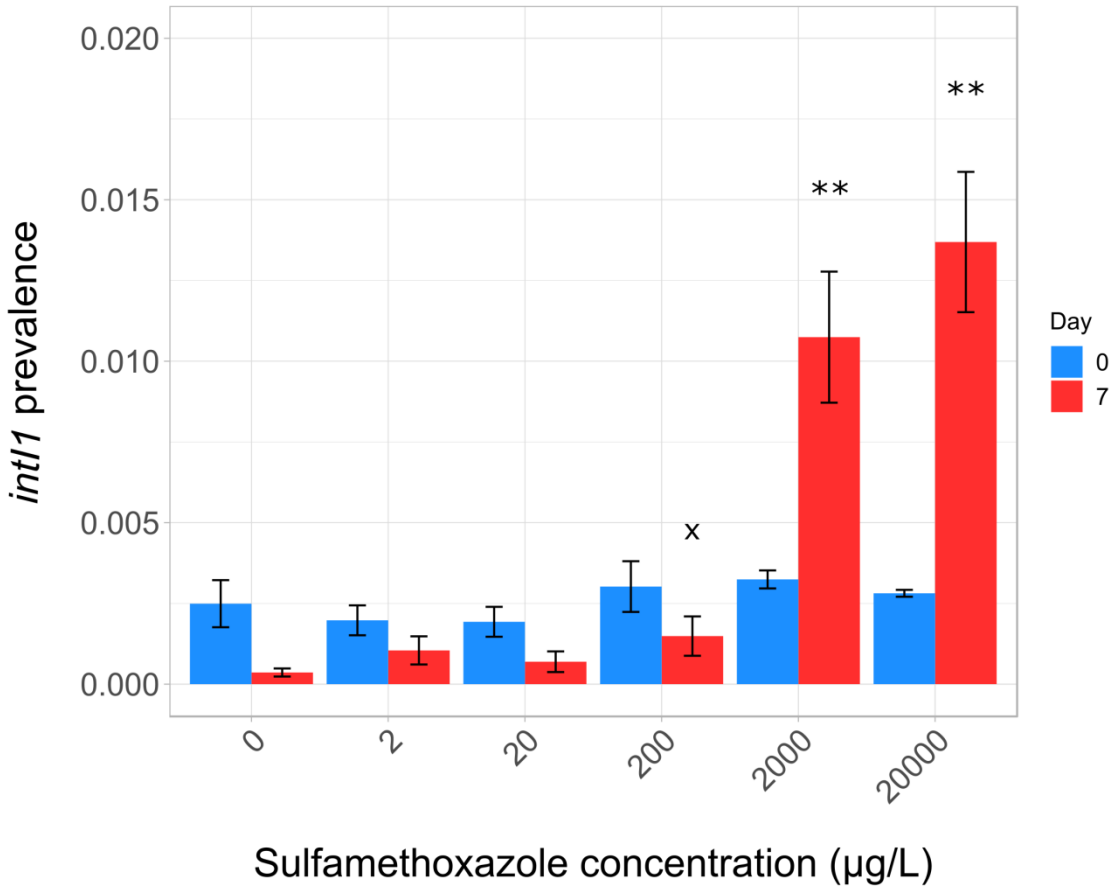


Figure 45: Selection of *intI1* by sulfamethoxazole. Standard error is represented by the error bars. x = significant increase, in comparison to no antibiotic control, to 90% confidence. ** = significant positive selection to 95% confidence.

A MSC of approximately 840 µg/L of SMX for selection of *intI1* was determined using a selection coefficient with a polynomial order 2 line of best fit ($y = -0.2 + 0.0047x + 7.6e^{-05}x^2$, $R^2 = 0.471$, standard error = 0.1543, x intercept = 839.6). Only an approximate MSC value can be calculated as a result of the natural variation between biological replicates. The selection coefficient graph can be seen in Figure 46.

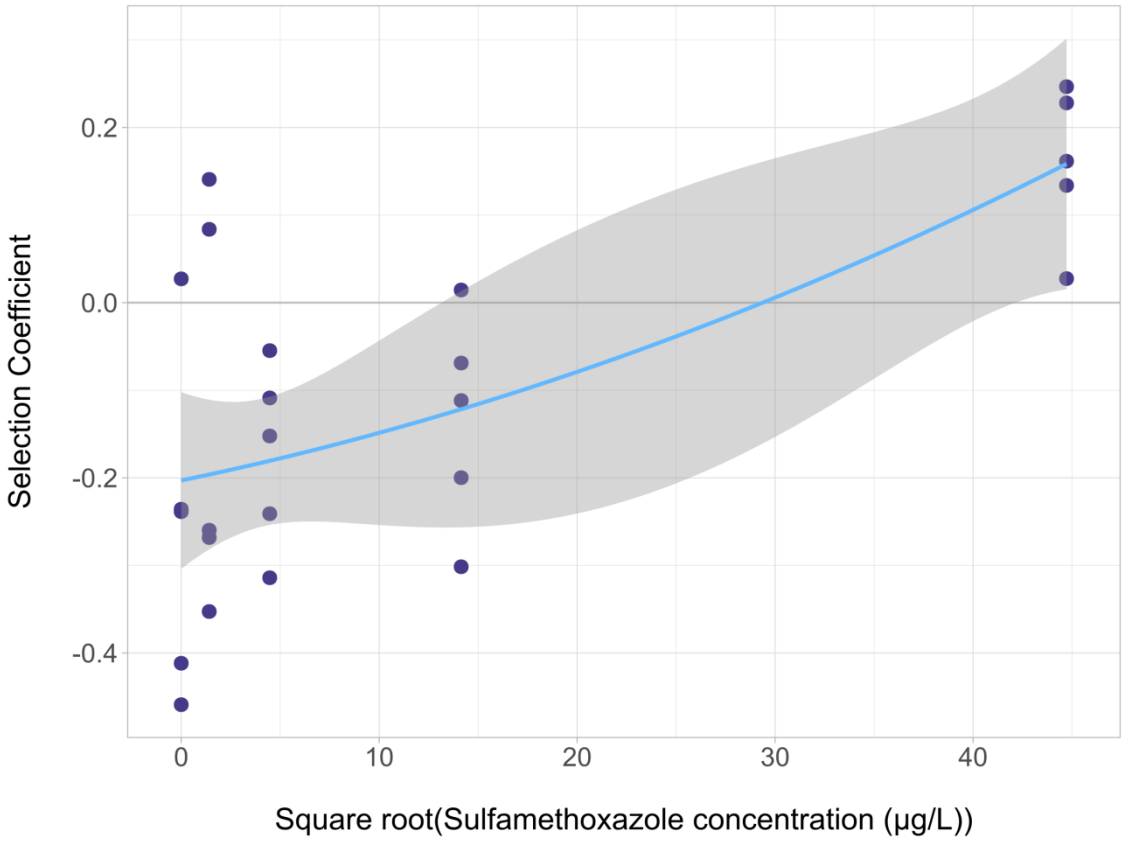


Figure 46: Selection coefficient of *intI1* in the presence of sulfamethoxazole. The concentrations plotted equate to the absolute values of 0, 2, 20, 200 and 2,000 µg/L. 20,000 µg/L was eliminated to determine the most accurate MSC. The blue line represents the line of best fit, the grey shading is the confidence intervals and the selection coefficient for each replicate is represented by the purple dots.

4.4.2.3 TRMP and SMX mixture experiment

4.4.2.3.1 Preliminary growth rate experiment

A growth rate experiment was set up, as in section 4.4.2.2, to determine if the planned concentration range was appropriate before undertaking the selection experiment. Graphs showing growth rate over the full 24 hour experiment can be found in Figures 90, 91 and 92 (Page 327 - Appendix) for TRMP, SMX and TRMP-SMX, respectively.

Trimethoprim

The highest variation in growth rate was seen at hour 6 (r = - 0.810284, Spearman's rank correlation) for TRMP. Dunn's test was then undertaken on this time-point. A significant difference, in comparison to the control, was first observed at 31.25 µg/L (p = 0.0454, Dunn's test) and at every subsequent higher concentration (62.5, 125, 250, 500 and 1,000 µg/L of TRMP (p = 0.0051, 0.0051, 0.0007, 0.0001 and <0.0001, respectively, Dunn's test)). Figure 47 shows the exponential growth phase of this experiment.

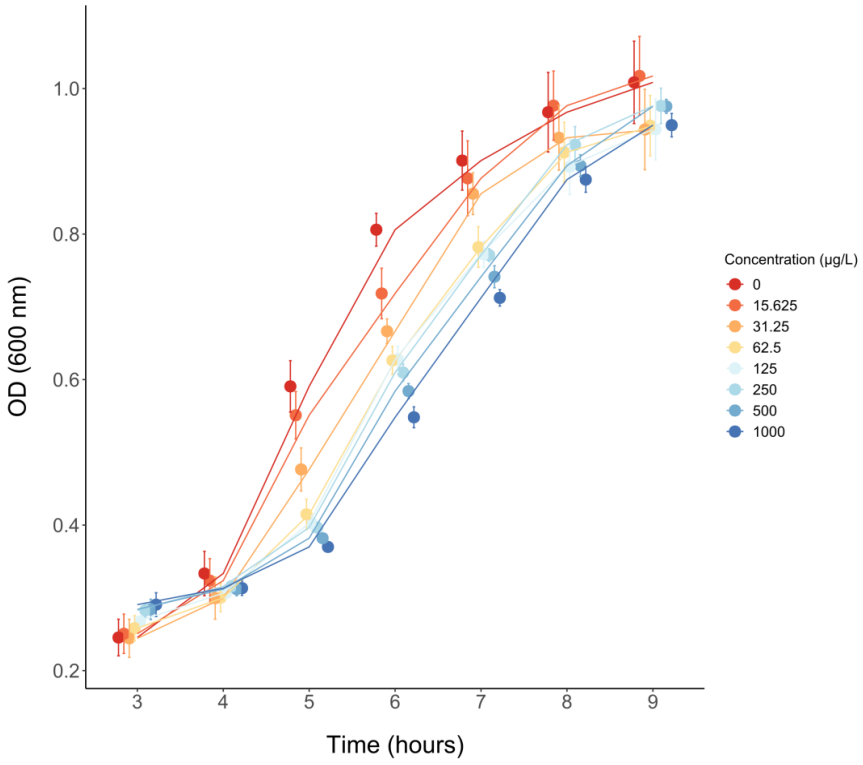


Figure 47: Growth rate as a function of TRMP concentration. Graph shows OD over a limited time frame to best show exponential phase. Standard error is represented by the error bars.

Sulfamethoxazole

For SMX, the greatest variation was also seen at hour 7 (r = - 0.872382, Spearman's rank correlation). A significant difference was observed to 90% confidence at 500 µg/L (p = 0.0759, Dunn's test) and to 95% confidence at 1,000 µg/L (p = 0.003, Dunn's test) and subsequent higher concentrations (2,000 and 4,000 µg/L (p = 0.0002 and <0.0001, respectively, Dunn's test)), Figure 48.

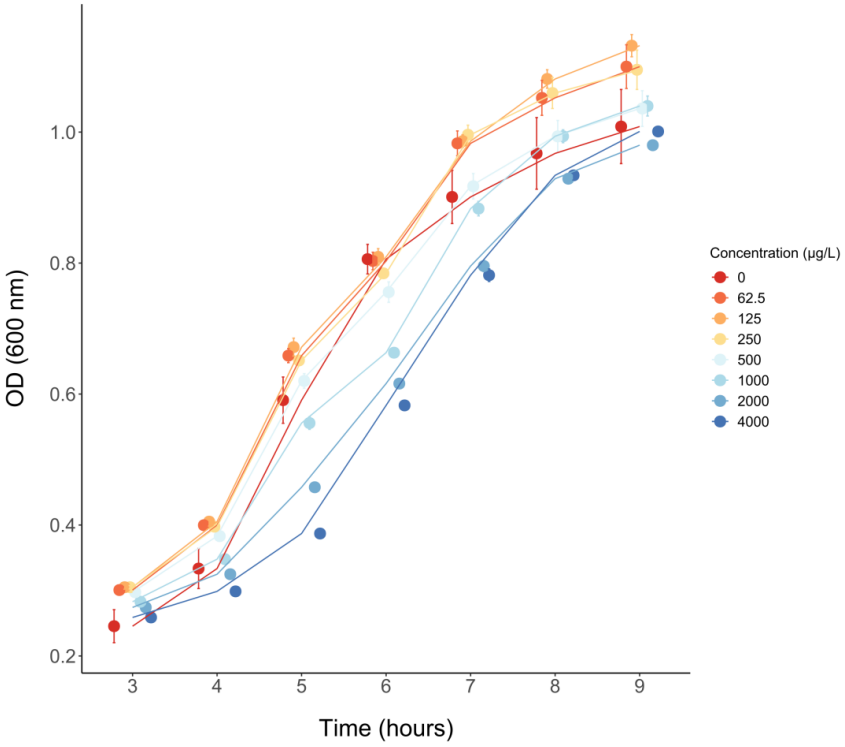


Figure 48: Growth rate as a function of SMX concentration. Graph shows OD over a limited time frame to best show exponential phase. Standard error is represented by the error bars.

Mixture

The greatest variation in samples was observed at hour 5 (r = - 0.9475483, Spearman's rank correlation). No significant difference was seen for the lowest concentration of total antibiotic (78.125 µg/L). A significant difference was first seen to 90% confidence at 156.25 µg/L (p = 0.0986, Dunn's test) of total antibiotic (31.25 µg/L of TRMP and 125 µg/L of SMX) and a significant difference to 95% confidence was observed at 312.5 µg/L (p = 0.0155, Dunn's test) of total antibiotic (62.5 µg/L of TRMP and 250 µg/L of SMX) and every subsequent higher concentration. A significant difference from the no antibiotic control was observed to 90% confidence at 156.25 µg/L (p = 0.0986, Dunn's test) and to 95% confidence at 312.5, 625. 1250. 2500 and 5000 µg/L of total

antibiotic (p = 0.0155, 0.0024, <0.0001, <0.0001 and <0.0001, respectively, Dunn's test). This can be seen in Figure 49.

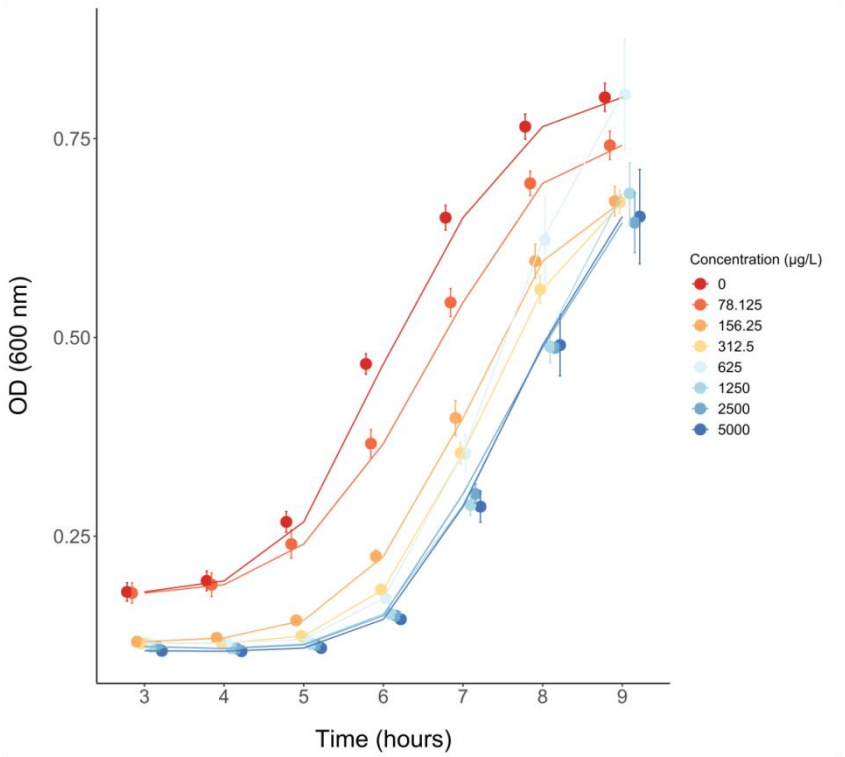


Figure 49: Growth rate as a function of TRMP-SMX concentration (in a 1:4 ratio of TRMP:SMX). Graph shows OD over a limited time frame to best show exponential phase. Concentrations are expressed as the total antibiotic concentration. They consist of TRMP and SMX in a 1:4 ratio. Standard error is represented by the error bars.

4.4.2.3.2 Selection experiment

As with the preliminary growth rate experiment (section 4.4.2.3.1), selection experiments with TRMP and SMX were undertaken in isolation as well as the mixture experiment in a 1:4 ratio of TRMP:SMX. This was to ensure that any difference seen with the mixture experiments, in comparison to the compounds

in isolation, was seen because of the mixing of compounds and not because of difference in inoculum (as the inoculum used was different to the two preliminary experiments presented above).

Trimethoprim experiment 2

No positive selection for *intI1* was observed at 15.625 and 31.25 µg/L of TRMP. Significant positive selection was observed to 95% confidence at 62.5 (p = 0.0186, Dunn's test) and every subsequent higher concentration of TRMP (125 (p = 0.0064, Dunn's test), 250 (p = 0.0021, Dunn's test), 500 (p = 0.0004, Dunn's test) and 1000 µg/L (p < 0.0001, Dunn's test)), Figure 50. The LOEC of TRMP was, therefore, determined to be 62.5 µg/L.

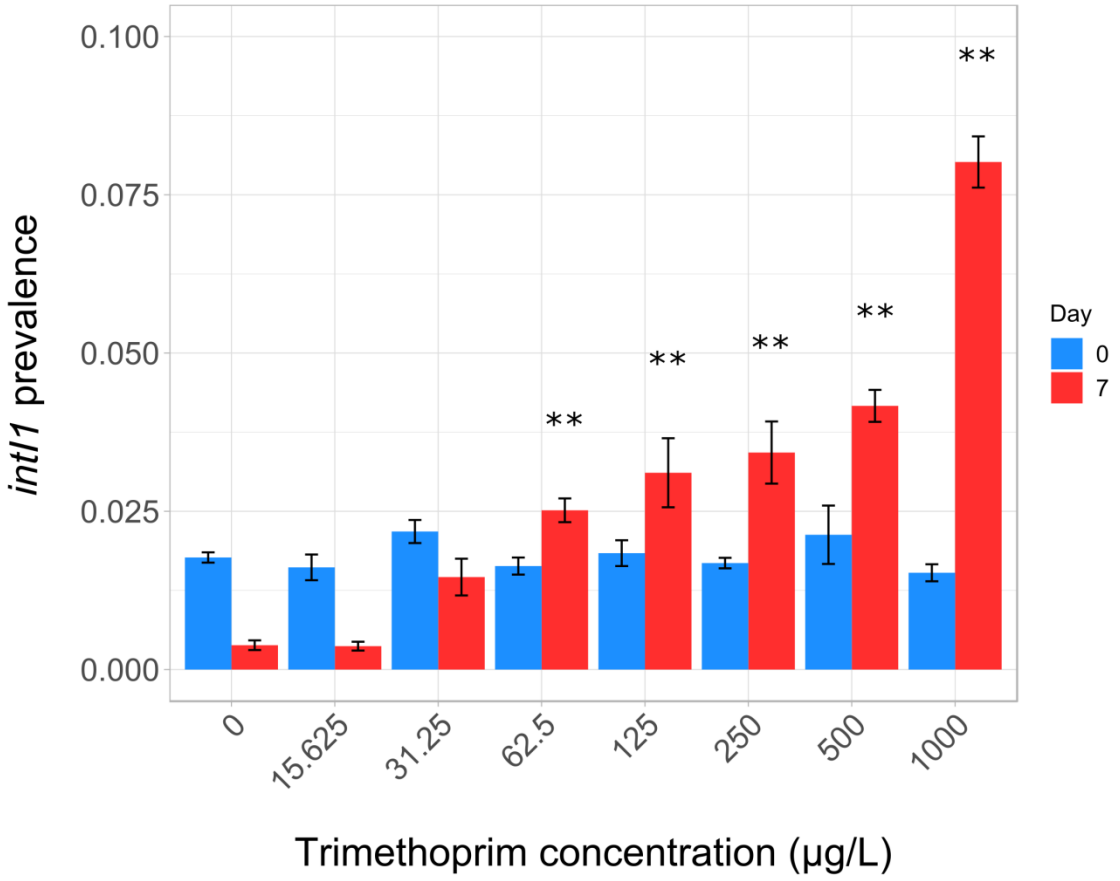


Figure 50: Selection for *intI1* by trimethoprim. Standard error is represented by the error bars. ** = significant positive selection to 95% confidence.

Sulfamethoxazole experiment 2

Significant variability was determined between prevalence at day 0 at various concentrations of SMX (p = 0.00285, ANOVA). The difference between prevalence at day 0 and day 7 was, therefore, used to determine where significant positive selection was observed. No positive selection of *intI1* was observed at 62.5, 125, 250 and 500 µg/L of SMX. Significant positive selection was first observed at 1,000 µg/L (p = 0.0186, Dunn's test (difference)), 2,000 (p = 0.0417, Dunn's test (difference)) and 4,000 µg/L (p = 0.004, Dunn's test (difference)) of SMX, see Figure 51.

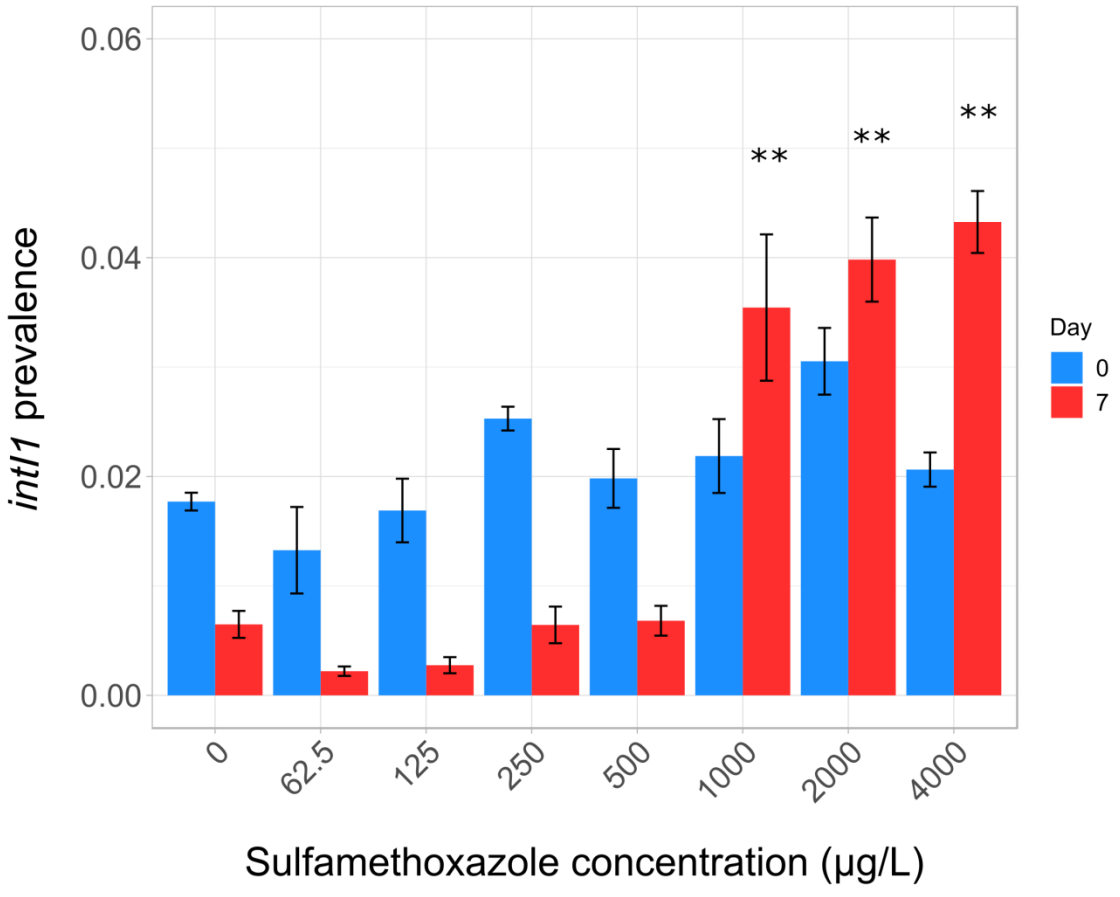


Figure 51: Selection of *intI1* by sulfamethoxazole. Standard error is represented by the error bars. ** = significant positive selection to 95% confidence.

Mixture

The selection experiment graph presented for the mixture experiment (Figure 52) displays the total antibiotic concentration (TRMP concentration + SMX concentration) used for the experiment. To clarify exact proportions of TRMP and SMX in the selection experiment, the table displayed immediately below the graph indicates the proportion of both TRMP and SMX at each total antibiotic concentration displayed.

When the compounds are mixed together, no significant selection was observed at 78.125 µg/L of total antibiotic concentration (15.625 and 62.5 µg/L of TRMP and SMX, respectively). Significant positive selection was first observed at 156.25 µg/L (p = 0.0017, Dunn's test) of total antibiotic concentration (31.25 µg/L of TRMP and 125 µg/L of SMX) and at all subsequent higher concentrations (312.5, 625, 1,250, 2,500 and 5,000 µg/L (p = 0.0025, 0.001, 0.0029, 0.0002 and 0.0037, respectively, Dunn's test) of total antibiotic).

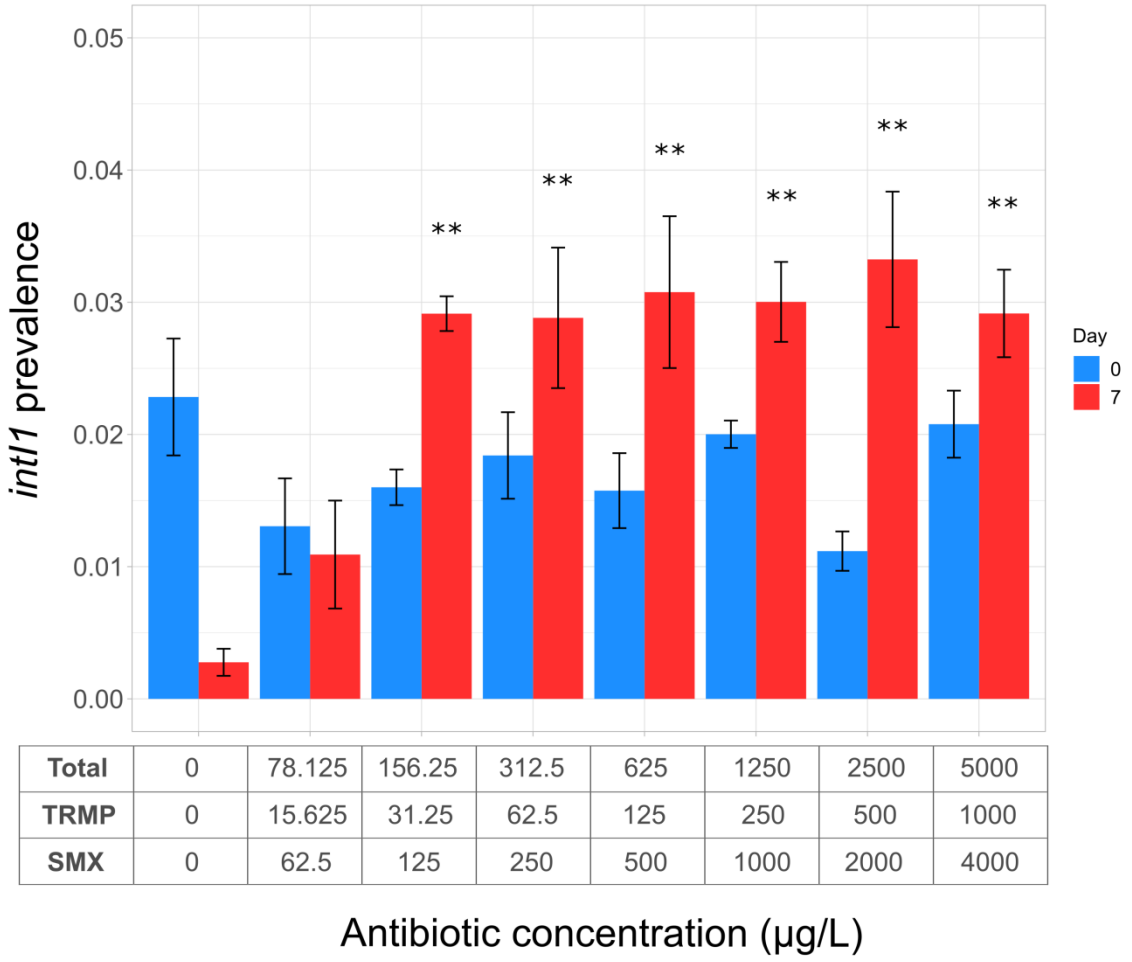
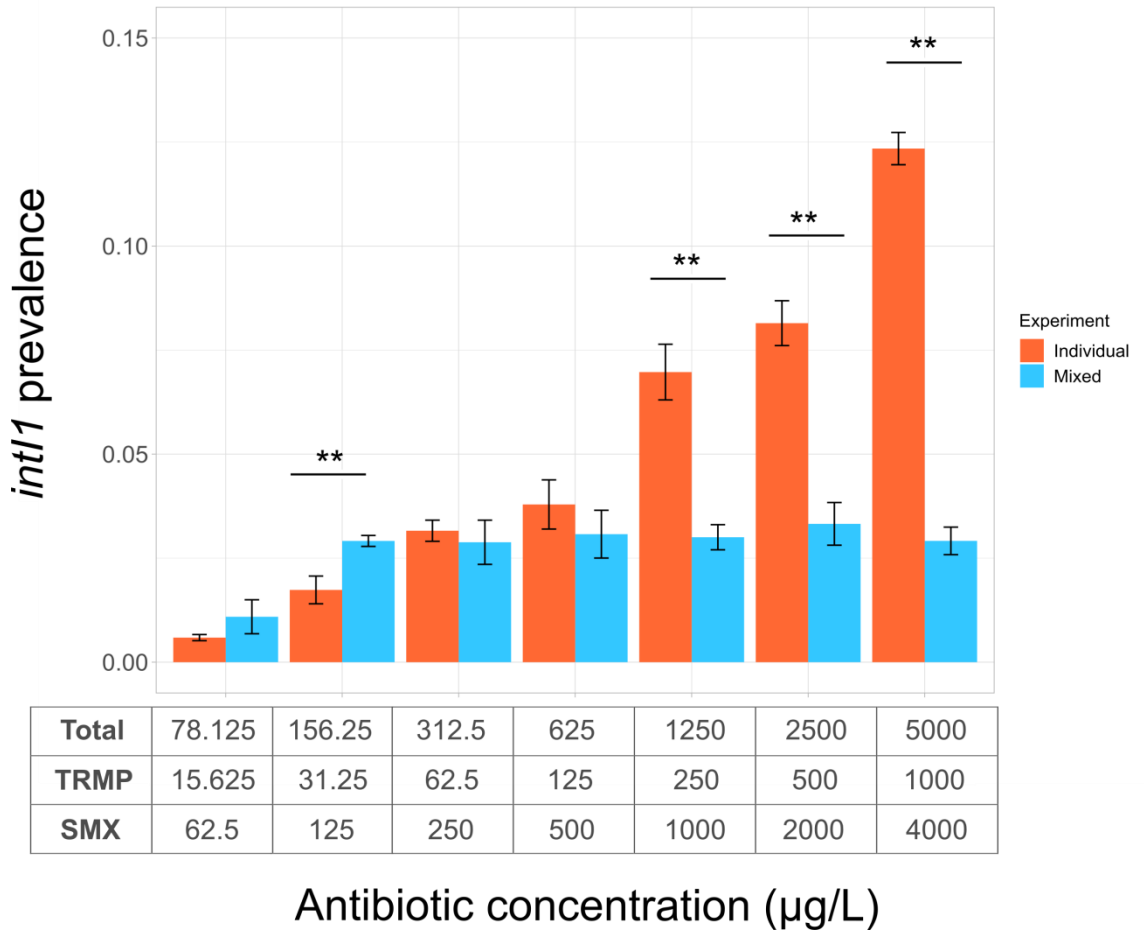


Figure 52: Selection of *intI1* by trimethoprim and sulfamethoxazole in a 1:4 ratio. Standard error is represented by the error bars. ** = significant positive selection to 95% confidence.

The prevalence from the individual experiments was totalled and compared to the prevalence in mixture experiments (Figure 53), no significant difference was observed at 78.125, 312.5, or 625 µg/L of total antibiotic, suggesting an additive effect. A significant difference was observed at 156.25 (p = 0.02055, t test), suggesting a synergistic effect. A significant difference was also observed at 1,250 (p = 0.002098, t test), 2,500 (p = 0.0001937, t test) and 5,000 µg/L (p = 0.007937, Mann Whitney U test), suggesting an antagonistic effect.



Total	78.125	156.25	312.5	625	1250	2500	5000
TRMP	15.625	31.25	62.5	125	250	500	1000
SMX	62.5	125	250	500	1000	2000	4000

Figure 53: A comparison of the total *intI1* prevalence from the individual compound experiments to the prevalence from the mixed experiment. ** = a significant difference to 95% confidence.

Selection coefficients

Selection coefficient graphs were used to determine MSCs for *intI1* selection by TRMP and SMX when used in isolation and for each of those when in combination.

For TRMP, when the experiment was run in isolation (TRMP experiment 2), the line of best fit (polynomial order 3, $y = - 0.232 - 0.0065x + 0.000571x^2 – 6.27e^{-06}x^3$, $R^2 = 0.7544$, standard error = 0.07675, x intercept = 37.8) estimated a MSC of approximately 38 µg/L. When in combination, the MSC of TRMP

decreased by approximately half and the line of best fit (polynomial order 3, y = -0.33 + 0.0259x – 0.000488x^2 + 2.82$e^{-06}x^3$, R^2 = 0.6759, standard error = 0.1282, x intercept = 18.5) estimated a MSC of approximately 19 µg/L. This can be seen in Figure 54. MSC values are approximations as a result of the natural variation between the biological replicates.

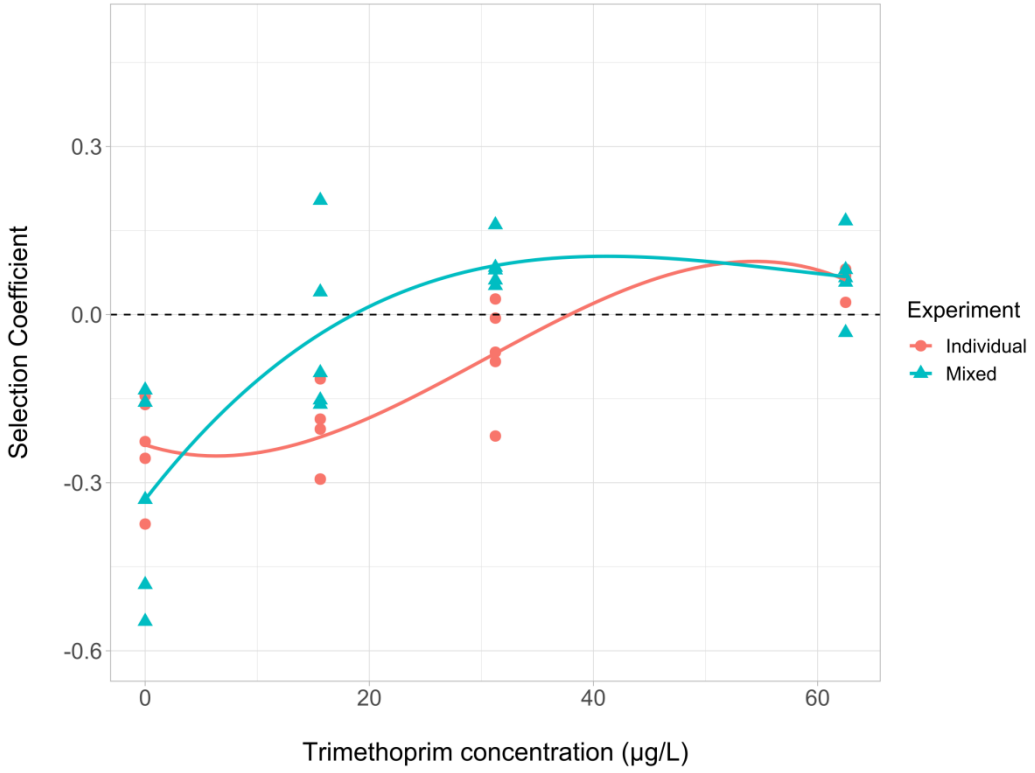


Figure 54: Selection coefficient of *intI1* in the presence of trimethoprim for the individual antibiotic and when mixed with sulfamethoxazole.

When SMX was used in isolation (SMX experiment 2), the line of best fit (polynomial order 3, y = -0.148 – 0.0305x + 0.00206x^2 – 2.87$e^{-05}x^3$, R^2 =0.698, standard error = 0.08743, x intercept = 841.2) determined the MSC as approximately 841 µg/L. In the mixed experiment, however, the MSC decreased by approximately 12 times to 69 µg/L (Figure 55), as defined by the line of best

fit (polynomial order 3, $y = -0.33 + 0.00647x - 3.05e^{-05}x^2 + 4.4e^{-08}x^3$, R^2 = 0.6758, standard error = 0.1282, x intercept = 68.5). The MSC values calculated are approximations as a result of the natural variation observed between the biological replicates.

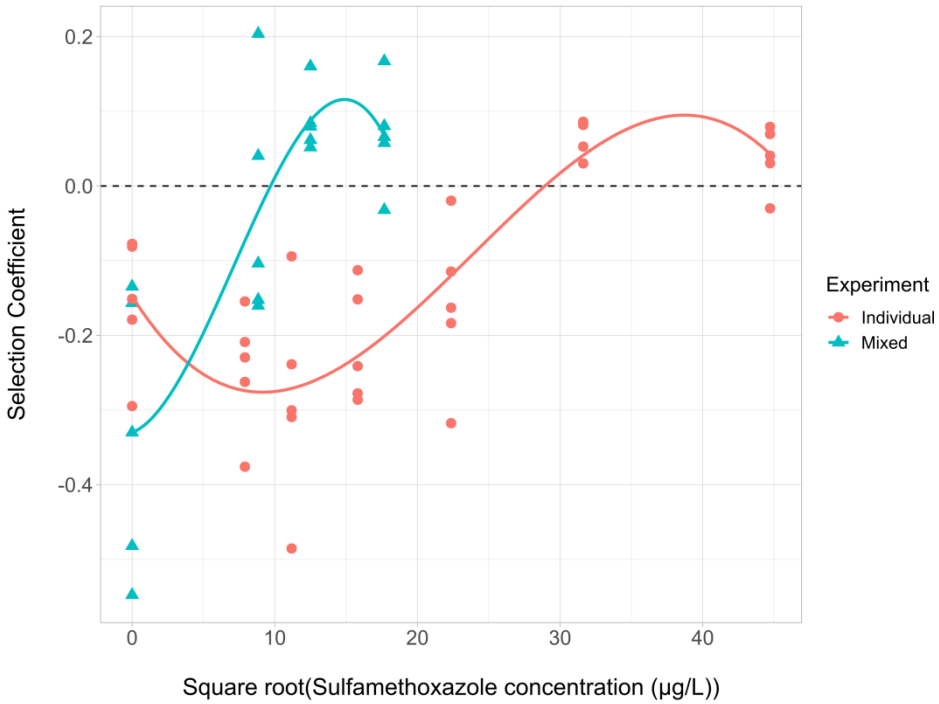


Figure 55: Selection coefficient of *intI1* in the presence of sulfamethoxazole for the individual antibiotic and when mixed with trimethoprim. The square root of SMX concentration was plotted. This equates to absolute concentrations of 0, 62.5, 125, 250, 500, 1000 and 2000 µg/L for SMX in isolation and 0, 62.5, 125 and 250 µg/L for the mixed experiment.

Confidence intervals have not been included on the two graphs that display the MSCs for TRMP and SMX when in combination and in isolation. Displaying both the individual and mixed lines of best fits on the same graph, for comparative purposes, meant that the confidence intervals overlapped and the graph became confusing to visualise. A graph for each dataset displaying the selection coefficient data points, the line of best fit and the confidence interval

area can be found in the supplementary data in Figures 93 – 96 (Page 329 - Appendix).

A MSC, defined as where the line of best fit (polynomial order 3, y = - 0.33 + 0.00517x – 1.95e-05x^2 + 2.25$e^{-08}x^3$, R^2 = 0.6795, standard error = 0.1282, x intercept = 92.9) crosses the x axis, of approximately 93 µg/L was determined for the total antibiotic concentration acting on *intI1* when TRMP and SMX are used in combination. Again, only an estimation of MSC can be calculated as a result of the natural variation between biological replicates. This can be seen in Figure 56.

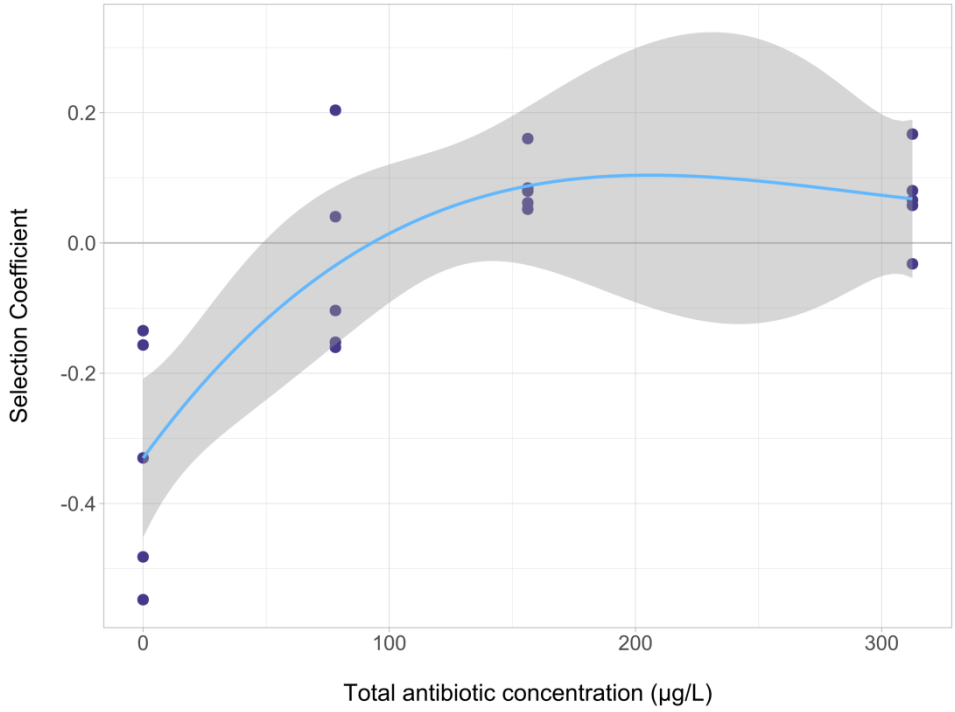


Figure 56: Selection coefficient of *intI1* in the presence of trimethoprim and sulfamethoxazole in a 1:4 ratio. The blue line represents the line of best fit, the grey shading is the confidence intervals and the selection coefficient for each replicate is represented by the purple dots.

4.5 Discussion

The data presented in this chapter suggests that antibiotics found in combination in the environment pose a greater risk than when found in isolation. This is cause for concern as current ecotoxicology testing only requires tests to assess the risks posed by active pharmaceutical ingredients (APIs) to be undertaken in isolation.

4.5.1 Synergistic, additive or antagonistic?

Macrolides

Defining the mixing effect on the selective endpoint when the macrolides are used in combination was difficult. A limited range of concentrations for each antibiotic and combination of antibiotics was undertaken in order to keep the size of the experiment manageable. This was to ensure that inoculum was homogenised and that the differences that were seen were as a result of the experimental treatment rather than an inoculum or experimental bias. For *ermF*, all concentrations of antibiotics showed a significant increase in prevalence compared to the no antibiotic control. This included the combination of the three antibiotics. Having not determined a NOEC, it was not possible to say with certainty what response occurred for the LOEC. By running a verification experiment, it was shown that ERY had a LOEC of 500 µg/L with this inoculum. It can be said with some certainty, therefore, that an antagonistic effect on the LOEC was not observed with the AZ-CLA-ERY. It cannot be determined, however, whether the AZ-CLA-ERY was acting synergistically or additively on the LOEC defined by *ermF*.

When investigating the effect on gene prevalence, an additive or synergistic effect (depending on the concentration tested) was observed when the

antibiotics were combined in a 1:1:1 ratio, in comparison to the average prevalence for individual antibiotics treatments.

For *intI1*, a LOEC of 10,000 µg/L was determined for AZ. A biological effect was seen, however, at concentrations lower than this. For CLA, a LOEC of 1,000 µg/L was determined but no significant selection was observed at the higher concentration of 10,000 µg/L. No significant selection was observed at any concentration tested for ERY. This matches the response of *intI1* observed to ERY in Chapter 3 (the LOEC defined here was 100,000 µg/L). No LOEC was determined for the combined antibiotics but significant selection to 90% confidence was observed at 10,000 µg/L. It was not possible, therefore, to determine whether AZ-CLA-ERY acts synergistically, additively or antagonistically on *intI1* LOEC as, currently, there was insufficient data.

An additive effect was observed, however, comparing average *intI1* prevalence in the presence of the individual compounds to the prevalence in the presence of the mixed antibiotics.

For *mphA*, a LOEC of 10,000 µg/L was determined for both CLA and ERY. A LOEC of 500 µg/L was determined for AZ, although as a concentration lower than this was not tested it was impossible to say with certainty that this was the lowest concentration at which selection would be observed. When the antibiotics were combined in a 1:1:1 ratio, the LOEC was 750 µg/L of total antibiotic (or 250 µg/L of each). This means the effect concentration for CLA and ERY has reduced in the mixture. Without testing lower concentrations of AZ it was impossible to determine whether the LOEC of AZ had also been reduced. One possible explanation for this reduction of LOEC in CLA and ERY was that the combined LOEC of 750 µg/L (defined by *mphA*) was being driven by AZ, as

this was more selective for *mphA* than CLA and ERY in isolation. Without testing pairs of antibiotic combinations, however, it was not possible to conclude whether an additive or synergistic effect occurred.

An additive effect was observed, however, comparing average *mphA* prevalence in the presence of the individual compounds to the prevalence in the presence of the mixed antibiotics.

Trimethoprim and sulfamethoxazole

Combining TRMP and SMX had a synergistic effect on the MSC of both antibiotics in comparison to when used in isolation (TRMP/SMX experiment 2). Approximately half the TRMP concentration was required to see the same response in *intI1* LOEC and MSC when in combination with SMX in comparison to the response when using TRMP on its own. The MSC of SMX when found in combination with TRMP was approximately $1/11^{th}$ and the LOEC was approximately $1/12^{th}$ of values determined in isolation.

When investigating the effect mixing TRMP and SMX had on prevalence a varied response was observed depending on the concentration. At the concentration defined as the LOEC for the mixing experiment, a synergistic effect was seen in comparison to the sum of the responses for the individual antibiotics. The response changed to an additive effect as concentration increases and to an antagonistic effect at even higher concentrations. This may be as a result of the complexity of class 1 integron ecology and genetics. For example, they may or may not carry a *sul1* gene in their backbone, the integrated gene cassettes will vary between integrons and host identity will vary.

Previous data suggests that, as a general rule, antibiotics act synergistically, in terms of bactericidal or bacteriostatic effect, when used in combination (Marx et al. 2015). Although whether this is true in terms of selection for resistance was previously unknown. Based on the preliminary data from the macrolide mixing experiment and the data from the TRMP-SMX mixing experiment, it would appear that the antibiotics tested here have an additive or synergistic effect on selective endpoints in combination which was particularly evident for the TRMP-SMX combination.

4.5.2 Comparison of individual and mixture selective endpoints with measured environmental concentrations

Macrolides

Limitations in experimental design (a limited concentration range was used) mean that no difference in LOEC (defined by *ermF*) could be reliably determined between single antibiotics and the mixture. The *ermF* prevalence data might suggest, however, that a synergistic effect is likely as the average prevalence was higher at each concentration in the mixture than the average for each antibiotic in isolation (Figure 39). From this preliminary data, therefore, it was clear that the macrolides have at least an additive (possibly synergistic) effect. As these antibiotics are often found in combination in environmental compartments, therefore, their predicted no effect concentration (PNEC) values should be adjusted accordingly. Table 6 shows the LOEC, NOEC and PNEC values for when ERY was used in isolation and in a 1:1:1 mixture combined with AZ and CLA and an additive effect was assumed. A NOEC of 250 µg/L (suggested by the verification experiment where a NOEC of 250 µg/L was

defined for ERY) was assumed as a “best case scenario” for the mixture experiment but this may actually be lower.

Table 6: Selective endpoints, NOECs and PNEC values for ERY defined by *ermF* when experiments are undertaken in isolation and with AZ and CLA in a 1:1:1 ratio. i = the values when the experiment was undertaken with the antibiotic in isolation. m = values when the experiment was undertaken when ERY was mixed with AZ and CLA in a 1:1:1 ratio. All values are in µg/L.

	LOEC		NOEC		PNEC	
	i	m	i	m	i	m
ERY	500	166.6	250	83.3	25	8.33

This PNEC value of ERY in the mixture experiment (8.33 µg/L) has been reduced by 3 times in comparison to the PNEC where ERY was used in isolation and is now in the same order of magnitude as the MEC_{max} of ERY (see Chapter 3 - 2.42 and 4 µg/L for ERY and ERY-H2O, respectively). The data presented here would suggest that, to mitigate the risk of development of resistance from the greater impact from the combination of antibiotics, measures may need to be taken to reduce concentrations of macrolides entering the aquatic environment. The limitations of this experiment means, however, the values for ERY in the mixture are speculative and may be significantly lower and even more of an environmental concern. Values for ERY in isolation have been confirmed by the verification experiment

Trimethoprim and sulfamethoxazole

Table 7 shows the MSCs, LOECs, NOECs and PNECs for TRMP and SMX when the experiment was undertaken with them in isolation (TRMP/SMX experiment 2) and when they were mixed.

Table 7: Selective endpoints, NOECs and PNEC values for TRMP and SMX defined by *intI1* when experiments are run in isolation and in combination with eachother in a 1:4 ratio. 1 = PNEC value derived from applying a 10-fold assessment factor to the MSC. 2 = PNEC value calculated when applying a 10-fold assesment factor to the NOEC. i = the values when the experiment was undertaken with the antibiotic in isolation. m = values when the experiment was undertaken with the antibiotics mixed in a 1:4 ratio. All values are in µg/L.

	MSC		PNEC[1]		LOEC		NOEC		PNEC[2]	
	i	m	i	m	i	m	i	m	i	m
TRMP	38	18.6	3.8	1.9	62.5	31.25	31.25	15.625	3.125	1.5625
SMX	863	74	86.3	7.4	1000	125	500	62.5	50	6.25

The MEC_{max} on the UBA database for TRMP is 27,680 µg/L found in untreated pharmaceutical wastewater (Umweltbundesamt 2016). This is significantly higher than all of the values shown in Table 7, but is also an unusually high environmental concentration. Removing the concentrations of residues found in pharmaceutical waste gives a more representative view of typical environmental concentrations so that the MEC_{max} was 95.1 µg/L found in hospital wastewater (Umweltbundesamt 2016). This is still higher than all of the values in Table 7

and means that environmental concentrations of TRMP are likely to select for resistance whether found in isolation or in combination with SMX.

For SMX, the MEC_{max} seen on the UBA database is 200,000 µg/L, again found in untreated pharmaceutical waste (Umweltbundesamt 2016). This concentration is, incidentally, the highest concentration of any antibiotic found on the database. Based on all of the selective endpoints determined here, this concentration is high enough to select for resistance in complex communities. It is again, however, significantly higher than typical concentrations found in the environment. Removing pharmaceutical waste from the UBA database the MEC_{max} is 81.1 µg/L in hospital wastewater (Umweltbundesamt 2016). This concentration is approximately 10 times lower than the LOEC and the MSC for the SMX experiment 2 and, therefore, selection for *intI1* in the environment if SMX is found in isolation would not be expected. This concentration is, however, between the LOEC and NOEC and is similar to that of the MSC of SMX when found in combination with TRMP. It is possible, therefore, that combining these two antibiotics in a 1:4 ratio means that current environmental concentrations of SMX are now a potential risk.

4.5.3 Previous work on selective endpoints for combinations

The only study investigating the selective endpoints of any combination of antibiotics is by Bengtsson-Palme and Larsson, 2016. This study used the clinical breakpoints from the EUCAST database and a mathematical approach to determine a $PNEC_R$ by an applying an assessment factor to breakpoints. The EUCAST database provides the clinical breakpoint of TRMP and SMX combination because it is a combination used in the clinic. It determined the breakpoint for a combination ratio of 1:19 (TRMP:SMX), deemed to be the

optimal serum ratio in terms of synergism between the two. The $PNEC_R$s determined by Bengtsson-Palme and Larsson, 2016 were also based on this ratio. In contrast, the work presented here used the environmental ratio of 1:4 (TRMP:SMX).

Bengtsson-Palme and Larsson, 2016 determined the $PNEC_R$s for TRMP and SMX in isolation to be 0.5 and 16 µg/L, respectively, which are 1-2 orders of magnitude lower than those generated in the current study. When they were combined in the 1:19 ratio, however, TRMP continues to have the same $PNEC_R$ of 0.5 µg/L whereas the $PNEC_R$ of SMX decreases to 9.5 µg/L. This suggests that TRMP was causing a synergistic effect on SMX, but no additive or synergistic effect was occurring to TRMP in the presence of SMX in contrast to the synergistic effect reported here.

The synergistic effect, in regards to the MSCs and LOECs, when these two compounds were combined in a 1:4 ratio (the environmental ratio (Straub 2013; Straub 2015)) was greater than the difference in $PNEC_R$s in the 1:19 ratio. The 1:19 or 1:20 ratio is used in the clinic as this is the ratio that produces the greatest synergistic effect. When investigating drug efficacy in humans and animals there are a host of additional factors to be taken into account, such as bioavailability, pharmacodynamics and pharmacokinetics (Queralt & Castells 1985; Schwartz & Rieder 1970; Günther et al. 1987) whereas in this work, however, the only relevant issue when combining the two antibiotics is the development of resistance.

4.5.4 Reproducibility of assay with different inoculum

For TRMP and SMX, two sets of experiments were run: the preliminary dataset run with the assistance of visiting students (TRMP/SMX experiment 1 - Section

4.4.2.1 and 4.4.2.2) and the compounds run in isolation as part of the mixing experiment (TRMP/SMX experiment 2 - Section 4.4.2.3). The preliminary data for determining MSCs for TRMP and SMX used different wastewater inoculum from each other and from the mixing experiment. It is interesting, therefore, to see how reproducible the assay is using the same compounds with differing microbial communities. In this case, two wastewater communities were collected from the same wastewater treatment plant but at a 2 year interval (TRMP experiment 1 in 2015 and TRMP experiment 2, SMX experiment 2 and TRMP-SMX experiment in 2017) and a third was collected from a different wastewater treatment plant (SMX experiment 1). The results obtained for the individual compounds as part of the mixing experiment align well to the previous preliminary data obtained.

For TRMP, in the mixing experiment the LOEC was 62.5 µg/L and the NOEC was 31.25 µg/L. In the preliminary experiment, the LOEC was 250 µg/L and the NOEC was 125 µg/L, although significant selection to 90% confidence was observed down to 62.5 µg/L. The MSCs, defined by selection coefficient graphs, were 23.9 µg/L and 37.9 µg/L for the preliminary data and the mixing experiment, respectively. These slightly different values were probably a result of variability in the replicates which may have skewed each dataset slightly differently and because the lines of best fit are different for the different datasets. These two MSC values were in the same order of magnitude as each other.

For SMX, slightly different concentration regimes were used for each experiment. For the experiment undertaken as preliminary work, concentrations were increased by 10-fold increments. This meant there was a 10-fold

difference between the LOEC (2,000 µg/L) and the NOEC (200 µg/L). In the individual compound data from the mixture experiment, however, the concentration was increase by 2-fold at each increment. The LOEC and NOEC from this experiment (1,000 and 500 µg/L, respectively) were in between the LOEC and NOEC of the preliminary experiment. The MSC for these two experiments were, as with TRMP, in the same order of magnitude as each other (786.24 µg/L and 862.5 µg/L for the preliminary and the mixture experiment, respectively). The similarities between the data obtained from the preliminary work and the subsequent work on the same compounds suggests that the assay is reasonably robust generating similar results with different inocula.

For the macrolide data, however, a decrease in MSC was observed with the inoculum used in the experiment presented in this chapter (Section 4.4.1) in comparison to the work undertaken in Chapter 3. The verification experiment was run to ensure that the LOEC of ERY did not decrease below 500 µg/L. Although the LOEC did not decrease below 500 µg/L, a biological effect was observed at lower concentrations of ERY. Significance to 90% confidence was seen at 100 µg/L, although there was no significant difference between the no antibiotic control and 250 µg/L. For *intI1,* previous work in Chapter 3 was not able to define a LOEC for AZ, although significance to 90% confidence was observed at 1,000, 10,000 and 100,000 µg/L. In work presented in this chapter, significant positive selection was observed at 500 µg/L of AZ but as no concentration below this was tested it is not possible to confirm that significant selection has reduced more than 2-fold.

4.5.5 Future work

Macrolides

As a result of a limited concentration range being tested for the macrolide antibiotics, it was not possible to define how combining the macrolides affects the LOEC/MSC. Further investigations into how resistance genes are affected at a more extensive range of concentrations could be important in understanding how these antibiotics behave in a mixture.

The presence of AZ might be having a stronger contributory effect than CLA and ERY. The LOEC for AZ was 500 µg/L (although no concentration was tested lower than this) and for CLA and ERY was 10,000 µg/L when the compounds were used in isolation. When they were used in combination, however, the LOEC of each was 250 µg/L (750 µg/L of total antibiotic was required to see a significant difference). It would, therefore, be interesting to determine the roles of each of the antibiotics through further investigations. Combining two antibiotics at a time in a 1:1 ratio may help to understand the effect each has on each other. Whilst it is possible to speculate that AZ causes a strong synergistic effect on CLA and ERY it, as it has been shown to be more potent than ERY (Jelić & Antolović 2016), this cannot be verified without investigating two compounds at a time.

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Three replicates from each concentration in the TRMP, SMX and TRMP-SMX mixing experiment have been sent for metagenome analysis. This includes samples from where TRMP and SMX were run in isolation as well as samples from when they were combined. Unfortunately, at the time of writing, the return of sequencing from Exeter Sequencing Service is still awaited and cannot, therefore, be presented here. The samples have been sent for sequencing as the only gene targeted in this experiment was *intI1.* No antibiotic resistance

genes specific to either class of TRMP or SMX were targeted using qPCR. The sequencing would be able to show what is occurring to individual class specific resistance genes and, in addition, compare the effect of mixing on these genes. As antibiotic resistance genes to these two compounds are regularly associated with *intI1* (Moura et al. 2009), the 3 replicates (of 5 run in the mixing selection experiment) with the highest *intI1* prevalence were sent for sequencing. It will also be interesting to determine how co-selection for other antibiotic classes is affected by the combination of the two antibiotics, as *intI1* is known to be associated with many different resistance genes of many classes (Gillings et al. 2015).

4.6 Conclusion

The mixtures tested demonstrated additive or synergistic effects on selective endpoints with reduced PNECs for individual antibiotics in mixtures. In general, an additive or synergistic effect on prevalence was also observed, although this became antagonistic with increasing concentrations of TRMP and SMX. The combination of antibiotics tested here was relatively simple in comparison to mixtures of pharmaceuticals found in the environment. This is important preliminary work that shows combinations of antibiotics in hotspots, such as WWTPs, need to be considered when determining risk of the development of resistance. It is a complex task to determine the effect that different combinations within the environment could have on selection for antibiotic resistance: there is an almost infinite number of possible combinations of different antibiotics interacting; of the ratios of those antibiotics involved in that interaction and of the concentrations of antibiotics that may be involved. In addition, antibiotic degradation products, other APIs and chemical compounds such as biocides and heavy metals may also interact with antibiotics and

change the effect they have on selection for resistance in the environment. Nevertheless, the work that has been undertaken is an important preliminary step to understanding the role interactions between compounds have in driving the selection for antibiotic resistance.

Chapter 5: The effect of temperature on selective endpoints of antibiotics

Author contributions

The author, under the supervision of named supervisors, undertook the growth rate experiments, the 24 hour sampling experiment to obtain samples for chemical degradation analysis and the selection experiment and subsequent analysis. Felicity Elder, University of Bath, analysed the degradation of AZ from the 24 hour experiment using mass spectrometry.

5.1 Abstract

A recent study investigated whether the difference in minimum temperatures across the United States correlated with an increase in the clinical rates of antimicrobial resistance seen. That study determined that a difference of 10 °C in those minimum temperatures resulted in significant increases in resistance in three common clinical pathogens. Whilst this study showed a correlation between the temperature increase and resistance, no causal link was determined. The aim of the work reported in this chapter was, therefore, to determine how an increase in temperature affected the selective endpoints of azithromycin (AZ).

Selection experiments were undertaken at 37, 28 and 20 °C. Isosensitest broth was inoculated with raw wastewater influent and passaged over a 7 (37 and 28 °C experiments) or 14 (20 °C experiment) day period with appropriate concentrations of AZ. QPCR targeted *ermF, mphA* and *intI1* to investigate the selective effect of AZ at different temperatures.

For all three genes targeted, the selectivity of AZ decreased with decreasing temperature and, therefore, an increase in AZ concentration needed for selection of the three genes was observed.

The data reported in this chapter represents a preliminary study into the effect of temperature on selective endpoints. More work needs to be undertaken to determine whether the selective potential of AZ is affected at different temperatures or whether there are other macrolide resistance genes, associated with different taxa, that were not targeted as part of this work. Metagenome analysis should be undertaken to determine candidate genes which would best evaluate whether the overall selective effect of AZ changes at 20 and 28 °C as the qPCR targets tested in this study were chosen based on selection by AZ at 37 °C.

5.2 Introduction

Climate change is defined as "*change in global or regional climate patterns"* (Lineman et al. 2015) and recent trends show that anthropogenic activities are implicated in current warming (IPCC 2018).

The Intergovernmental Panel on Climate Change (IPCC) stated in their 2018 report that since the industrial revolution, anthropogenic activities have already been responsible for an increase of 1 °C of global temperature. If this trend continues at its current rate (approximately 0.2 °C a decade), the critical value of an increase of 1.5 °C will be reached between 2030 and 2052 (IPCC 2018).

In the 2011 Annual report of the Chief Medical Officer, Dame Sally Davies stated that the threat of antimicrobial resistance is "*arguably as important as climate change for the world"* (Davies 2013). There is very limited data on the link between rates of AMR and anthropogenic climate change and, until 2018,

no direct link had been identified. A 2010 paper suggested that increasing natural disasters caused by climate change might lead to an increase in unsanitary conditions and hence, an increase in infection rate. Treating infections would then increase antibiotic use and subsequently exert an increase in selective pressure for antibiotic resistant bacteria to emerge. This study, therefore, showed that climate change would indirectly increase antibiotic resistance levels (Gould 2010).

A study published in 2018, however, linked an increase in local temperature and increasing antibiotic resistance rates (MacFadden et al. 2018). This study investigated whether differences in local temperature across the United States affected rates of antibiotic resistance in the bacterial pathogens *E. coli, K. pneumoniae* and *S. aureus*. By using patient information from 2013 to 2015, the authors determined that an increase of 10 °C of minimum local temperature was associated with an increase in resistance rates of 4.2%, 2.2% and 2.7%, respectively, for the pathogens mentioned above. The relationship of resistance in *E. coli* and minimum temperature across the United States can be seen in Figure 57.

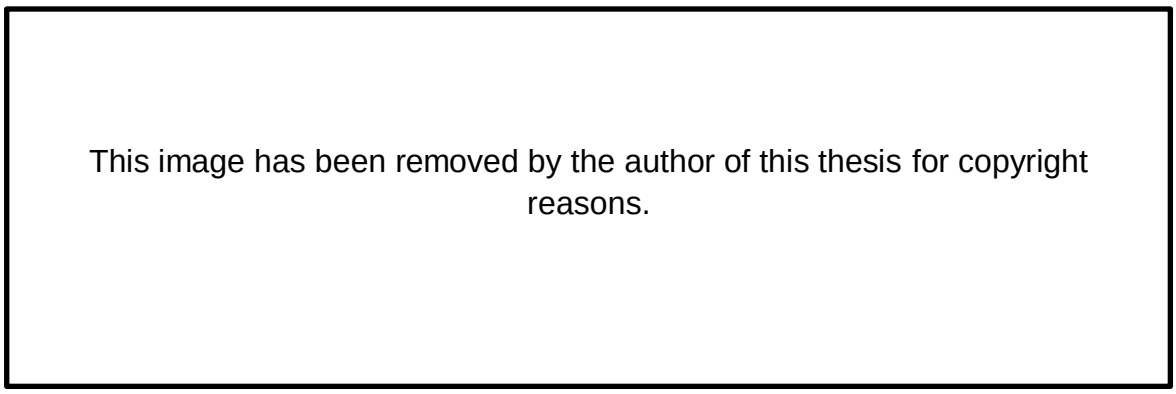


Figure 57: Graph reproduced from MacFadden *et al.*, 2018. A: Normalised prevalence of resistant *E. coli* in the United States. B: Minimum temperatures in the United States. A correlation is observed between temperature and prevalence of resistant *E. coli* across the United States.

The authors of the 2018 study put forward a number of hypotheses for why this correlation was observed. First, it was suggested that increasing temperature may increase the rate of horizontal gene transfer (HGT), and, therefore, facilitate the spread of resistance genes through bacterial populations. Secondly, the authors suggested that the correlation could be a result of the fact that bacterial growth rate is highly dependent on temperature: an increase in temperature, and hence growth rate, may result in increased carriage of pathogens in and increased transfer events between human and animal hosts. Finally, it was suggested that temperature may enhance the growth rates of resistant bacteria residing in the environment which may, subsequently, lead to an increase in transmission of these strains from environmental sources. The authors did note, however, that their study only shows a correlation between antibiotic resistant bacteria and local temperature and did not prove any causal link between the two. They suggested that further experimental work would

need to be undertaken to determine whether a causal link can be shown (MacFadden et al. 2018).

To date, no work has investigated the effect of temperature on selective endpoints of antibiotics. Limited work has, however, been undertaken on the effect of temperature on minimum inhibitory concentration (MIC). One study, published in 1985, investigated the effect of incubation temperature on the MIC of a number of antibiotics in three clinical bacterial pathogens. No effect on MIC was observed for any antibiotic tested with *Enterobacteriaceae* or *P. aeruginosa.* A difference was seen, however, with *Pseudomonas maltophilia* (later reclassified as *Strenotrophomonas maltophilia* (Conly 1996)) where antibiotics either had lower MICs at a higher temperature (37 °C) in comparison to the lower temperature (30 °C) or no difference between the two was observed (Wheat et al. 1985). This is, however, only one clinical strain and it cannot, therefore, be assumed that the selective endpoints of any particular antibiotic will behave in the same way (decreasing with increasing temperature) when investigating this in a diverse bacterial population.

The aim of the work presented here was, therefore, to determine whether increasing temperatures have an effect on the minimal selective concentrations (MSCs)/lowest observed effect concentrations (LOECs) of an antibiotic. For this experiment, azithromycin (AZ) was used as the study compound and three different temperatures were used. This would show whether a correlation exists between temperature and selective endpoints. In addition, investigating whether a relationship could be seen between different temperatures could show how selective endpoints are affected in different environmental and non-environmental compartments that have different temperatures.

5.3 Methods

5.3.1 Antibiotics

AZ (Sigma-aldrich) was the chosen antibiotic for this experiment and was dissolved in ethanol (Acros Organics).

5.3.2 Temperatures

Three temperatures were chosen to undertake this series of experiments. These were 37, 28 and 20 °C. The temperature 37 °C was chosen as a comparison for the experiments undertaken in Chapter 3 and 4. 20 °C is the current guideline temperature used in the OEDC guidelines for the activated sludge respiration inhibition test (ASRIT) for ecotoxicology guidelines (OECD 2010). Finally, 28 °C was chosen as an intermediate in order to see whether a trend would be able to be observed with change in temperature.

5.3.3 Growth rate experiment

Growth rate experiments were undertaken to determine the doubling time of the mixed community bacteria. This enabled a passage period to be determined.

Growth rate plates were set up with 6 replicates of 200 µl of iso-sensitest broth (Oxoid) and inoculated with washed wastewater at 10% v/v and 6 replicates of a broth control.

For 37 °C and 28 °C treatments, plates were placed in the BioTek Synergy 2 plate reader. These were set to the appropriate temperature, set to read optical density (OD) at 600 nm and to shake at a medium speed. The 37 °C plate was read every hour for 24 hours and the 28 °C plate was read every hour for a total of 48 hours.

As the plate reader was unable to hold a stable temperature until it reached 5 °C above room temperature, and as the room temperature of the laboratory used fluctuated from 18 °C to 23 °C, the 20 °C plate was placed in an incubator at 140 rpm and regular readings were taken. In order to capture the exponential phase, readings were taken, using the BioTek Synergy 2 plate reader, at 600 nm every hour from hour 14 to 23 in addition to obtaining an initial and final OD.

Growth rate was calculated using the equation: R = (LN[OD2/OD1])/(T2-T1). Two timepoints were chosen in the exponential phase (T1 and T2) and the corresponding OD values (OD1 and OD2, respectively) were used in this calculation.

5.3.4 Chemical degradation of AZ

As antibiotics degrade over time, it was important to determine whether temperature affects the speed at which this occurs. Further, to ensure that results determined from selection experiments are comparable, it is important that the bacterial community is exposed to the same concentration of antibiotic.

A 24 hour experiment was conducted to investigate the extent of degradation at the different experimental temperatures. Iso-sensitest broth was inoculated with 10% v/v washed raw wastewater and with 10,000 µg/L of AZ. Five replicates of broth, wastewater and antibiotics were left shaking at 180 rpm at each temperature (20, 28 and 37 °C). In addition, 5 replicates of just iso-sensitest broth and AZ were run at 37 °C to test if bacterial resistance elements, such as enzymes, were degrading the AZ over time or if the degradation of AZ was happening irrespective of the presence of bacteria.

AZ was chosen as the antibiotic for this experiment as it was studied extensively in the work presented in Chapter 3. It is also known to be more

stable than the original macrolide antibiotic, erythromycin (although this is based on pH stability) (Lode et al. 1996) and for its low removal rates during the wastewater treatment plant (WWTP) process (Čizmić et al. 2019). It was hoped, therefore, that minimal degradation would occur and no additional measures would need to be undertaken in the selection experiment set-up to account for degradation.

Sampling occurred at the beginning of the experiment and then at every hour up until 6 hours. After 6 hours, samples were taken every 3 hours until 24 hours. 50 µl of sample was added to 400 µl of methanol (Fisher) and 50 µl of an internal standard (LGC Standards). Methanol was used to kill bacteria and dilute the broth so that this did not block the chemical analysis machinery. An internal standard was used at a set concentration to ensure that, if degradation occurred during storage or transportation, a calculation could be undertaken to ensure an accurate measurement of AZ.

Samples were stored at -80 °C for 24 hours and were then centrifuged at 16,200 xg for 10 minutes to pellet the bacteria. The supernatant was removed and placed in polypropylene LCMS vials (Waters). This was then stored at -80 °C (or on ice packs when travelling) until the analysis was undertaken.

The chemical analysis was undertaken by Felicity Elder, a PhD student based at the University of Bath on three of the five replicates at hour 0 and 24. Mass spectrometry was used, as per the methods in Lopardo, Rydevik and Kasprzyk-Hordern, 2019, to determine the degradation rate of AZ over the 24 hour period.

5.3.5 Selection experiment

The concentration range of AZ was the same as that used in the final selection experiments in Chapter 3. These were 0, 100, 250, 500, 750, 1,000, 10,000 and 100,000 µg/L.

5.3.6 qPCR targets

The qPCR targets used here were the *ermF*, *mphA*, *intI1* and 16S rRNA genes. These targets were chosen based on the results presented in Chapter 3, as these genes were positively selected for at 37 °C.

5.4 Results

5.4.1 Growth rate of a mixed community at three temperatures

To ensure that the experiments were comparable, it was necessary to determine that the same number of generations of bacteria was exposed to the antibiotic before passage. Growth rate experiments were, therefore, undertaken at three temperatures to determine the rate of growth during the exponential phase.

The rate of growth is shown in Table 8, along with the passage period chosen for the selection experiment.

Table 8: Results from growth rate experiments. Shown here is the rate at which the bacterial community grew at a specific temperature as well as the passage period determined for subsequent selection experiments.

Temperature	Rate	Passage period
37 °C	0.145302	24 hours
28 °C	0.12359	24 hours
20 °C	0.063118	48 hours

Growth at 28 °C was shown to be 1.18 times slower than that at 37 °C. Stationary phase, however, was reached before 24 hours (as with 37 °C) and passage would occur, therefore, at the same time as the 37 °C experiment.

Growth rate at 20 °C was shown to be approximately 2.3 times slower than at 37 °C. Stationary phase was reached by 48 hours and reached a similar OD as the other two temperatures. There will have been, therefore, a similar number of bacterial generations by 48 hours at 20 °C as with the other two temperatures at 24 hours. Passaging of this experiment was, therefore, undertaken every other day for 7 passages (14 days).

Growth rate curves can be seen for each temperature in Figures 97, 98 and 99 (Page 331 - Appendix).

5.4.2 Chemical degradation of AZ at three temperatures

No degradation of AZ was observed in the presence of bacteria at any of the three temperatures over a 24 hour period. In addition, no difference was observed in the absence of the bacterial community at 37 °C. As there was no change, intermediate timepoints were not analysed. Data showing the concentration at the beginning and end of the 24 hour degradation experiment can be seen in Figure 58.

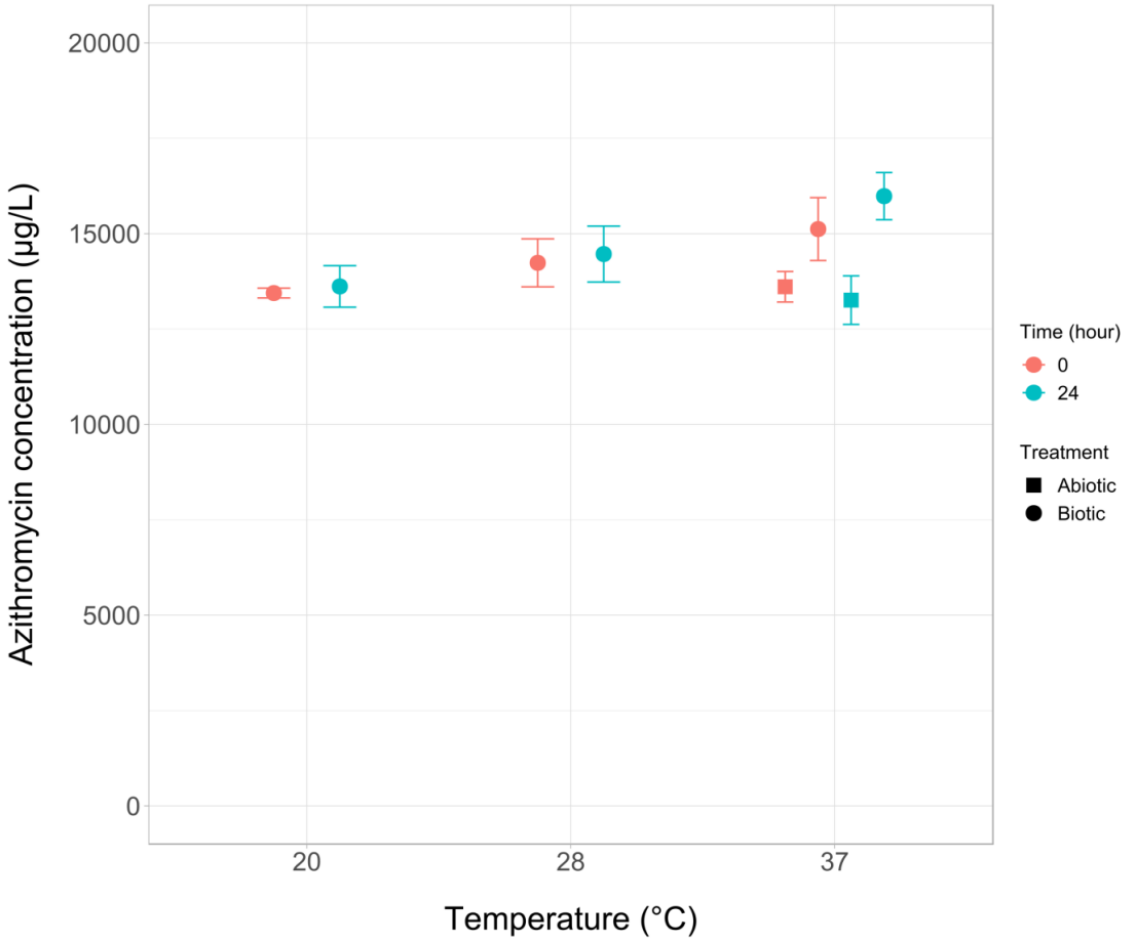


Figure 58: Degradation of AZ over a 24 hour period at 3 temperatures. No degradation of AZ was observed at any of the temperatures tested (20, 28 and 37 °C). Presence or absence of bacteria did not affect the degradation. Three of five replicates were analysed and are presented here. Standard error is represented by the error bars.

5.4.3 The effect of temperature on the selective endpoints of AZ

ermF

Figure 59A, B and C shows *ermF* as a function of AZ at 37, 28 and 20 °C, respectively. No LOEC was determined for *ermF* selection by AZ at 37 °C although significant selection to 90% confidence was observed at 10,000 µg/L (p = 0.0720, Dunn's test). A significant increase in prevalence was observed in

comparison to the no antibiotic control at 100, 250, 500, 750 and 1,000 µg/L (p = 0.035, 0.0142, 0.0107, 0.0027, 0.0442, respectively, Dunn's test) although none of these concentrations elevated the prevalence of *ermF* above the initial starting prevalence. At 20 and 28 °C, no significant positive selection was seen to occur at any concentration of AZ used. For 20 °C, however, a significant increase in prevalence, in comparison to the no antibiotic control, was observed to 95% confidence at 750 and 10,000 µg/L (p = 0.0371 and 0.0152, respectively, Dunn's test) and to 90% confidence at 1,000 µg/L (p = 0.0971, Dunn's test).

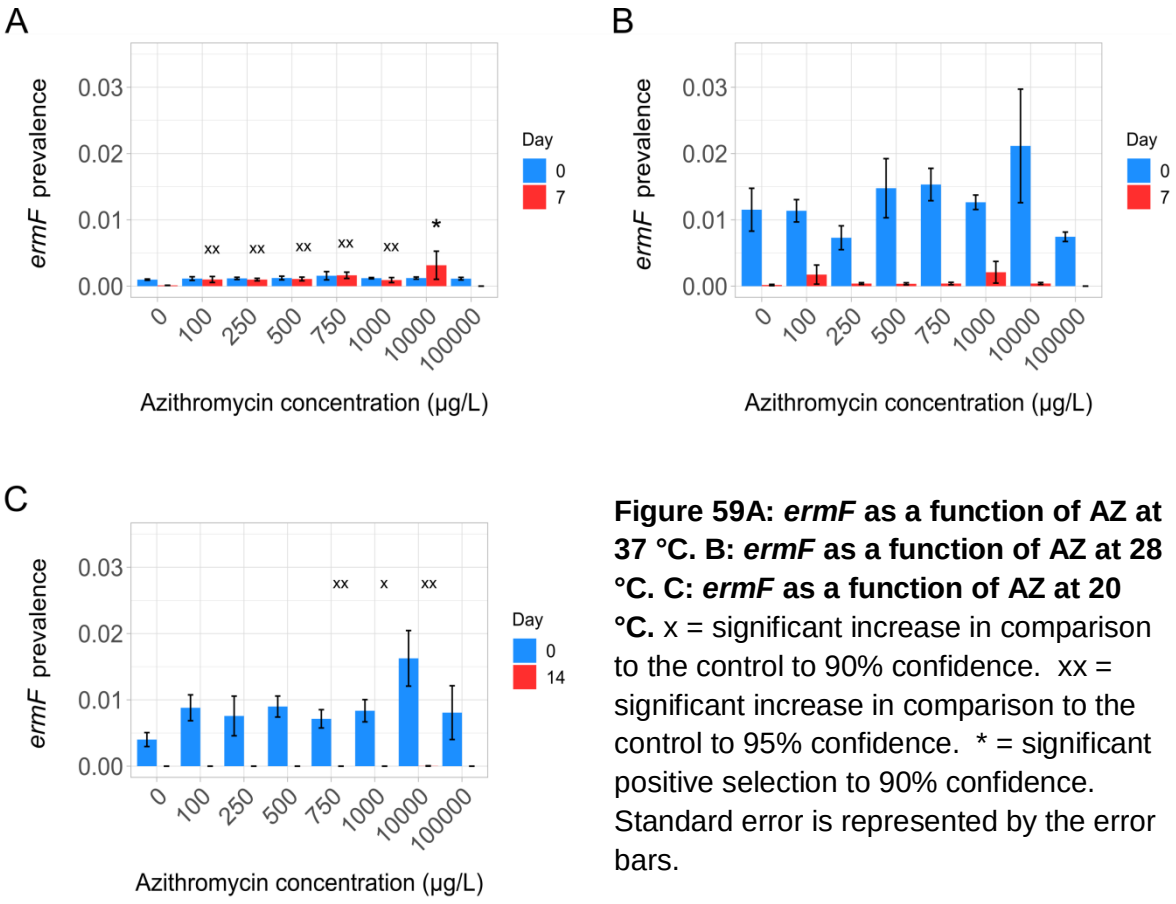


Figure 59A: *ermF* as a function of AZ at 37 °C. B: *ermF* as a function of AZ at 28 °C. C: *ermF* as a function of AZ at 20 °C. x = significant increase in comparison to the control to 90% confidence. xx = significant increase in comparison to the control to 95% confidence. * = significant positive selection to 90% confidence. Standard error is represented by the error bars.

mphA

Figure 60A, B and C shows the selection of *mphA* by AZ at 37, 28 and 20 °C, respectively. At 37 °C a LOEC of 750 µg/L of AZ was determined (p = 0.0212, Dunn's test). Significant selection was also observed at every subsequent concentration tested. Running the experiment at 28 °C increased the LOEC to 1,000 µg/L (p = 0.0174, Dunn's test). A significant increase in prevalence to 90% confidence in comparison to the no antibiotic control was, however, observed at 750 µg/L (p = 0.0758, Dunn's test) although this did not increase over the initial prevalence of *mphA* at day 0. A LOEC of 10,000 µg/L was observed when the experiment was undertaken at 20 °C (p = 0.0003, Dunn's test). Prevalence of *mphA* at day 14 at concentrations of 500, 750 and 1,000 µg/L was significantly higher than the prevalence of *mphA* in the no antibiotic control (p = 0.0371, 0.0291 and 0.008, respectively, Dunn's test), as with the 28 °C experiment. This was not, however, significantly higher than the starting prevalence of *mphA.*

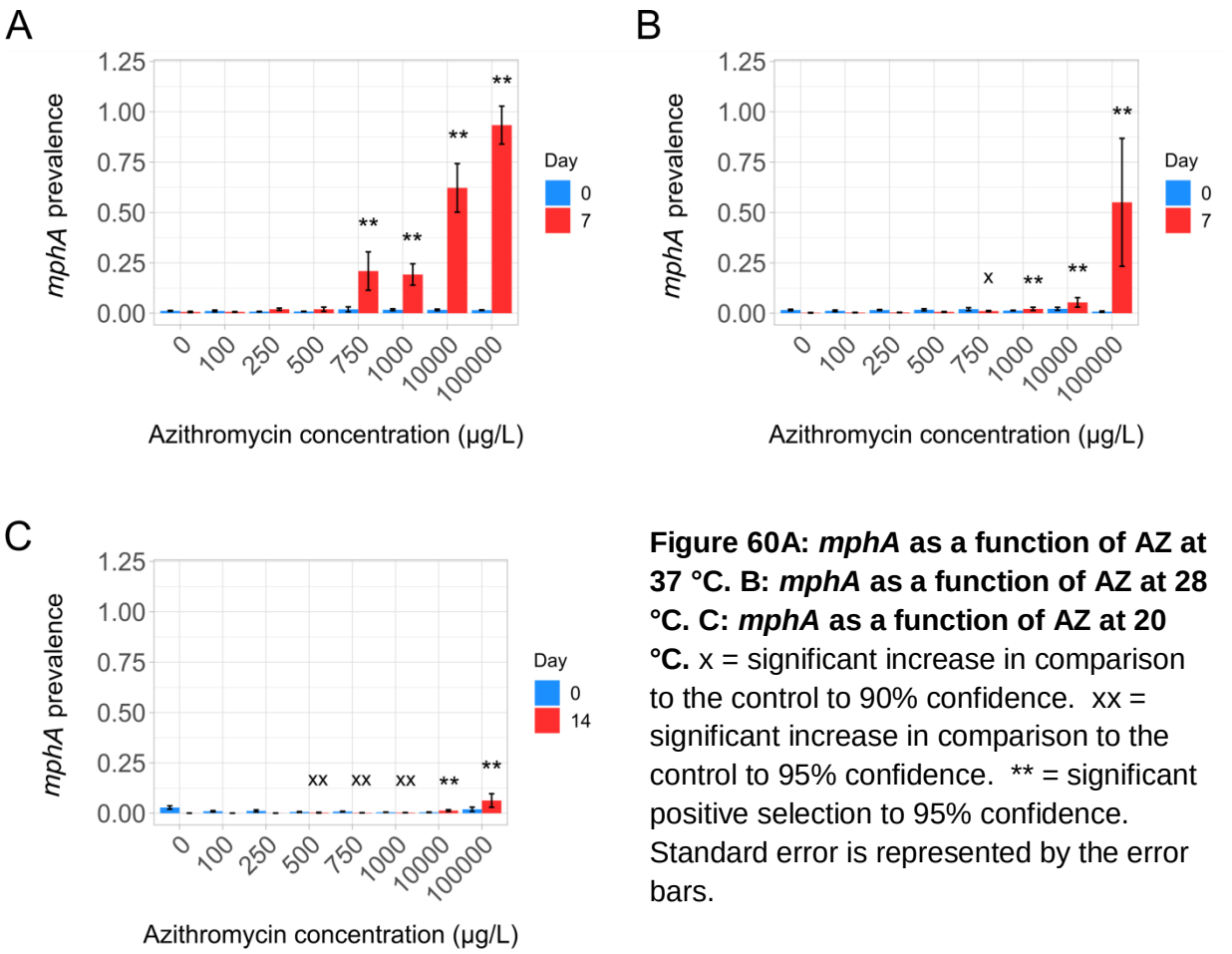


Figure 60A: *mphA* as a function of AZ at 37 °C. B: *mphA* as a function of AZ at 28 °C. C: *mphA* as a function of AZ at 20 °C. x = significant increase in comparison to the control to 90% confidence. xx = significant increase in comparison to the control to 95% confidence. ** = significant positive selection to 95% confidence. Standard error is represented by the error bars.

When only data from day 7 (28 and 37 °C experiments) or day 14 (20 °C experiment) from a limited range of concentrations are plotted, the correlation between the significant increase over the no antibiotic control at 20 and 28 °C and the positive selection observed for *mphA* observed at 37 °C is more evident. This can be seen in Figure 100 (Page 333 - Appendix).

intI1

Figure 61A, B and C shows *intI1* selection by AZ at 37, 28 and 20 °C, respectively. At 37 °C, a LOEC of 750 µg/L was determined (p = 0.0274, Dunn's test). Significant selection was also observed at the higher concentrations of 1,000, 10,000 and 100,000 µg/L (p = 0.0186, 0.0008 and 0.0417, respectively, Dunn's test). At 28 °C, no significant selection was observed until 100,000 µg/L

(p = 0.0394, Dunn's test) and this, therefore, was defined as the LOEC. A LOEC of 100,000 µg/L of AZ was also determined for the experiment run at 20 °C (p = 0.0350, Dunn's test).

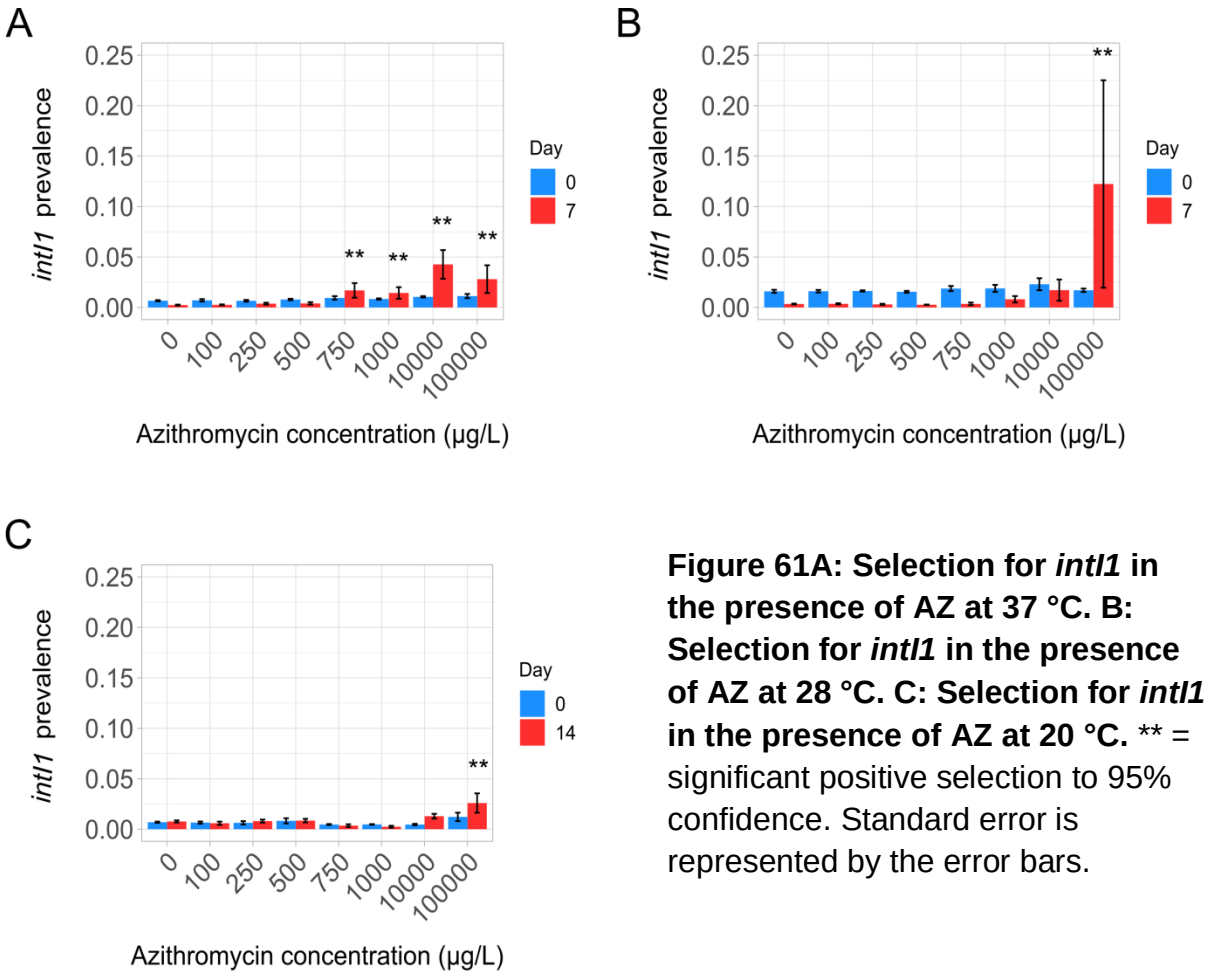


Figure 61A: Selection for *intI1* in the presence of AZ at 37 °C. B: Selection for *intI1* in the presence of AZ at 28 °C. C: Selection for *intI1* in the presence of AZ at 20 °C. ** = significant positive selection to 95% confidence. Standard error is represented by the error bars.

5.5 Discussion

5.5.1 Stability of AZ at three different temperatures

The concentration of AZ did not decrease in any of the treatments undertaken. Temperature did not affect the degradation rate of AZ in this system as no degradation was observed at all over the 24 hour period. Furthermore, the presence or absence of a microbial community did not affect degradation rate.

AZ is synthesised as a semi-synthetic derivative of erythromycin with greater stability than the parent compound, although this is in regards to stability in acidic conditions and not temperature effect (Lode et al. 1996). Furthermore, AZ is known for its low removal rates in WWTPs (Čizmić et al. 2019). It could, therefore, be expected that AZ would be extremely stable with minimal degradation over a 24 hour period regardless of the temperature it was exposed to.

There are many genes that confer macrolide resistance. A number of these resistance mechanisms work by enzymatically modifying the compound (Golkar et al. 2018) and it would be expected, therefore, that more degradation would be observed in the samples with bacteria present in comparison to the abiotic control. This, however, proved not to be the case. It could be assumed, therefore, that other macrolide resistance mechanisms, such as efflux pumps (Roberts 2008) or base substitution in the 23S rRNA subunit of the ribosome (Vester & Douthwaite 2001), play a larger role in phenotypic resistance in this system than resistance genes that enzymatically degrade AZ.

5.5.2 Effect of temperature on selective endpoints

When investigating the selection of *ermF, mphA* and *intI1* by AZ there appeared to be a trend with all three of these genes: that the selective ability of AZ decreased with decreasing temperature. The selective endpoint for 20 and 28 °C, therefore, was higher than that at 37 °C in regards to all genes tested. This, therefore, appears to be consistent with the data presented in MacFadden *et al.*, 2018 which determined that an increase in clinical resistance was observed with higher local temperatures within the United States (MacFadden et al. 2018). It also is consistent with another study that observed a decrease in MIC

of various antibiotics to *Pseudomonas maltophilia* with increasing temperature to 37 °C from 30 °C (Wheat et al. 1985).

These results suggest, therefore, that the assay used in the experiments presented here and in Chapters 3 and 4 may be most protective in terms of selective endpoints for bacteria that are likely to be human associated being favoured by growth at 37 °C. However, only 2 macrolide resistance genes and *intI1* were targeted by qPCR in the experiment reported here. There are many other genes that confer macrolide resistance that were not targeted (Roberts 2008). Previous data has shown that bacterial community structure is significantly affected by temperature. For example, one study determined that temperature had a significant effect on bacterial diversity from biomat, soil and sediment samples obtained from geothermal sites in New Zealand and Canada. The highest bacterial diversity was observed at 24 °C with decreasing diversity observed with both an increase and decrease in temperature (Sharp et al. 2014). A difference in the bacterial species dominating in the experiments run at different temperatures may mean that different resistance genes are harboured in these communities at 20 and 28 °C in comparison to those in the 37 °C experiment. Whilst *mphA, ermF* and *intI1* were not selected for at similar concentrations in the lower temperature experiments, other resistance genes may be.

5.5.3 An inoculum effect on selective endpoints

As with the macrolide work undertaken in Chapter 4, an inoculum effect was observed when using the Falmouth/Penryn 2017 wastewater here in comparison to the Falmouth/Penryn 2015 wastewater used in Chapter 3. For the *mphA* and *intI1* data presented in this chapter, LOECs were determined to

be 750 µg/L of AZ at 37 °C, whereas a LOEC of 1,000 µg/L was observed for *mphA* in Chapter 3 and although no LOEC was determined for *intI1,* significant selection to 90% confidence was observed at 1,000 µg/L. The LOECs run as part of the experiments presented here, therefore, have reduced by a quarter, but were still significantly higher than environmental concentrations.

For *ermF,* a lower initial prevalence was observed in the 37 °C experiment in comparison to the 20 and 28 °C experiments, although steps were undertaken when setting up the selection experiment to ensure that the starting inoculum for all three experiments was homogenised. No LOEC was able to be determined and significant positive selection was only observed to 90% confidence at 10,000 µg/L. This was 10-fold higher than the LOEC determined in Chapter 3 for the selection of *ermF* by AZ. The lower starting prevalence of *ermF* in the 37 °C experiment might explain the higher concentration needed to see positive selection. A low starting prevalence of the bacterial species harbouring these genes may mean that they were outcompeted by other species carrying different resistance genes during the experimental period.

5.5.4. Sub-MSC trends

When changes in *mphA* prevalence at the end of the selection experiment, relative to the no antibiotic control, were examined in more detail (Figure 100, Page 333 - Appendix), significant changes in prevalence were observed at 20 and 28 °C at some of the same concentrations at which positive selection was observed at 37 °C. For example, significant positive selection was observed at 750, 1,000, 10,000 and 100,000 µg/L of AZ at 37 °C. At 28 °C, significant positive selection was first observed at 1,000 µg/L, but a significant increase in prevalence, in comparison to the no antibiotic control, was seen at 750 µg/L.

This was not higher than the starting prevalence. Similarly, at 20 °C, significant positive selection was observed at 10,000 and 100,000 µg/L of AZ, but a significant increase in prevalence, in comparison to the no antibiotic control, was observed at 500, 750 and 1,000 µg/L. Again, this was not higher than the starting prevalence.

This suggests that some form of selection for *mphA* at 20 and 28 °C was occurring at concentrations similar to where positive selection was observed at 37 °C but at a reduced level and not above the starting prevalence of *mphA*. This may be as a result of competition with other resistant species which may carry different resistance mechanisms that survive better at the different temperatures at which the experiments were undertaken.

5.5.5 Future work

In order to further understand the effect of temperature on selective endpoints the next steps undertaken should be to use metagenome analysis on the DNA from the selection experiment presented above. From this, it will be possible to identify additional candidate gene targets for qPCR that are enriched at different temperatures in the presence of AZ. The qPCR targets chosen so far and presented here were based on results from the experiments run at 37 °C that are presented in Chapter 3. As different bacterial species are able to survive under different conditions (such as different temperatures) it follows that different resistance genes may be harboured in these different species of bacteria. By undertaking metagenome analysis it will be possible to determine overall MLS resistance as a function of the AZ concentrations and to determine changes in species diversity with temperature (as was undertaken in Chapter 3, Section 3.4.4.4). It will then be possible to identify the specific gene or genes

responsible for this response at 20 and 28 °C. Only then will it be possible to determine how temperature effects the selection for total resistance elements of a bacterial community.

5.6 Conclusion

Although preliminary data appears to determine that AZ becomes less selective with decreasing temperature, more work needs to be undertaken to investigate the effect AZ has on other macrolide resistance genes at the three temperatures tested. Examination of differential rates of negative selection illustrated an increase in several gene targets at 20 and 28 °C, relative to the no antibiotic control, at the same AZ concentration as observed LOECs for positive selection at 37 °C. This also raises questions relating to LOEC/MSCs being the most appropriate endpoint for environmental protection. This is discussed further in Chapter 6.

Chapter 6: A comparison of methods for determining selective endpoints for antibiotic resistance

Author contribution

The author undertook all experiments in this chapter, under the supervision of named supervisors. Samples for metagenome sequencing were sent to Exeter Sequencing Service where library preparation and sequencing was undertaken. Raw sequence reads had adaptor sequences removed by Exeter Sequencing Service. All subsequence analysis was undertaken by the author.

6.1 Abstract

A number of studies have investigated the selective effects of low concentrations of antibiotics. Two of these studies investigated the selective effect of tetracycline (Lundström et al. 2016) and of ciprofloxacin (Kraupner et al. 2018) in a complex microbial community and used experimental methods attempting to replicate environmental conditions, such as low temperature and low nutrient content. The aim of the research reported in this chapter was, therefore, to investigate whether the high temperature / nutrient method used here was comparable to the more environmentally relevant, but less controlled, methods of previously published studies.

A selection experiment using a range of tetracycline concentrations was undertaken to compare selection experiments presented in this thesis to the biofilm system used in Lundström et al. 2016. When analysing the data using just *tetG* prevalence at day 7, a significant difference, compared to the no antibiotic control, was seen at 1, 10 and 100 µg/L of tetracycline. This correlates with the Lundström et al. 2016 data. When investigating the data using both day

0 and day 7, however, as undertaken in investigations reported in this thesis, a decrease in prevalence of *tetG* was observed at every concentration tested. Instead of positive selection occurring, a decrease in negative selection or an increase in persistence of *tetG* was observed to be occurring at 1, 10 and 100 µg/L of tetracycline.

As a result of this lack of positive selection, a larger selection experiment was carried out to determine a minimal selective concentration (MSC) and lowest observable effect concentration (LOEC) of tetracycline. The LOEC determined by *intI1* was 2,000 µg/L and the MSC was approximately 651 µg/L. Metagenome analysis showed an increase in total TET resistance genes at 125 µg/L and at every higher concentration tested. No samples from lower concentrations were sent for sequencing, so it is conceivable, therefore, that a significant increase over the control is also occurred at lower concentrations. Metagenome data was also used to identify significant increases for individual TET resistance genes not targeted by qPCR. Further analysis of these genes will need to be undertaken to establish an accurate LOEC and MSC for TET.

Although the phenomenon of increased persistence is less of an environmental concern than if positive selection occurs, it does increase the total number of resistant bacteria found in the environment relative to no antibiotics being present. The potential for horizontal gene transfer and the risk of human exposure to these genes are, therefore, also increased. A new selective term is proposed – minimal increased persistence concentration (MIPC).

6.2 Introduction

A minimal selective concentration (MSC) is defined as the lowest concentration of an antibiotic or co-selective agent *"where the resistant mutant is enriched*

over the susceptible strain" (Andersson & Hughes 2011). In other words, the concentration at which positive selection for the resistant strain or resistance gene is observed.

There are a small number of studies, described briefly in Chapter 1, which used a variety of methods to investigate antibiotic MSCs. This introduction sets out a detailed description of currently published methods and a discussion of the limitations surrounding each assay used to determine selective endpoints. The rationale behind these studies was to investigate whether positive selection occurs in the environment, at environmental concentrations of antibiotics, and whether protective measures need to be implemented to reduce current measured environmental concentrations (MECs) and, in turn, reduce selection for antibiotic resistance.

The first two studies to investigate the potential selective effects of sub-minimum inhibitory concentrations (MICs) of antibiotics were Gullberg et al. 2011 and Gullberg et al. 2014. In the 2011 study, *Escherichia coli* and *Salmonella enterica* were used in competition assays where susceptible and resistant strains of the same bacteria were competed at various concentrations of antibiotics in rich media. The antibiotics tested were aminoglycosides, fluoroquinolones and tetracyclines with corresponding resistant mutants which carried an appropriate chromosomal mutation. Both susceptible and resistant strains were tagged with different fluorescent markers and were quantified by flow cytometry. The authors were able to determine a MSC for each resistant strain at various concentrations of antibiotics by calculating selection coefficients, plotting these against antibiotic concentration, finding the line of best fit and calculating where this line crossed the x axis. The MSCs ranged

from 1/4 to 1/230 of the MIC of the susceptible bacterium. The absolute MSC values ranged from 1,000 µg/L of streptomycin to 0.1 µg/L of ciprofloxacin.

The 2014 study used the same approach, competing a susceptible *E. coli* against a resistant *E. coli* harbouring plasmid borne resistance genes, instead of chromosomal resistance. This study tested 4 antibiotic compounds: erythromycin, kanamycin, tetracycline and trimethoprim. Both susceptible and resistant strains were fluorescently tagged and change in prevalence of resistance in the population was observed over time using flow cytometry. As with the 2011 study, selection was observed at sub-MIC concentrations for all antibiotics test. These concentrations ranged from 1/17 to 2/3 of the MIC for trimethoprim and kanamycin, respectively. This equated to concentrations of 33 µg/L and 470 µg/L of antibiotic, respectively.

Limitations of this work are centred on the environmental relevance of both studies which comprised experiments that used a single species and competed a susceptible bacterial strain against a resistant mutant. Single species are never found in isolation, with mixed communities of varied species found throughout environmental settings. The broth used (Muller Hinton) was nutrient rich and not representative of the nutrient content of many environmental settings. Although these studies were a valuable first step in understanding selection at low, environmentally relevant concentrations of antibiotics, this data may not be representative of what happens in the natural environment. It may, therefore, not provide the most appropriate endpoints when determining safe release levels of antibiotics from wastewater treatment plants (WWTPs) although it does represent a relatively time efficient, transferable assay for assessing risk.

A study by Bengtsson-Palme & Larsson in 2016 used a mathematical approach to calculate concentrations that could potentially select for resistance and suggested these could be used as safe release concentrations of antibiotics into the environment. This mathematical methodology obtained MIC values for all antibiotics found in the EUCAST database and identified the lowest 1%. Values were adjusted where a limited number of species were tested and, by applying a 10-fold assessment factor to all values, predicted no effect concentrations for the selection of resistance ($PNEC_R$s) were calculated. The application of an assessment factor was to allow for the difference between MICs and MSCs. This study calculated $PNEC_R$ values for 111 antibiotics found on the EUCAST database and 11 combinations of antibiotics. The $PNEC_R$s ranged from 0.008 µg/L to 64 µg/L for itraconazole and nitrofurantoin, respectively. This concentration range was lower than the range of MSCs established by both Gullberg *et al.*, 2011 and Gullberg *et al.*, 2014 (0.1 to 1,000 µg/L), although there was crossover between the two approaches.

This method defined endpoints for all antibiotics and combinations of antibiotics on the EUCAST database and is intended, according to the study, to inform and aid regulators in implementing mitigation strategies to reduce the risk of the development of environmental antibiotic resistance. The EUCAST MIC values, and hence the $PNEC_R$ values calculated, are, however, based on inhibitory thresholds for clinical bacterial strains, rather than selective thresholds in environmental bacteria. This, therefore, brings into question the relevance of these values with reference to environmental discharge limits. Further, the method used is a purely mathematical approach and, as the authors did note, experimental work determining MSCs for individual compounds, using complex microbial communities, should also be undertaken to better refine safe antibiotic

discharge limits and validate the values calculated in the Bengtsson-Palme & Larsson 2016 study. The same group has continued this work by undertaking such experiments using complex microbial communities (Lundström et al. 2016; Kraupner et al. 2018).

Lundström et al. 2016 used a variety of methods (metagenome analysis, colony forming unit counts, MIC of isolates, pollution induced community tolerance assay, functional diversity and qPCR) to investigate the effect of low tetracycline (TET) concentrations on a complex microbial community. QPCR was reported as the most sensitive assay to calculate the MSC of TET. The authors used a complex community from sewage effluent to establish a biofilm in a flow through system. The experiment was conducted at 20 °C with a constant inflow of nutrient, antibiotic and inoculum and the resulting nutrient concentration in the aquaria was approximately 1/60 R2A broth. Biofilms were harvested after 9 days and *tetA* and *tetG* prevalence was quantified using qPCR (normalised to the 16S rRNA gene). An increase in both *tetA* and *tetG* prevalence was observed at 1 and 10 µg/L compared to the no antibiotic controls. These concentrations are in the same range as current MECs of tetracycline (Lundström et al. 2016).

Kraupner et al. 2018 investigated the selective potential of ciprofloxacin. They investigated both a test tube system (run at 25°C in 10X diluted R2A broth) and the same biofilm flow through system described above, to compare the results of each system. Both in the biofilm and test tube system, plating was used to isolate *E. coli* from the community and to calculate the percentage of resistant *E. coli* to find which concentration of TET was selecting for resistance in comparison to the no antibiotic control. Using the biofilm system, a significant

difference in the percentage of resistant *E. coli* was observed at 10 µg/L of ciprofloxacin, but not at 1 µg/L. Although the test tube system saw a significant reduction in community diversity, similar MSCs were determined for ciprofloxacin in comparison to the biofilm system. In the test tube system, a significant difference was observed at 5 µg/L but not at 1µg/L but it should be noted that 5 µg/L was not tested in the biofilm experiment.

The main limitation of both studies was that the prevalence of the resistance genes at the beginning of their experiment in the original inoculum were not quantified (Murray et al. 2018). As a result, they were unable to determine whether positive selection was occurring. A comparison to the no antibiotic control is important; as key information about the change of prevalence of the genes tested over time is missing from these studies. Another flaw in their methodology is that in order to maintain the biofilm it was necessary to constantly input bacteria from the raw, unevolved inoculum into their experimental system. It is not, therefore, possible to determine whether the increase in resistance observed, relative to the no antibiotic control, is due to selection in the biofilm or introduction of resistant bacteria into the biofilm population during the experiment.

Two studies, Murray et al. 2018 and Stanton *et al.*, 2019, both used the same methods. These methods were selection experiments using a complex microbial community as described in Chapter 2. Both studies analysed class specific resistance genes and, in the case of Stanton *et al.*, 2019, *intI1* gene prevalence using qPCR. The change in prevalence of these genes at the beginning and the end of the selection experiment was determined.

The study by Murray et al. 2018 investigated the selective potential of cefotaxime in a complex microbial community. Following metagenome analysis to identify a suitable gene target, the authors quantified *bla*$_{CTX-M}$ prevalence using qPCR (normalised to 16S rRNA copy number) and determined a MSC for cefotaxime of 0.4 µg/L.

Stanton *et al.*, 2019 investigated the selective potential of 2 classes of compounds that have been placed on the European Commission's Water Framework Directive's Priority Watch List (European Commission 2015a; European Commission 2018) due to their toxicity to aquatic organisms. These were three macrolide antibiotics (azithromycin (AZ), clarithromycin (CLA) and erythromycin (ERY)) and ciprofloxacin. LOECs, using the macrolide specific *ermF* resistance gene, were determined for AZ, CLA and ERY as 1,000, 750 and 1,000 µg/L, respectively, and a MSC of 514 µg/L was determined for ERY (Chapter 3). This study also investigated the selective potential of ciprofloxacin. The *intI1* gene was used to determine a MSC of 10.5 µg/L and a LOEC of 15.625 µg/L of ciprofloxacin.

The methods used in Murray et al. 2018 and Stanton *et al.*, 2019 and throughout this thesis, although more environmentally relevant than Gullberg et al. 2011, Gullberg et al. 2014 and Bengtsson-Palme & Larsson 2016 could be argued to be less environmentally relevant than the biofilm flow through system used in Lundström et al. 2016 and Kraupner et al. 2018 which used a low growth medium and lower temperatures, more representative of environmental conditions, as opposed to the experiments reported in this thesis, all of which used a rich nutrient broth at 37 °C. However, as described previously there are major limitations to the biofilm flow through system.

The aim of research reported in this chapter was to determine whether the experimental set up used here gave comparable results to the more "environmentally realistic" method used by Lundström et al. 2016 and Kraupner et al. 2018. The initial part of the work presented in this chapter was conducted in 2016, before the study from Kraupner et al. 2018 was published, and, therefore, replicated the experiment in Lundström et al. 2016 using TET as the study compound. This work aimed to determine a MSC for TET using the resistance gene *tetG* as an endpoint using the selection experiment methodology described in Chapter 2.

A subsequent aim of this chapter was to determine a MSC for TET.

6.3 Methods

6.3.1 Antibiotics and antibiotic concentrations tested

6.3.1.1 Comparison of in vitro tests for determining MSCs

Tetracycline hydrochloride (Fisher), dissolved in water and sterilised using a 0.22 µm filter, was used at concentrations of 0.1, 1, 10 and 100 µg/L. This was to test a more extended range of TET concentrations than that used in the study by Lundström et al. 2016.

6.3.1.2 Determining a MSC for tetracycline

Tetracycline hydrochloride was prepared and sterilised by the same method described in Section 6.3.1.1. In this experiment, a wide concentration range was targeted. The literature was searched in order to find an MIC or breakpoint for *Enterobacteriacae* as a highest concentration. The article "Determination of minimum inhibitory concentrations" suggested a concentration range of TET at which MICs of TET for *Enterobacteriacae* might be expected to be seen (Andrews 2001). 128,000 µg/L was the top concentration suggested and was,

therefore, used as the top concentration of TET in this experiment and a 2-fold serial dilution was undertaken down to the concentration 0.9765625 µg/L (comparable to the concentration range undertaken in Lundström *et al.*, 2016).

6.3.2 Selection experiment

As the concentration range (presented in Section 6.3.1.2) was large, it was necessary to split this experiment into two halves. The high TET concentrations were set up in the morning and the low concentrations in the afternoon of the same day. This enabled the period of time between the bacteria being exposed to the concentration of antibiotic and the day 0 samples being frozen to be minimised, which therefore minimised the variation in prevalence of genes tested in the day 0 samples. Sufficient bacterial culture for both halves of the experiment was defrosted. These were mixed together to ensure a homogenised inoculum. Whilst the first half of the experiment was being set up, the rest of the inoculum was stored at 4 °C to minimise growth and change in community structure.

Each half of this experiment had its own no antibiotic control to account for any community structure change whilst the inoculum was stored.

6.3.3 QPCR targets

6.3.3.1 Comparison of in vivo tests for determining MSCs

The only qPCR targets used here were the tetracycline resistant *tetG* gene, alongside the 16S rRNA gene to determine prevalence. This was one of two of the genes targeted by qPCR in the Lundström et al. 2016 study.

6.3.3.2 Determining a MSC for tetracycline

TetG was, once again, tested alongside *tetM* (which encodes a ribosomal protection protein) and *intI1. TetG* was chosen as it was used in Lundström *et*

al., 2016. Lundström et al. 2016 chose *tetG* as a qPCR target as this gene showed one of the strongest correlations to TET concentration from metagenome data produced and presented in the same study. *TetM* was chosen as it has been previously described as a genetic indicator for tetracycline resistance in the environment (Berendonk et al. 2015). *IntI1* was chosen as it has been described as a good proxy for the presence of antibiotic resistance genes in the environment (Gaze et al. 2011; Gillings et al. 2015). Two sets of primers for *tetA* were also tested, the first from Lundström et al. 2016 (*tetA (1)* – Table 2, Section 2.4.1) and the second from Zhu *et al.*, 2017 (*tetA (2)* - Table 2, Section 2.4.1). Neither of these primer sets are presented here as it was not possible to determine specific melt curves for either despite trying different primer concentrations and annealing temperatures.

6.3.4 Data analysis

The post-hoc tests (Dunnett's/Dunn's) were undertaken in relation to each half of the experiment described in Sections 6.3.1.2 and 6.3.2, comparing each half to its own no antibiotic control.

6.4 Results

6.4.1 Comparison of in vitro tests for determining MSCs

An experiment targeting a small range of concentrations was undertaken to determine if the selection experiment, used for all experiments presented in thesis, was comparable to the biofilm system presented in Lundström et al. 2016.

The results from this experiment can be seen in Figure 62. When the data was interrogated in the same way as Lundström et al. 2016 (without plotting the initial *tetG* prevalence) a dose response between *tetG* prevalence and

tetracycline was seen. This is presented in Figure 62A. Statistical analysis showed a significant difference in prevalence, compared to the no antibiotic control, to 90% confidence at 1 µg/L (p = 0.0784, Dunn's test), 10 µg/L (p = 0.0658, Dunn's test) and 100 µg/L (p = 0.0784, Dunn's test). If, however, the starting prevalence of *tetG* is plotted alongside the day 7 prevalence (Figure 62B) no significant positive selection was observed over the 7 day period for *tetG* at any TET concentration tested (0.1, 1, 10 & 100 µg/L). The average prevalence at day 0, in all of the replicates of every concentration, was 0.0096 and the highest prevalence of all of the replicates at any concentration at the end of the 7 day experimental period was 0.0000043 (>1,000 times decrease). As the prevalence at day 7 is vastly lower than that at day 0, it cannot be visualised on Figure 62B, although it has been plotted. The significant difference between those samples treated with 1, 10 and 100 µg/L of TET and the no antibiotic control showed a decreased rate of negative selection; in other words an increase in *tetG* persistence.

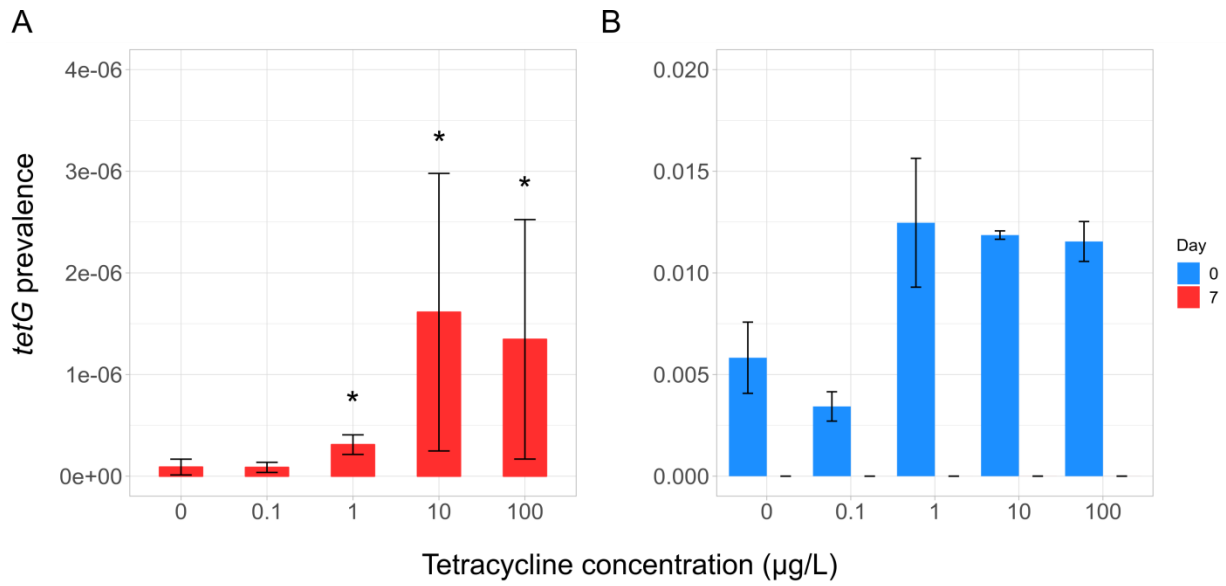


Figure 62: *TetG* as a function of TET concentrations. A: Graph shows only the day 7 prevalence data. B: Graph shows the prevalence data from both days, although prevalence of *tetG* at the end of the experiment is so low that this cannot be observed on the graph. Please note that these graphs are on different axes as the day 7 data (A) cannot be visualised otherwise. Standard error is represented by the error bars. x = a significant increase in prevalence relative to the no antibiotic control to 90% confidence.

Plotting only the day 7 prevalence of *tetG* (Figure 62A) gave comparable results to the effect observed and presented in Lundström *et al.*, 2016 (Figure 63) although as the prevalence of *tetG* in the starting inoculum was not quantified in their biofilm experiment, it is not possible to say with certainty that what they were observing was increased persistence or positive selection.

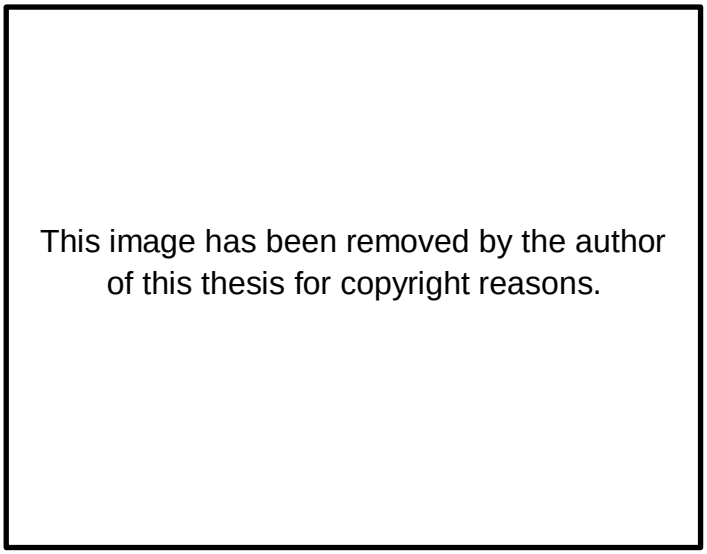


Figure 63: *TetG* as a function of TET concentrations in a biofilm flow through system. Reproduced from Lundström et al. 2016. * = p <0.05, *** = p < 0.001. A one – tailed Student's *t*-test was used on $\log_2$ transformed data to determine significance.

If the selection experiment data is plotted as a selection coefficient, the line of best fit (polynomial order 2, $y = -1.7 + 0.038x - 0.0038x^2$, $R^2 = 0.007067$, standard error = 0.4041) never crosses the x axis as no positive selection was occurring. This can be seen in Figure 64.

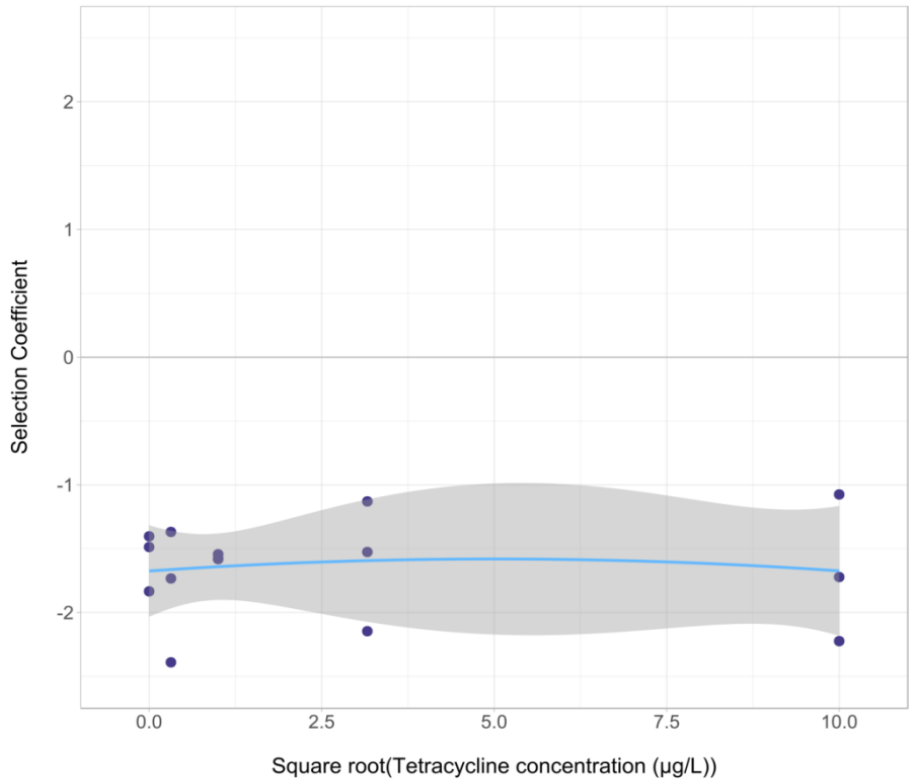


Figure 64: Selection coefficient of *tetG* by TET. The square root of tetracycline concentration was plotted. Absolute concentrations represented by this graph are 0, 0.1, 1, 10 and 100 µg/L of TET. The blue line is the polynomial line of best fit (order 2), the grey shading represents the confidence intervals and the purple points represent the selection coefficients for each replicate.

It was impossible, therefore, to determine a MSC or LOEC for positive selection from this data set.

6.4.2 Determining a MSC for tetracycline

As the definition of a MSC is the concentration at which "the resistant mutant is enriched over the susceptible strain" (Andersson & Hughes 2011), the results presented above and in Lundström et al. 2016 may not be true MSCs. No positive selection was demonstrated; rather differential rates of negative selection or increased persistence are likely to account for the TET result. A

wider range of TET concentrations were, therefore, tested in order to determine the MSC.

6.4.2.1 qPCR analysis

A number of targets were used for qPCR analysis. These were *intI1, tetM* and *tetG.* The qPCR data sets presented here are split into the two halves in which they were undertaken and statistical comparison is to the relevant no antibiotic control. For determining selection coefficients, the datasets from the two halves of the experiment were grouped together where appropriate. This allowed a line of best fit and a MSC to be determined.

tetG

TetG was also tested for this dataset with the aim of determining a LOEC and a MSC for this gene at higher concentrations than those tested in Section 6.4.1.

For both halves of this experiment, however, no positive selection was observed for *tetG*. This can be seen in Figure 65. It was impossible, therefore, to determine a LOEC or MSC for this gene.

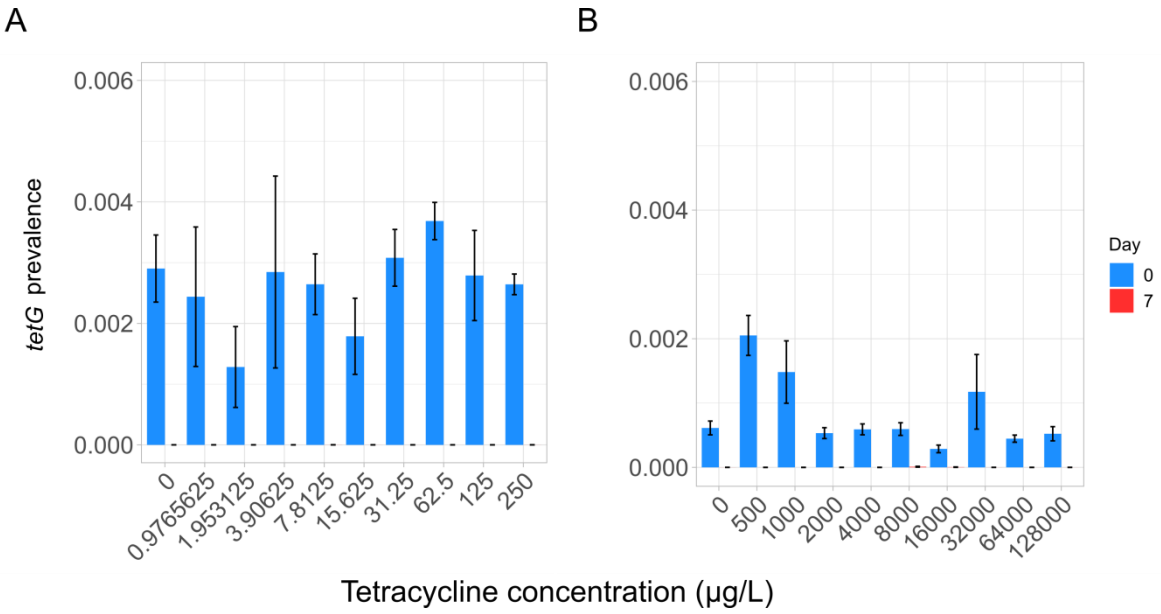


Figure 65: *TetG* as a function of a large range of TET concentrations. The low range of concentrations are represented in 65A and the high in 65B. Two high outlier replicates have been removed from the 3.90625 µg/L sample and one from the 7.8125 µg/L sample. Standard error is represented by the error bars.

If only the day 7 data are displayed, as in Section 6.4.1, increase in persistence, or decreased negative selection, was once again observed. This time, however, there was no distinct dose response pattern to this increase. At the low concentrations of TET, a significant increase in persistence was observed compared to the no antibiotic control at 3.90625, 7.8125, 15.625, 31.25 and 250 µg/L (p = 0.0254, 0.0003, 0.0009, 0.0230 and 0.0310, respectively, Dunn's test). No increase in comparison to the no antibiotic control was observed at 0.9765625, 1.953125, 62.5 and 125 µg/L. See Figure 66.

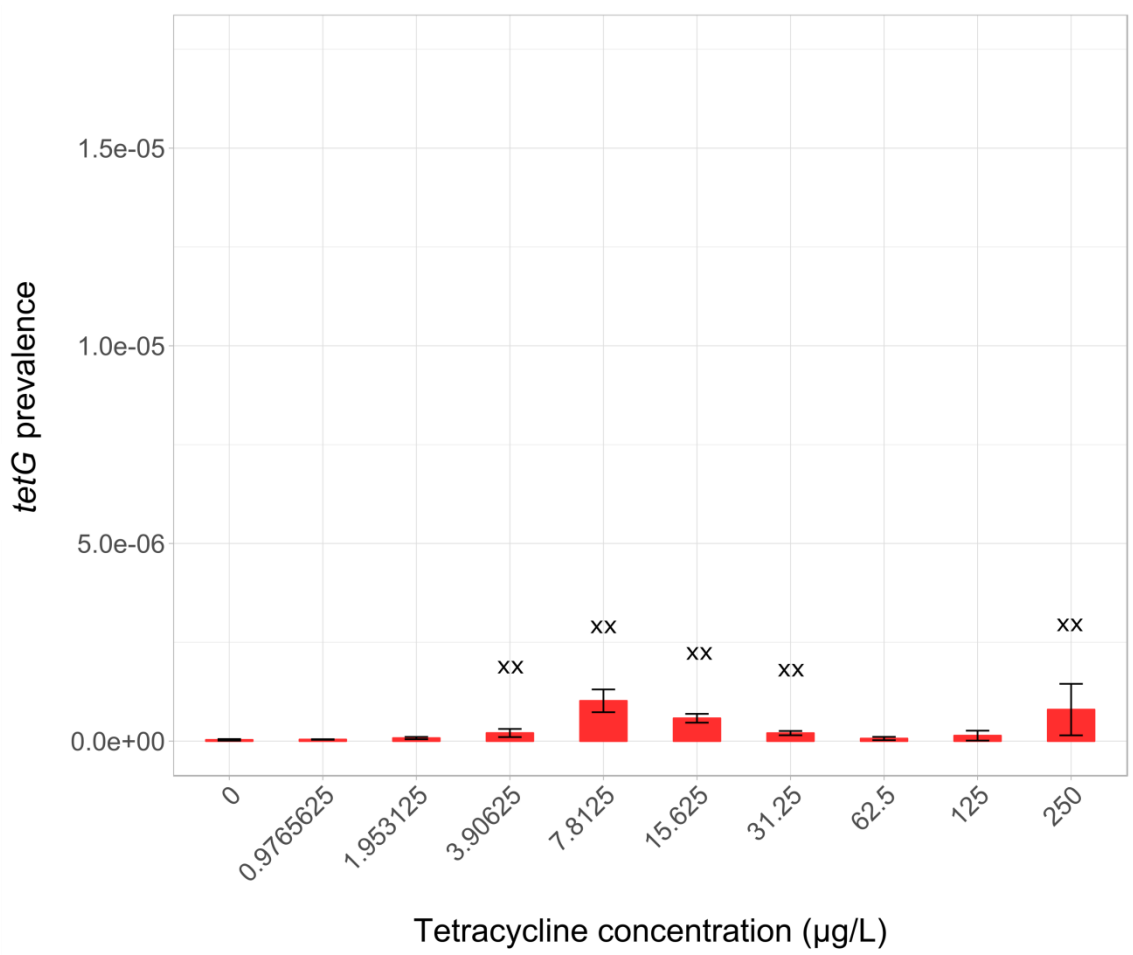


Figure 66: *TetG* as a function of low TET concentrations – day 7 only. Standard error is represented by the error bars. xx = significant persistence to 95% confidence.

At higher concentrations of TET, a significant increase compared to the no antibiotic control was only observed at 8,000 µg/L (p = 0.0413, Dunn's test). This can be seen in Figure 67.

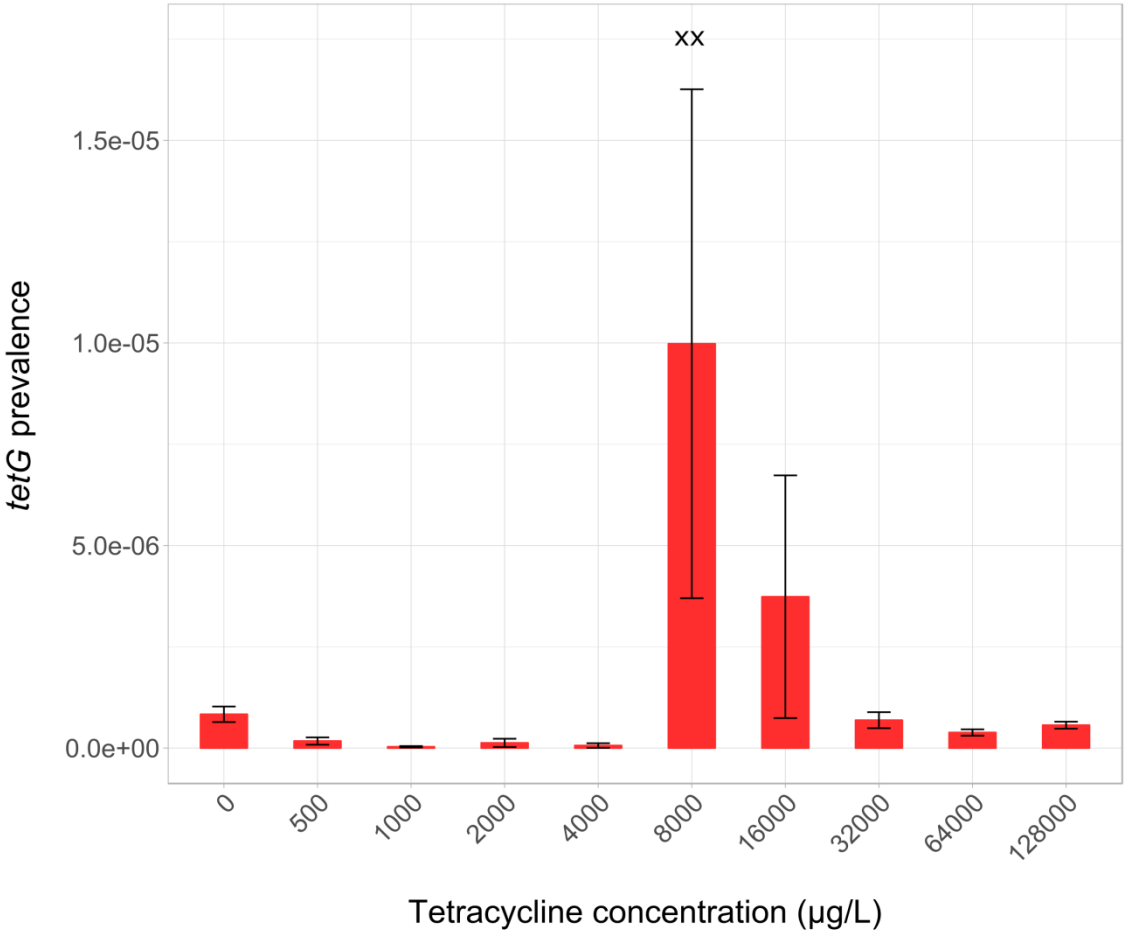


Figure 67: *TetG* as a function of high TET concentrations – day 7 only. Standard error is represented by the error bars. xx = significant persistence to 95% confidence.

If a selection coefficient graph is plotted, the line of best fit never crossed the x axis and, therefore, as in Section 6.4.1, positive selection was never occurring. This can be seen in Figure 101 (Page 334 - Appendix).

tetM

As with *tetG*, no positive selection was observed for *tetM* as a result of exposure to lower TET concentrations. An increase in prevalence was seen, however, over time in all samples (Figure 68).

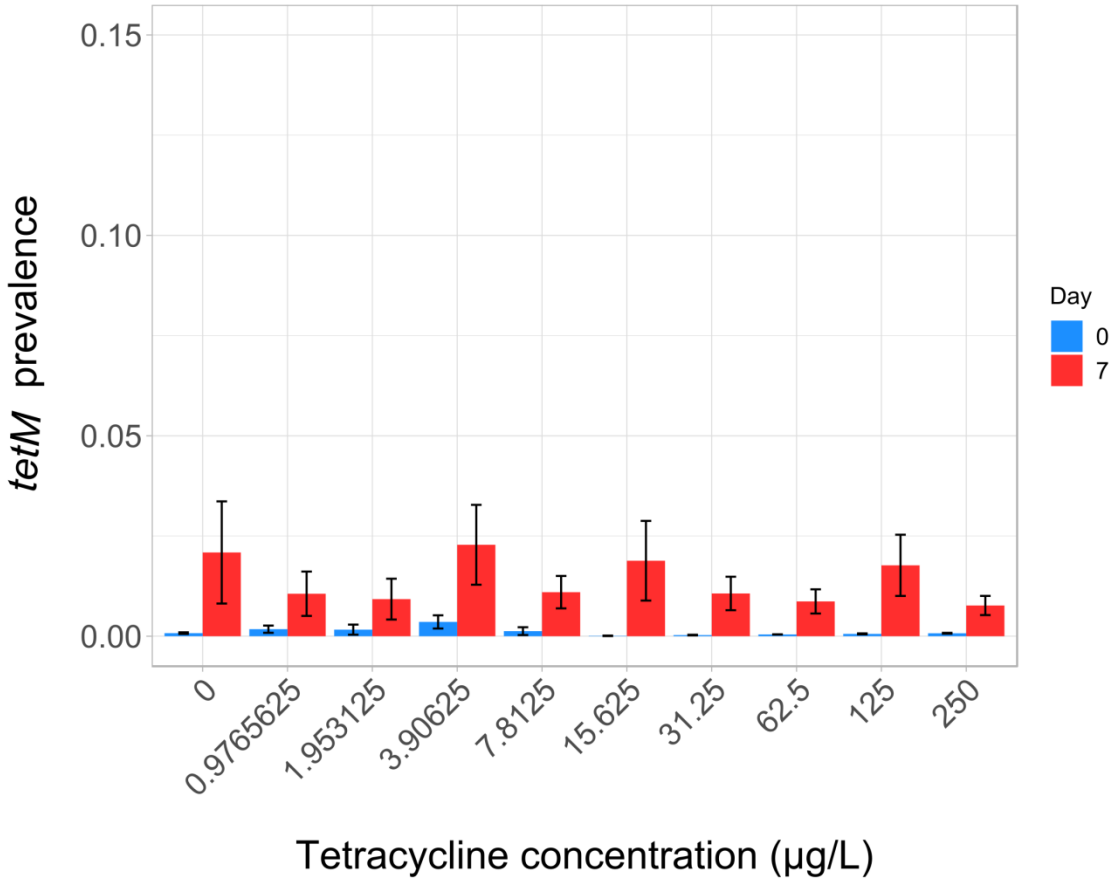


Figure 68: *TetM* as a function of low TET concentrations.
Standard error is represented by the error bars.

With the higher TET concentrations, no significant selection was seen at any of the TET concentrations, compared to the no antibiotic control, except at 128,000 µg/L (p = 0.0376, Dunn's test). The LOEC (lowest observable effect concentration) was 128,000 µg/L and a NOEC (no observable effect concentration) was 64,000 µg/L. Again, an increase in *tetM* over time was seen in all samples, including the no antibiotic control (Figure 69).

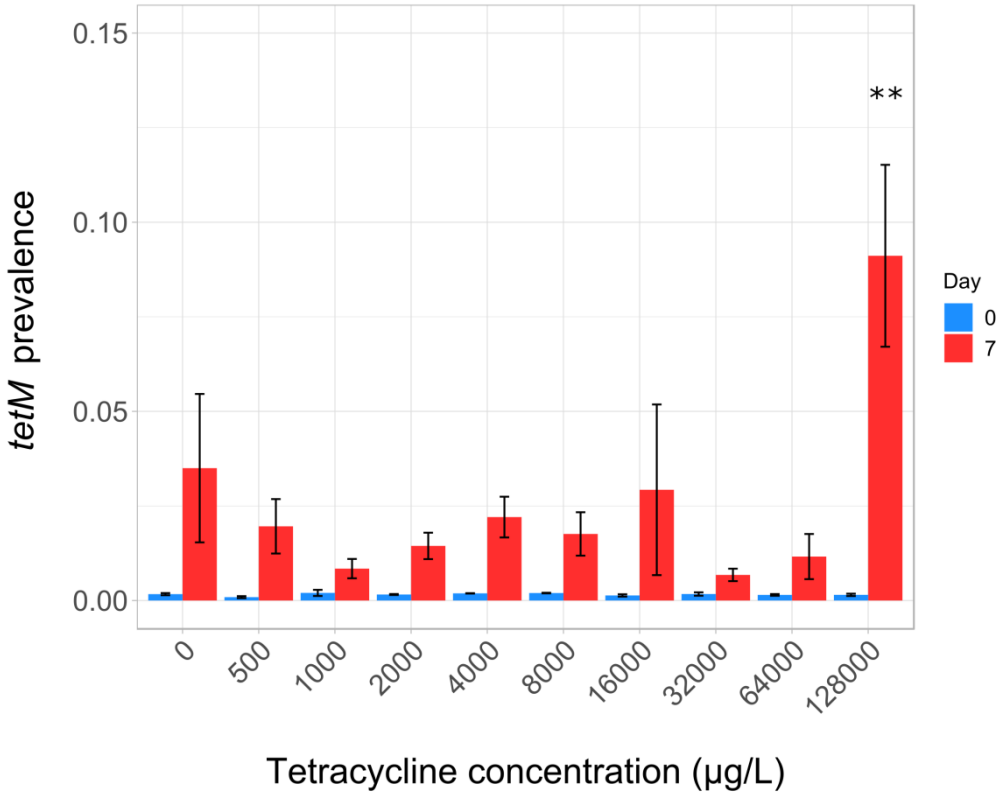


Figure 69: *TetM* as a function of high TET concentrations. Standard error is represented by the error bars. ** = significant positive selection to 95% confidence.

No MSC was able to be determined because an increase was observed at each concentration, including the no antibiotic control. The line of best fit, therefore, never crossed the x axis. The selection coefficient graph can be seen in Figure 102 (Page 335 - Appendix).

intI1

No significant increase in *intI1* was seen in comparison to the no antibiotic control at any concentration of TET tested in the low concentration experiment, although a biological effect appears to occur at 250 µg/L. As a result of variability of prevalence between the replicates, this is not statistically significant (Figure 70).

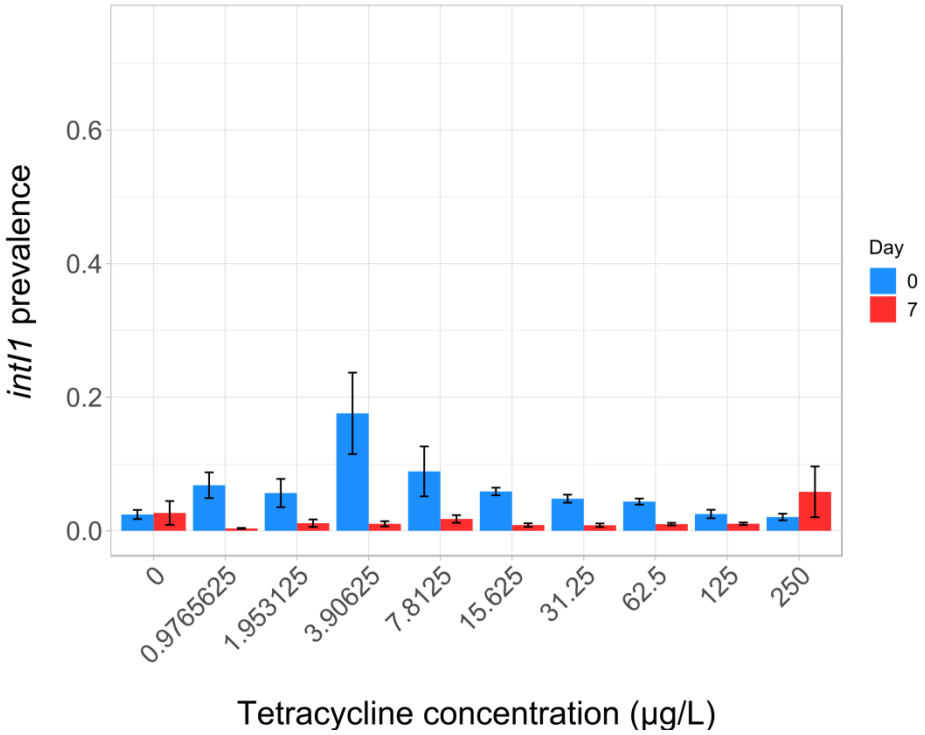


Figure 70: *IntI1* as a function of low TET concentrations. Standard error is represented by the error bars.

Figure 71 shows the response of *intI1* to the higher concentrations of TET tested. No significant increase in *intI1* was seen at concentrations of 500 and 1,000 µg/L. A significant increase was seen to 95% confidence at the concentrations of TET of 2,000, 4,000, 8,000, 16,000, 32,000 and 128,000 (p = 0.0127, 0.0207, 0.0008, 0.0015, 0.0342 and 0.0001, respectively, Dunn's test)

and to 90% confidence at 64,000 (p = 0.0859, Dunn's test). The LOEC was, therefore, 2,000 µg/L and the NOEC was 1,000 µg/L.

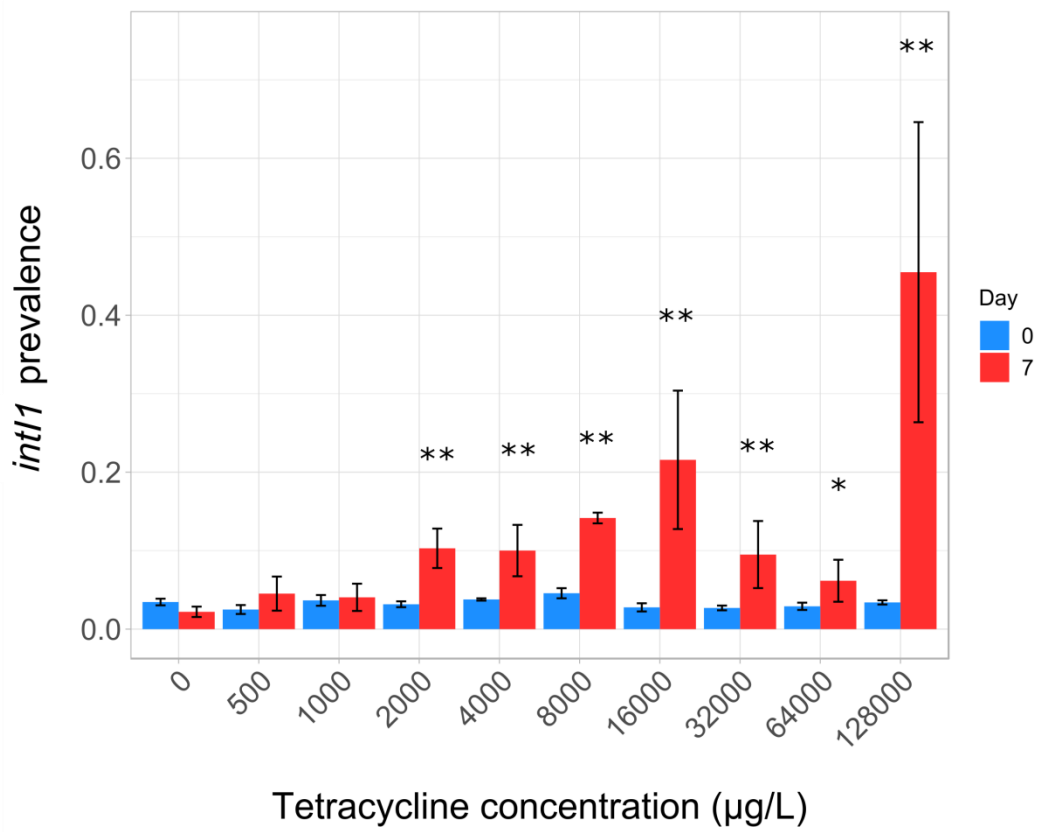


Figure 71: *IntI1* as a function of high TET concentrations. Standard error is represented by the error bars. * = significant positive selection to 90% confidence, ** = significant positive selection to 95% confidence.

A MSC was determined as approximately 651 µg/L (Figure 72). To get the most accurate and protective MSC, only a subset of concentrations were used and the line of best fit (polynomial order 2, y = -0.15 + 0.00027x – $6.1e^{-08}x^2$, R^2 = 0.3024, standard error = 0.156, x intercept = 651.43) was plotted. The concentrations of TET plotted here are 62.5, 125, 250, 500, 1,000 and 2,000 µg/L. As a result of the natural variation in the biological replicates, it is only possible to determine an approximation for the MSC value.

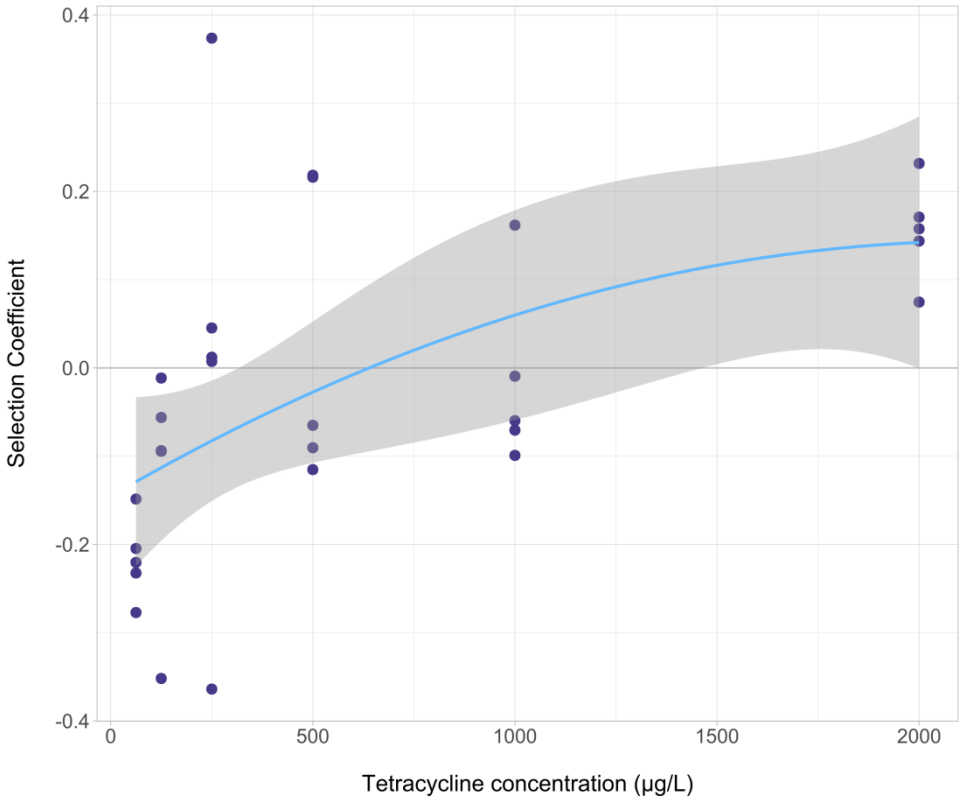


Figure 72: Selection coefficient of *intI1* by TET. A reduced range of concentrations ranging from 62.5 to 2,000 µg/L of TET is plotted here to make the line of best fit more accurate and the most protective. The blue line is the polynomial line of best fit (order 2), the grey shading represents the confidence intervals and the purple points represent the selection coefficients for each replicate.

6.4.2.2 Metagenome analysis

As the lowest MSC and LOEC were both determined by *intI1*, and not by a class specific resistance gene, a subset of samples from the selection experiment were sent for metagenome sequencing to identify possible new class specific targets that were being selected for but which had not been targeted by qPCR. Three replicates were chosen with the highest *intI1* prevalence from the concentrations 125, 250, 500, 1,000, 2,000, 4,000, 8,000 and 16,000 µg/L as well as three replicates from the no antibiotic controls from each half of the experiment. As *intI1* showed the lowest effect, it was presumed

that selection for other class specific genes was occurring around this concentration range and that these had not previously been targeted by qPCR. By undertaking metagenome analysis, it was hoped that other TET specific resistance genes could be identified.

Overall tetracycline resistance

ARGs-OAP identifies antibiotic class and subtype. First, the sum of all detected tetracycline genes was analysed. A significant increase in TET resistance genes occurred at every concentration of TET tested, relative to the appropriate no antibiotic control. Significant increase was seen to 90% confidence at 2,000 µg/L (p = 0.06782, Dunnett's test) and to 95% confidence at 125, 250, 500, 1,000, 4,000, 8,000 and 16,000 µg/L (p = 0.0107, 0.050, 0.01283, 0.02658, 0.01006, 0.00609, 0.00884, respectively, Dunnett's test). This can be seen in Figure 73.

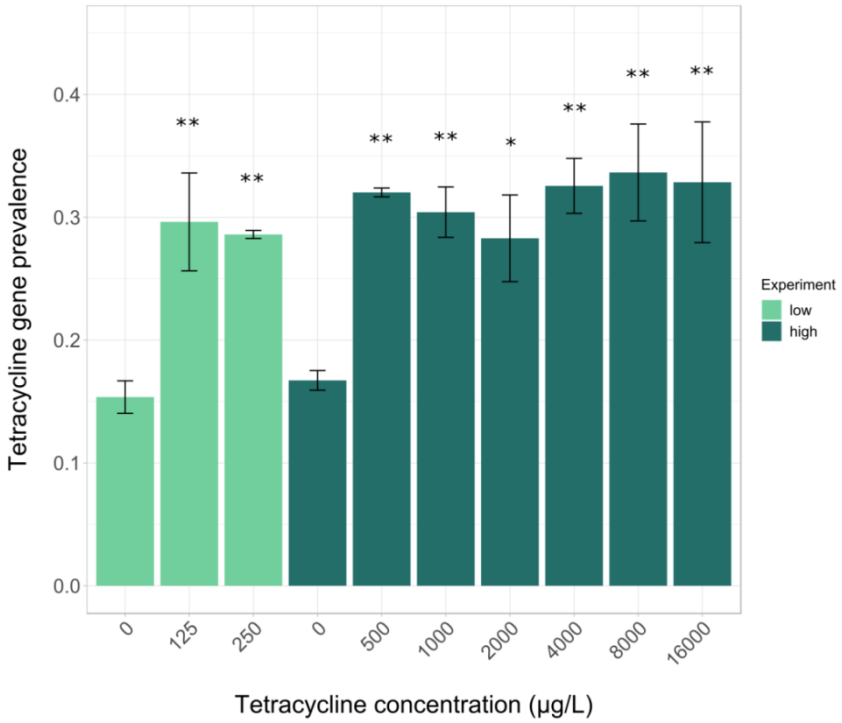


Figure 73: Tetracycline resistance gene prevalence from metagenome analysis as a function of TET concentration. Standard error is represented by the error bars. ** = significance to 95% confidence.

Tetracycline resistance genes by subtype

A number of specific TET resistance genes increased in prevalence, compared to the no antibiotic control, at concentrations lower than the current qPCR LOEC determined by *intI1* (2,000 µg/L). The results showing the effect on all genes on the ARG-OAP database can be seen in the heatmap in Figure 103 (Page 336 - Appendix).

The genes that significantly increased at the lowest concentration of TET were *tetW* (which encodes a ribosomal protection protein) (Figure 74)*, tetA* (Figure 75) and *tet32* (which encodes a ribosomal protection protein) (Figure 76)*. TetW* saw a significant increase at the lowest concentration sequenced at 125 µg/L (*p* = 0.0032, Dunn's test) and at every concentration tested except 2,000 µg/L, although a biological effect was observed here. Without testing the lower concentrations, it is unclear if this was the lowest concentration at which *tetW* may have been selected for, so a LOEC could not be defined.

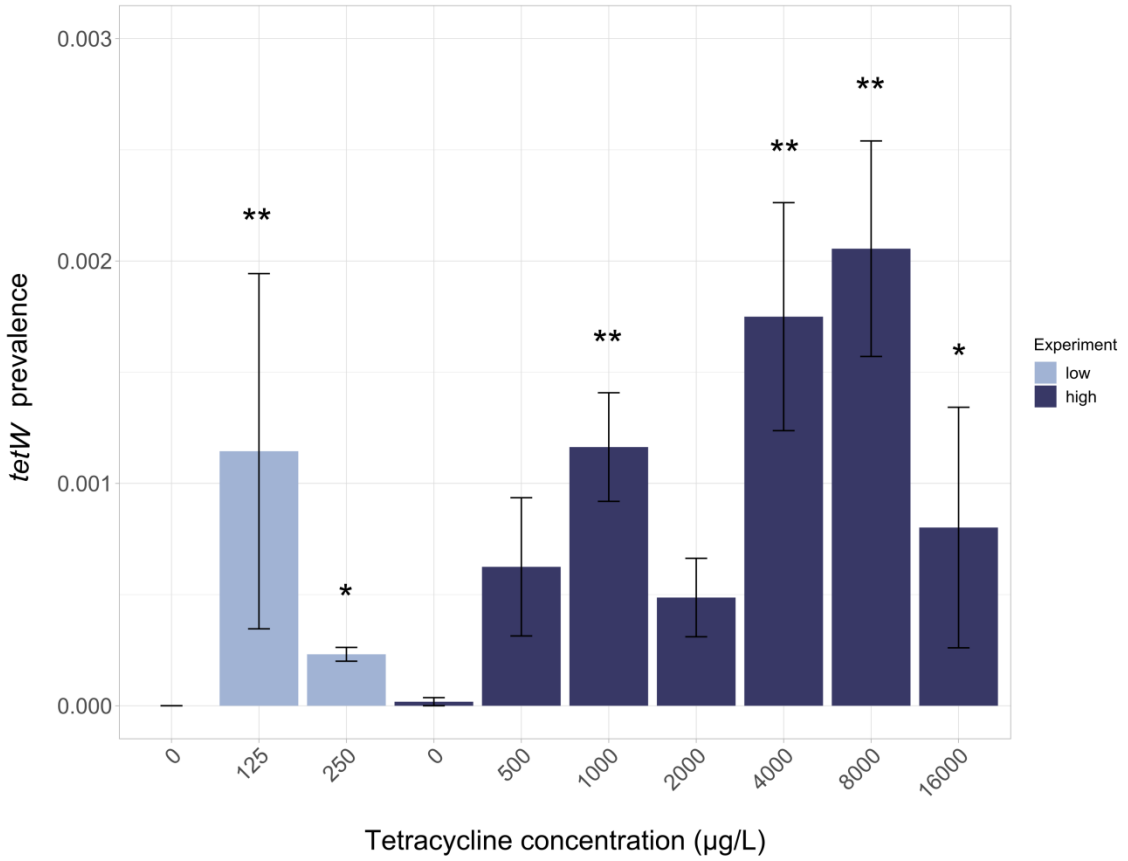


Figure 74: *TetW* as a function of TET concentration. Standard error is represented by the error bars. * = significance to 90% confidence. ** = significance to 95% confidence.

TetA (p = 0.00245, Dunnett's test) and *tet32* (p = 0.0169, Dunn's test) both significantly increased in prevalence at 250 µg/L of TET, compared to the no antibiotic control. Biological effects were also seen for both of these genes at 125 µg/L, but statistical significance was not observed. The response that *tetA* had to TET best mimics the response observed to overall TET resistance (Figure 73). A significant increase was seen at every subsequent concentration of TET except 4,000 and 8,000 µg/L, although biological effects were observed at these concentrations in comparison to the no antibiotic control.

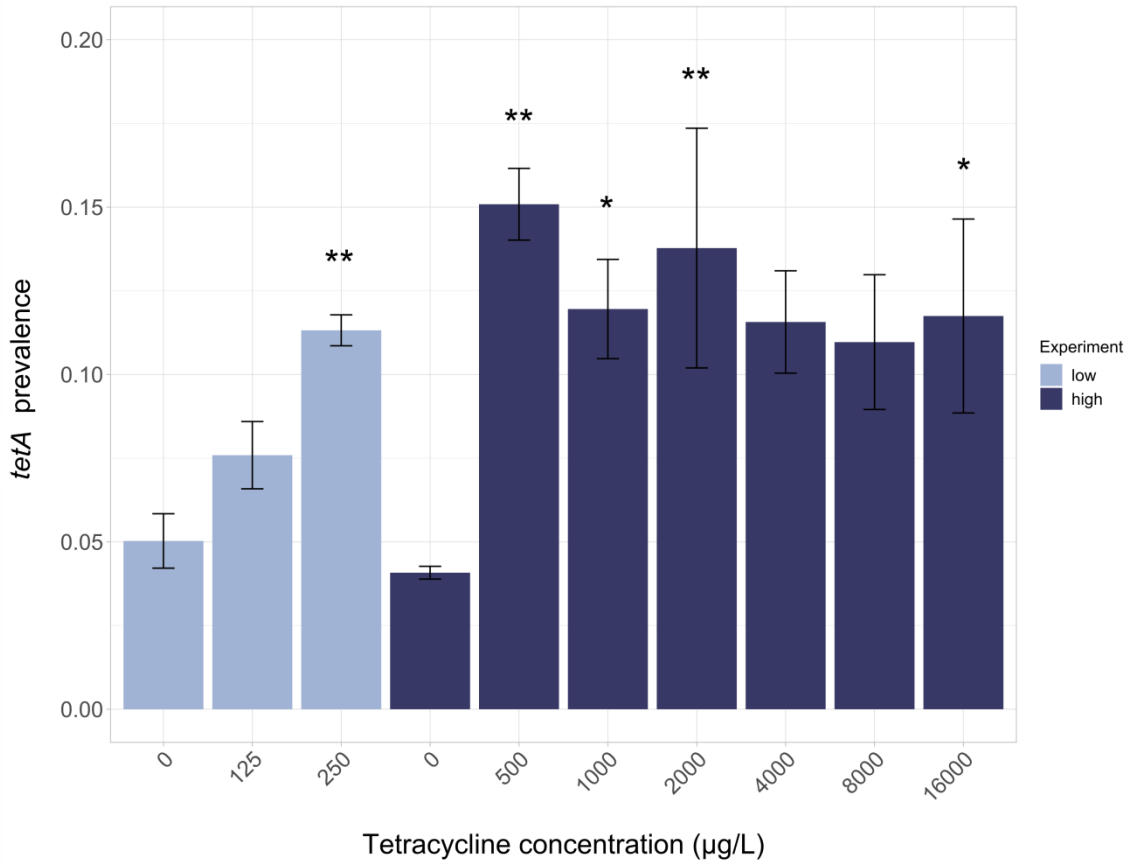


Figure 75: *TetA* as a function of TET concentration. Standard error is represented by the error bars. * = significance to 90% confidence. ** = significance to 95% confidence.

For *tet32,* a significant increase in prevalence was observed in comparison to the no antibiotic control at 250 µg/L and every higher concentration except 1,000 µg/L. A biological effect was observed at 1,000 µg/L in comparison to the no antibiotic control but, as variation between replicates was high, it was not found to be statistically significant.

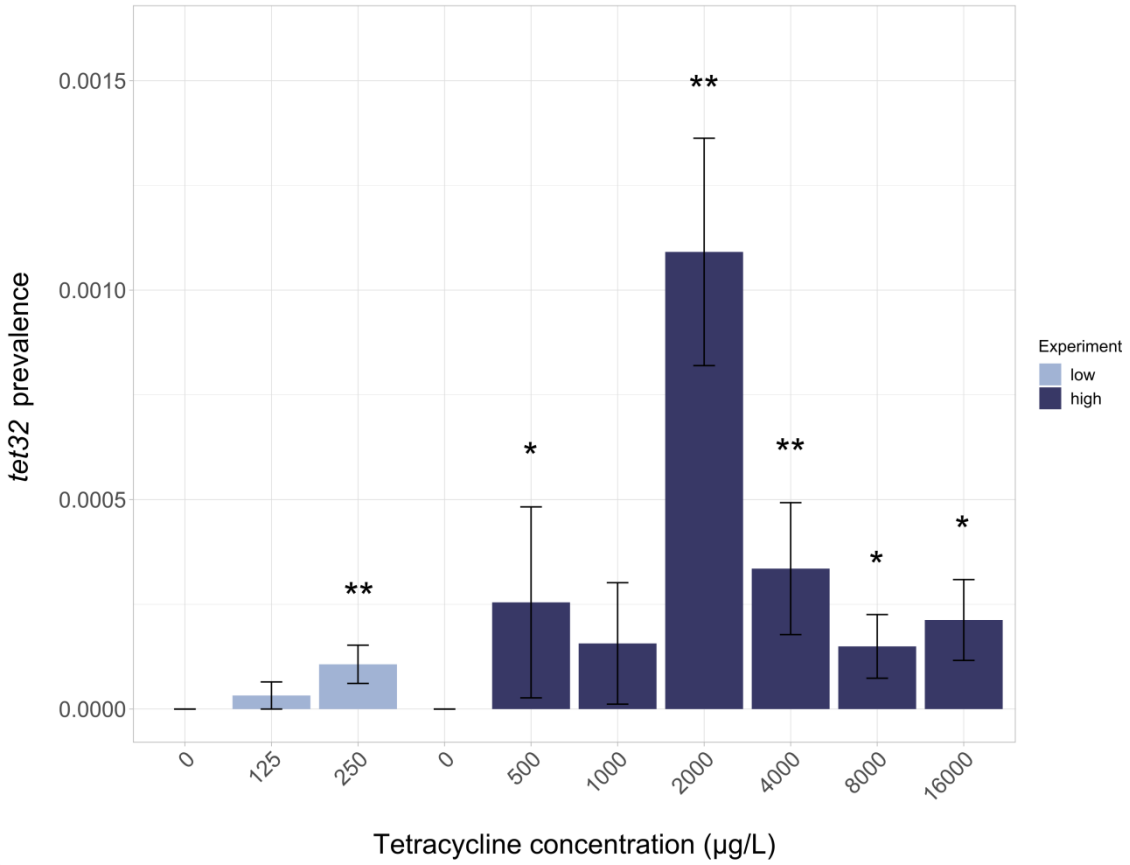


Figure 76: *Tet32* as a function of TET concentration. Standard error is represented by the error bars. * = significance to 90% confidence. ** = significance to 95% confidence.

Other TET specific genes significantly increased at concentrations lower than the current LOEC (based on the *intI1* qPCR data). These were *tetJ* and *tetL*, both of which encode efflux pumps, and *tetO* and *tetQ,* both of which encode a ribosomal protection protein. *TetJ* significantly increased at 1,000 µg/L only (to 90% confidence) (p = 0.0941, Dunn's test). *TetL* and *tetO* significantly increased, in comparison to the control, at 500 µg/L (p = 0.0242 and 0.0436, respectively, Dunn's test) and, for *tetO* at most, and for *tetL* at all, TET concentrations higher than this. The prevalence of *tetQ* significantly increased

(to 90% confidence), compared to the no antibiotic control, at 1,000 µg/L (p = 0.05, Dunn's test) and at all higher concentrations except 2,000 µg/L.

Co-selection for antibiotic resistance gene classes by TET

Other data that can be determined from the metagenome datasets was the co-selective potential of TET, see Figure 104 (Page 337 - Appendix). Co-selection for aminoglycosides, bleomycin and trimethoprim is determined at the lowest concentration of TET sequenced (125 µg/L).

6.5 Discussion

6.5.1 A comparison of *in vitro* experiments

The biofilm flow through system used by Lundström et al. 2016 and Kraupner et al. 2018 is a complicated method to determine selective endpoints. As demonstrated in the paper by Bengtsson-Palme & Larsson 2016, there are a high number of antimicrobial compounds and a significant number of clinical combinations of antimicrobials that require testing for their selective potential. To perform biofilm experiments with enough replication would be a labour intensive task. In addition, the flow cells are "open" with inoculum continually entering the biofilm. The selection experiment methodology presented in this thesis, and in Murray et al. 2018 and Stanton *et al.*, 2019, are simpler and less labour intensive making it more appropriate for routine risk assessment. It was important, however, to compare this method to the biofilm methodology used previously.

When only the prevalence at day 7 was considered, the TET selection experiment reported here produced comparable data to that reported in Lundström et al. 2016. The TET resistance gene *tetG* illustrated a significant

increase in prevalence at 1, 10 and 100 µg/L compared to the no antibiotic control. In Lundström et al. 2016, a significant increase was also seen at 1 and 10 µg/L compared to the control. When interpreting the *tetG* data, by including the starting prevalence of the gene, however, a significant reduction in prevalence was observed at every TET concentration tested over the 7 day experimental period. To fully understand the data and the selective effect of the test antibiotic, it is clearly important to determine the prevalence of the resistance gene in the original inoculum. What was being observed here can be described in two ways: either, a reduction in the rate of negative selection of *tetG* at the concentrations of 1, 10 and 100 µg/L of TET relative to the no antibiotic control; or the increased persistence of *tetG*. The results from both the preliminary experiment that was undertaken for comparative purposes to Lundström *et al.*, 2016 reported in Section 6.4.1 and that in the full TET experiment presented in Section 6.4.2 aligned reasonably well with each other at low TET concentrations. A significant increase in prevalence was first observed at 1 µg/L of TET in the preliminary experiment, whereas in the full TET experiment reported in Section 6.4.2 it was observed at 3.90625 µg/L. A slight difference in where significance is first observed may be expected as these two experiments were run using different inocula and so would have had different community compositions. Significant increase, compared to the no antibiotic control, did not occur at many of the higher concentrations in the experiments presented in this section. It is unclear whether this lack of increase in *tetG* prevalence would have been observed at higher concentrations in the initial experiment (Section 6.4.1) and in Lundström *et al.*, 2016 as these concentrations were not tested in either.

The first part of the work reported in this chapter was undertaken in 2016, before the study by Kraupner et al. 2018 was published. By the time that study was published, Aimee Murray had already undertaken a selection experiment to determine the MSC and LOEC of ciprofloxacin using the selection experiment method. This data, which can be seen in supplementary data Figures 87 and 88 (Appendix), is due to be published in Stanton *et al.*, 2019 alongside the macrolide data, as all of these compounds have been included on the 2018 European Commission's Water Framework Directive's priority watch list (European Commission 2018). The Kraupner et al. 2018 study determined a selective endpoint for ciprofloxacin of 5 µg/L for the test tube system and 10 µg/L for the biofilm system (although 5 µg/L of ciprofloxacin was not tested in the biofilm system). The test tube system used in Kraupner et al. 2018 used a lower temperature and lower nutrient content and was, therefore, more environmentally relevant than the methods used in this thesis. The ciprofloxacin data from Stanton *et al.*, 2019 determined a MSC of approximately 11 µg/L and a LOEC of 15.625 µg/L using the *intI1* gene. This, again, aligns well with the biofilm approach used by Kraupner *et al.*, 2018 and validates the selection experiments used here against more environmentally relevant conditions. It may be coincidental, however, that on this occasion their results align well with the ciprofloxacin data determined by Aimee Murray using our experimental approach. Without determining the starting prevalence of the resistance genes tested, there is no clear evidence that what Kraupner *et al.*, 2018 observed was, in fact, positive selection, or whether it is actually reduced negative selection/increased persistence, as we hypothesise was observed with *tetG*.

6.5.2 Increased persistence and the MIPC

The MSC is defined as the concentration at which “the resistant mutant is enriched over the susceptible strain” (Andersson & Hughes 2011). The MSC was not, therefore, determined for tetracycline by Lundström et al. 2016 in all likelihood as no positive selection was seen to occur when repeating the experiment even though their observations could be approximately replicated (Figure 62A). This cannot be confirmed, however, without repeating their study and quantifying the prevalence in the starting inoculum. This raises an issue surrounding observations of significant effects within a sub-MSC selective window and a new selective endpoint is, therefore, proposed. The concentration at which a significant increase in persistence (or a reduced rate of negative selection) is observed could be defined as the minimal increased persistence concentration (or the MIPC). This is the lowest concentration at which there is an increase in resistance gene prevalence in comparison to when no antibiotic is present. However, the prevalence of the resistance gene is lower than the starting prevalence of the gene in the original inoculum and decreases over time.

Although the MIPC is less concerning than the MSC when quantifying environmental risk, it will still increase the numbers of resistance genes and resistant bacteria found in the environment, in comparison to where no antibiotic is present. This will, in turn, increase the probability of horizontal gene transfer events and will also increase human exposure risk in the environment. As mentioned in Chapter 1, there can be human exposure to antibiotic resistance genes / bacteria in environmental settings, such as coastal waters, and this may, in turn, lead to gut colonisation by resistant bacteria (Leonard et al. 2018). Moreover, the increased prevalence of resistance genes and resistant bacteria

at concentrations above the MIPC (compared to when no antibiotic is present) may prime the community in the environment so that if the concentration of antibiotic were to increase to above the MSC, at a later date, a significant surge in resistance may be seen. If no antibiotic had been present, or had only been present at concentrations lower than the MIPC, there would be fewer, or no antibiotic resistant bacteria and resistance genes in the environment for selection to act upon. It could be reasoned, therefore, that the MIPC, rather than the MSC or the LOEC, should be used when determining discharge limits that protect against the development of resistance in the environment, as this will also minimise human exposure to resistant bacteria in the environment and reduce probability of further resistance evolution.

The graph from the Gullberg et al. 2011 study, shown in Figure 3 in Chapter 1, has been revised to include the MIPC and is shown in Figure 77. As can be seen from this revised graph, both at sub-MIPC concentrations and throughout the sub-MSC persistence window, susceptible bacteria out-compete resistant bacteria. When a threshold concentration of antibiotic is reached (the MIPC) the growth rate of the susceptible bacteria starts to decrease. This reduces the susceptible to resistant bacterial ratio in the community, and hence increases the prevalence of the resistant bacteria, in comparison to when no antibiotic is present. Only when the MSC is reached, and the growth rate of the susceptible bacteria decreases below the growth rate of the resistant, is the resistant strain enriched over the susceptible and positive selection is observed.

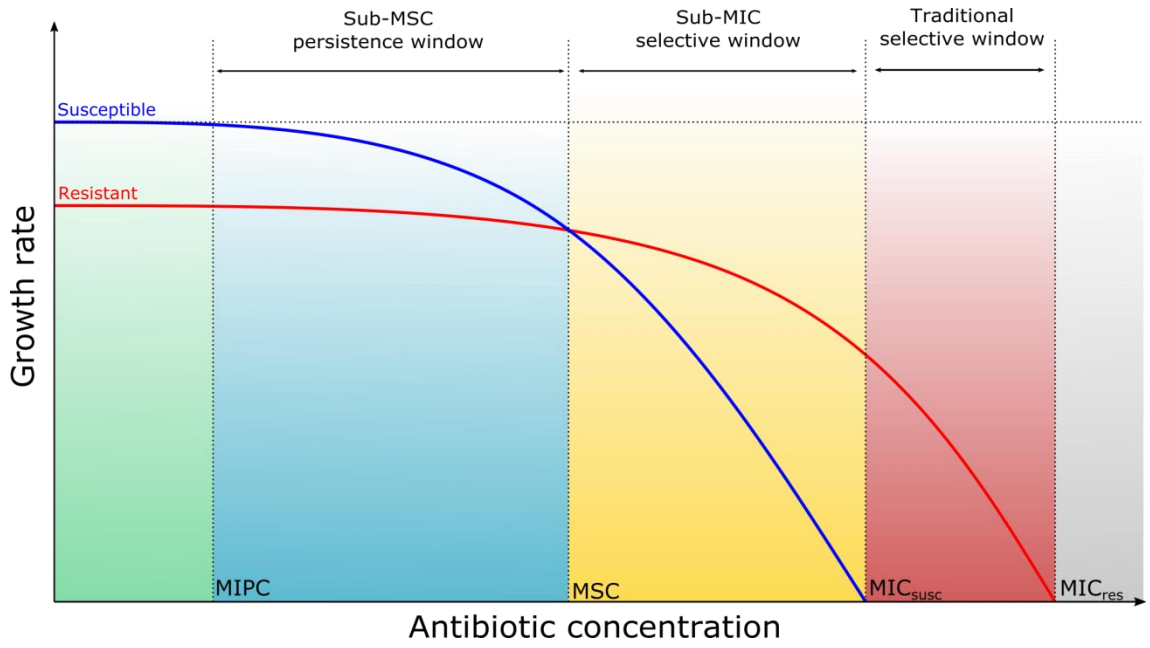


Figure 77: Bacterial growth rate as a function of antibiotic concentration. Graph adapted from Gullberg et al. 2011 to include the sub-MSC persistence window (blue area) and the MIPC. The blue line indicates the change of growth rate of susceptible bacteria with increasing concentrations of antibiotic. The red line shows the change in growth rate of resistant bacteria with increasing antibiotic concentrations.

6.5.3 Comparison to environmental concentrations of TET

The LOEC and MSC reported here were based on *intI1* prevalence and not on a TET specific resistance gene. These were 2,000 µg/L (LOEC) and 651 µg/L (MSC) of TET. PNECs (predicted no effect concentrations) of 100 and 65.143 µg/L were calculated by applying a 10-fold assessment factor to the NOEC and MSC, respectively. The metagenome analysis found that a significant increase in prevalence of total TET resistance genes was observed at 125 µg/L, although no lower concentration was sequenced. This was lower than the LOEC and the MSC defined by qPCR, but as sequencing was not undertaken on the day 0 samples it cannot be said with certainty that this was positive selection as

opposed to increased persistence. All of the values calculated here were, however, significantly higher than typical MECs of TET.

Environmental concentrations taken from the UBA's 2016 database determined a MEC_{max} of 2,260 µg/L of TET in surface water in China. This is only slightly higher than the LOEC but significantly higher than the MSC determined for *intI1.* This MEC is, however, highly unusual. The MEC_{mean} and MEC_{median} are much more indicative of the typical range of environmental concentrations of TET. These were 17.035 µg/L and 0.108 µg/L, respectively, and do not take into account the many studies where TET was below the limit of detection. The range of typical MECs (i.e. MEC_{mean} and MEC_{max}) are lower than the LOEC, MSC and PNECs determined here (Umweltbundesamt 2016).

6.5.4 Comparison to previously defined selective endpoints

TET MSCs determined by single species competition assays were 15 µg/L (from Gullberg et al. 2011) and 45 µg/L for plasmid based resistance, but decreased to 30 µg/L when this resistance element was transferred to a chromosome (Gullberg et al. 2014). The $PNEC_R$ calculated by Bengtsson-Palme & Larsson 2016 was 1 µg/L. It is expected that there would be differences between results from single species experiments and mathematical models on the one hand and the complex community experiments on the other hand. However, these results are significantly different to the selective endpoints determined in this chapter. The results from the work presented here would suggest that LOEC and MSC (defined here by *intI1*) may be underestimating selection of TET, although recent data suggests that a complex community context increases the MSC for gentamicin by over 40 times in comparison to single species assays (Klümper et al. 2019). TET resistance

genes from the metagenome data, illustrate a significant increase in prevalence over the control at every concentration tested. It is important, therefore, to use the information obtained by the metagenome analysis to inform further work using qPCR analysis to quantify other TET specific resistance genes. Results from that might then better align with previously defined selective endpoints.

6.5.5 *tetG* and *tetM*

No positive selection was observed for *tetG* at any concentration of TET in either of the selection experiments undertaken and reported in this chapter.

One explanation is that *tetG* is not maintained well in environmental bacteria. In a study by Wang et al. 2014, however, *tetG* was strongly correlated with *intI1* in reclaimed wastewater. It is possible to conclude, therefore, that *tetG* might follow a similar selective pattern to *intI1* and that these environmental bacteria are able to maintain this gene. This, however, was not observed in the experiments reported here. Another explanation is that the bacterial species carrying these resistance genes were not favoured by the experimental conditions. This does not, however, negate the MIPC and importance of quantifying the prevalence at day 0. If day 0 had not been tested at these concentrations, positive selection may have been determined as the outcome at 1 µg/L of TET. To fully understand what is occurring in experimental systems in relation to selection, quantifying the prevalence at day 0 is of great importance.

Increased persistence is not only observed for *tetG* selection by TET. In Chapter 3, Section 3.4.3, a significant increase in prevalence of *ermB* was observed in comparison to the no antibiotic control for the all three macrolide antibiotics tested. This increase in prevalence was never higher than the original prevalence in the inoculum indicating increased persistence. There was

not, however, a dramatic drop in *ermB* prevalence from the beginning to the end of the experimental period, as was observed with change of *tetG* prevalence. The bacteria that are harbouring the *ermB* genes do not, therefore, appear to be impacted by the experimental conditions. It is concluded, therefore, that an increase in persistence is a true phenomenon that can occur and that positive selection was not observed for *tetG* because of an inability to adapt the laboratory conditions.

For *tetM,* positive selection was observed at every concentration tested and in the no antibiotic control. This may be because the bacteria harbouring these genes are highly adapted to the experimental conditions. The increase observed at the no antibiotic control was, therefore, as a result of *tetM* harbouring bacteria outcompeting other bacteria less well adapted to these growth conditions. Only when a concentration of 128,000 µg/L of TET was reached was a significant increase in *tetM* genes seen in comparison to the no antibiotic control. This was determined, therefore, that this concentration of TET that is having a selective effect on this gene.

To fully understand whether growth and shift in the community structure is behind the results for *tetG* and *tetM,* a method such as epicPCR (Spencer et al. 2016) could be used in the future to link the resistance genes to their bacterial hosts. This could be used, in conjunction with 16S sequencing to determine diversity of the community, to fully understand the underlying mechanisms involved in the evolution of this community and the resistance genes it harbours.

6.5.6 Future work

Metagenome analysis identified alternative TET resistance genes that could be used to determine a more accurate LOEC and MSC than the *intI1* endpoint and that may align better with selective endpoints from previous studies. However, a point to note is that when the community context is considered the *intI1* MSC of approximately 651 µg/L is 14.5 times greater than the 45 µg/L reported by Gullberg *et al.*, 2014, which aligns closely to the 13-fold difference observed for a focal species in the absence/presence of a community under kanamycin selection (Klümper et al. 2019).

Three TET specific gene targets were promising candidates for future work on determining the MSC and LOEC of TET. *Tet32, tetA* and *tetW* all saw a biological effect at the lowest concentration of TET sequenced, with *tetW* determined to have a statistically significant increase at this concentration (125 µg/L). *Tet32* and *tetA* first saw a significant increase at 250 µg/L, the next concentration tested. QPCR should be undertaken on these genes to determine if this was positive selection, or significant increased persistence. *TetA* was tested in Lundström *et al.*, 2016. Whilst attempts were made to use the qPCR primers from this study, it was found that they were unable to determine specific melt curves and new primers would, therefore, need to be designed for further work.

6.6 Conclusion

The methods used in experiments for this thesis; in Stanton *et al.*, 2019 and Murray *et al.*, 2018 are comparable to the more environmentally relevant methods used in Lundström *et al.*, 2016 and Kraupner *et al.*, 2018. This has been demonstrated by the TET work presented in this chapter and by the work

undertaken by Aimee Murray on ciprofloxacin. The ciprofloxacin work determined a comparable MSC and LOEC to the LOECs determined in Kraupner *et al.*, 2018. The TET work, when analysing the data looking at day 7 only, also aligned well to the MSC value given in Lundström *et al.*, 2016.

The TET work has also provided a valuable insight into a sub-MSC persistence window phenomenon. It has demonstrated the value of quantifying the prevalence of genes at the beginning of the experimental period in order to fully understand the changes of antibiotic resistance gene prevalence at varying concentrations of antibiotics over time. This was also the first time that a concentration at which increased persistence (i.e. reduced rates of negative selection) has been shown. The concentration at which this phenomenon first occurs can be defined as the minimal increased persistence concentration (or MIPC).

An increase in persistence would lead to an increase in the absolute number of resistance genes found in the environment (compared to the situation where no antibiotic was present) and could potentially increase human exposure events to antibiotic resistance genes. It could be argued that the MIPC should be used (instead of the MSC) as the endpoint for defining safe release concentrations from wastewater treatment plants.

Metagenome analysis revealed a significant increase, in comparison to the control, at the lowest concentration of TET sent for sequencing. Work to calculate a true MSC/LOEC for TET is still being undertaken with potential targets for qPCR being identified from the metagenome analysis. If the significant increases in these resistance genes, observed in the metagenome analysis, are from an increase in persistence, rather than positive selection, the

MSC of TET, currently defined by *intI1*, would be significantly higher than typical measured environmental concentrations.

Chapter 7: Discussion

7.1 Real world implications and applications

The work presented in this thesis provides novel insights into selection for antibiotic resistance at environmentally relevant antibiotic concentrations. Work presented in Chapter 3 is the first study to determine experimentally derived selective endpoints in complex microbial communities for the three macrolide antibiotics on the European Commission's Water Framework Directive's priority watchlist (European Commission 2015b; European Commission 2018). This work showed that current measured environmental concentrations (MECs) of macrolide antibiotics are not likely to select for resistance whether genotypic or phenotypic. In addition, Chapter 4 presents the first work showing the effects that mixtures of antibiotic compounds have on selective endpoints, suggesting that these tend to have additive or synergistic effects on selective endpoints. Furthermore, there has been no previous work investigating temperature effects on selective endpoints. Preliminary data presented in Chapter 5 illustrates that certain macrolide resistance genes are under weaker selection at lower temperatures and that minimal selective concentration (MSC) increases with decreasing temperature. Finally, Chapter 6 defined the term minimal increased persistence concentration (MIPC) and described the phenomenon of increased persistence for the first time in a comparison to previous work by Lundström *et al.*, 2016.

The findings presented in this thesis can be used to inform policy makers about the risk antibiotic residues pose to human health via selection for resistance in bacteria found in the environment. With this information, informed decisions can

be made to decide whether implementation of mitigation strategies is needed to reduce or eliminate antibiotic residues from entering the environment.

7.1.1 Selective endpoints vary for different antibiotics

From data produced and presented in this thesis and from other work which investigated selection for resistance in complex microbial communities, such as that by Murray *et al.*, 2018 and Kraupner *et al.*, 2018, it is clear that a “one size fits all” approach is not appropriate for determining the selective endpoints of antibiotics. Antibiotics such as cefotaxime (Murray et al. 2018), ciprofloxacin (Kraupner *et al.*, 2018 and Figure 87 and 88 (Page 325 - Appendix)) and trimethoprim (TRMP) (Chapter 4) were selective at concentrations similar to those found in the environment when in isolation, whereas the macrolides (Chapter 3) and sulfamethoxazole (SMX) (Chapter 4) have selective endpoints that were significantly higher than current typical environmental concentrations.

It appears that the variation in antibiotic potency leads to different selective ability. If the potency of the antibiotic is high and a lower concentration is required to see an effect, it follows that there will also be a strong selective pressure for resistance to develop at these lower concentrations. For example, AZ is known to be more potent and to have lower minimum inhibitory concentration (MIC) values than erythromycin (ERY) (Retsema et al. 1987) and it was typically more selective than ERY as can be seen from *intI1* and *mphA* data from the qPCR analysis and total MLS (macrolide-lincosamide-streptogramin) resistance from metagenome analysis (Chapter 3). It is unclear whether mode of action influences the potency of an antibiotic and whether a correlation is observed between mode of action and selective ability. Based on the results from antibiotics that select at low concentrations, such as TRMP

which disrupts synthesis of folic acid (Eliopoulos & Huovinen 2001); ciprofloxacin which prevents DNA replication (Redgrave et al. 2014) and cefotaxime which inhibits cell wall synthesis (LeFrock et al. 1982), and those that select at higher concentrations, such as SMX which disrupts folic acid synthesis (Eliopoulos & Huovinen 2001) and the macrolides which inhibit protein synthesis (Mazzei et al. 1993; Kannan & Mankin 2011), there is no clear correlation between mode of action and selective ability. A correlation may, however, become evident if the selective ability of more antibiotics is tested as the selective endpoints of only a limited range of antibiotics have been tested to date.

Another explanation could be that there are differing fitness costs associated with the resistance genes tested. It is clear from the investigation in Chapter 3 that different macrolide resistance genes behave differently at the same concentrations of antibiotic. The lower the fitness cost of the resistance gene and the genetic material associated with it, the lower the concentration needed for selection. For example, integrons have relatively low fitness cost (Lacotte et al. 2017) whereas plasmids are known for their high fitness cost (San Millan & MacLean 2017). The *intI1* gene was quantified for all antibiotics tested. Concentrations of antibiotic needed to select for *intI1* varied greatly, ranging from a lowest observable effect concentration (LOEC) of 62.5 µg/L of TRMP (Chapter 4) to LOECs of 100,000 µg/L for clarithromycin (CLA) and ERY (Chapter 3). The *intI1* gene, encoding the class 1 integron, is often found in association with antibiotic resistance genes, and depending on the identity of the cassette genes associated with *intI1*, and where the integron is located (for example embedded in a plasmid (Gillings et al. 2008)), a different fitness cost may occur.

It is clear from the data presented in this thesis, and by other studies investigating selective endpoints, that environmental risk assessments must be undertaken on a compound by compound basis.

7.1.2 Designing a risk assessment for selective endpoints

Data presented in this thesis can be used to help inform the design of effective risk assessment protocols to help minimise selection for resistance in the environment. A number of factors should be taken into account:

1. It is important to use a mixed microbial community when undertaking these risk assessments. Mixed community experiments provide more realistic insights into how an antibiotic affects a range of bacterial species, as well as the resistance genes and mobile genetic elements (MGEs) associated with those species, and how competition within a community impacts selection (Klümper et al. 2019). Although using mixed communities is important, this method does come with limitations. Natural variation between replicates can often be high, presumably related to small founder effects and stochastic processes, which may mean that a biological effect is seen but statistical significance is not determined at the lower concentrations where effect size is small. This variation is also evident when determining the line of best fit for selection coefficient graphs, as the highest R^2 values are often relatively low. Increasing the number of replicates in these types of assays may be a way to minimise variation when undertaking risk assessments.

2. It is clear that compounds cannot be assessed on an individual basis to provide information about selection in the environment as, based on data provided in Chapter 4, mixing of antibiotics always produces a stronger selective effect than when compounds are found in isolation. An effective risk

assessment must include complex mixtures if it is to mitigate selection in the environment. Current work has only investigated the effect of mixtures on selection for class specific antibiotic resistance genes or the *intI1* gene. It is conceivable that a mixing effect of antibiotics could also increase co-selection for resistance genes effective against other antibiotic classes.

3. Using qPCR has been reported as the most sensitive method for assessing selection for resistance (Lundström et al. 2016; Murray et al. 2018), although choice of candidate gene can greatly influence the results observed (as can be seen from the different results observed between *ermF, mphA* and *intI1* for the macrolide antibiotics – Chapter 3). This would need to be taken into account when selecting gene targets for risk assessment. Metagenome sequencing can provide a useful tool to identify candidate genes. However, as has been seen with *ermF* (Chapter 3), the gene that responds at the lowest concentration does not always give the greatest response in terms of increase in prevalence. This could mean that, although a response is observed for a gene at a "lowest" concentration, other genes (such as *mphA* and *intI1*) may influence phenotypic resistance of the whole community on a greater scale. Quantifying *intI1* using qPCR may be a useful tool to compare different antibiotics when investigating selective endpoints, as it is independent of specific gene identity. It may be a useful starting point to understand the concentration range at which selection may occur when there are a number of genes that are able to confer resistance to a particular antibiotic.

4. It is critical to quantify the prevalence of the candidate gene in the inoculum or in the day 0 samples in order to fully understand the change in the prevalence of gene over time. Knowing the starting prevalence will mean it will

be possible to determine if positive selection is occurring or if it is, in fact, a significant increase in persistence. Without this, it is possible to misinterpret the data as a significant increase in positive selection when this may not, in fact, be the case.

5. Once there is sufficient data on how temperature affects selective endpoints, temperature may need to be taken into consideration when undertaking risk assessments. If there is found to be a temperature effect, the temperature at which the experiment is undertaken may need to be adjusted to best mimic the environmental conditions for which the risk assessment is being undertaken, although arguably 37 °C is more likely to reflect selection in human adapted strains which are of greatest concern. However, it is possible that further work will reveal that selective endpoints are independent of temperature: ciprofloxacin selection experiments at 25 °C (test tube system) and 20 °C (biofilm system) in Kraupner *et al.*, 2018 and at 37 °C (see Figure 87 and 88 (Page 325 - Appendix)) reveal similar selective endpoints. Although, as Kraupner *et al.*, 2018 did not quantify the starting prevalence of resistance in their inoculum, this similarity may be coincidental.

6. Finally, it will also be important for regulators to determine which selective endpoint to use for risk assessment. For positive selection, LOECs and MSCs are used throughout the work presented here. MSCs often provide a more protective selective estimate than LOECs. With natural communities, however, variation between biological replicates can often mean that a line of best fit (determined by statistical means) does not correlate well to the selection coefficient data. In addition, it is not always possible to determine a MSC value from a selection coefficient graph. If an increase in prevalence is observed over

time in the no antibiotic control (presumably as the bacteria harbouring these genes have a competitive advantage over other species in the starting inoculum), the line of best fit never crosses the x axis, which is the point that is defined as the MSC. Whereas the MSC might provide a more protective estimate, a LOEC value might provide a more realistic endpoint. Another consideration would be whether to use the MIPC as the selective endpoint as, although positive selection of resistance genes is not occurring, increased persistence (reduced rate of negative selection) could lead to increased numbers of resistant bacteria / resistance genes in the environment and, therefore, increased exposure risk and probability of horizontal gene transfer (HGT) and emergence of novel resistance mechanisms.

7.1.3 Holistic approach for environmental risk assessment

Work produced for this thesis, and by Murray *et al.*, 2018, shows that implementing mitigation strategies to minimise selection for resistance would be more protective than existing ecotoxicology test endpoints. For example, a MSC of 0.4 µg/L was determined for cefotaxime by Murray *et al.*, 2018. By applying a 10-fold assessment factor to this value, a predicted no effect concentration (PNEC) of 0.04 µg/L was calculated. This is lower than the PNEC for the environment provided by the "AMR Industry Alliance Antibiotic Discharge Targets" document which lists 0.1 µg/L as the PNEC calculated from traditional ecotoxicology data (AMR Industry Alliance 2018). This is, however, not always the case. For example, LOECs of 1,000 µg/L were determined for AZ and ERY and 750 µg/L of CLA in Chapter 3. By applying a 10-fold assessment factor to the no observable effect concentrations (NOECs), PNECs of 75, 50 and 75 µg/L are calculated for AZ, CLA and ERY, respectively. These values are significantly higher than the PNEC values for surface water presented in the

meta-analysis study by Le Page *et al.*, 2017 which were 0.019, 0.084 and 0.2 µg/L for AZ, CLA and ERY, respectively.

When undertaking risk assessment for antibiotics, and other co-selecting compounds, it is, therefore, important to determine the potential concentration thresholds for effects on non-target aquatic organisms in order to understand where, and at what concentration, mitigation needs to be implemented to protect the environment and human health.

7.2 The importance of sub-MSC selection and the MIPC

In Chapter 6, the persistence of antibiotic resistance genes was discussed and the MIPC defined. Increased persistence was identified for the first time when comparing the assay used in experiments presented in this thesis to the biofilm experiment reported in Lundström *et al.*, 2016. It was suggested in Chapter 6 that the MIPC might be a more appropriate and conservative estimate to use for environmental risk assessment, as opposed to using the MSC or LOEC, as it would be the most protective value to use when attempting to prevent the maintenance or enrichment of resistant bacteria and resistance genes in environmental microbial communities.

The work presented in Chapters 3, 4 and 5 used thresholds for positive selection (LOECs/MSCs) to define selective endpoints and compared these endpoints to current MECs. If the MIPC is used as the selective endpoint, there is a question of whether mitigation strategies would need to be implemented to reduce the risk of increased persistence where they were not previously required. Further, for antibiotics where mitigation strategies are already needed, because current MECs are similar to LOEC/MSCs, there is a question of whether more stringent mitigation measures would be needed to prevent

persistence of resistance genes. It was considered appropriate, therefore, to reanalyse the data presented in Chapters 3, 4 and 5 using the MIPC as the selective endpoint to determine whether there is potential for those genes tested in the previous chapters to persist at current MECs.

7.2.1 Selective endpoints of macrolides – work from Chapter 3

Table 9 shows a comparison of LOECs, MIPCs and MEC_{max} from data presented in Chapter 3.

Table 9: Comparison of LOECs, MIPCs and MEC_{max}s (data presented in Chapter 3). 1 = PNECs derived by applying 10-fold assessment factor to the NOEC. 2 = Gene used to define LOEC. 3 = PNEC calculated by applying a 10-fold assessment factor to MIPC. 4 = Gene used to define MIPC. 5 = MEC_{max} values presented in Table 5 (Chapter 3). Concentrations are in µg/L.

	LOEC	NOEC	PNEC[1]	Gene[2]	MIPC	PNEC[3]	Gene[4]	MEC_{max}[5]
AZ	1,000	750	75	*ermF*	10,000	1,000	*ermB*	1.5
CLA	750	500	50	*ermF*	100	10	*mef*	1
ERY	1,000	750	75	*ermF*	0.1	0.01	*ermB*	2.42

The MIPCs of CLA and ERY were lower than the LOECs. The MEC_{max} of CLA was 6.6 times lower than the PNEC derived from the MIPC, as opposed to 33.3 times when using the PNEC calculated from the LOEC. This may mean that strict monitoring of concentrations released from wastewater treatment plants (WWTPs) may need to occur and mitigation strategies implemented in the future. The MIPC of 0.1 µg/L of ERY, even without deriving a PNEC, was, however, lower than the MEC_{max}. Mitigation strategies would, therefore, need to be implemented, which is not the case for the PNEC calculated from the LOEC.

7.2.2 Effect of mixtures on selective endpoints – work from Chapter 4

Significant persistence of *intI1* was observed to 90% confidence at 200 µg/L of SMX in SMX experiment 1 (p = 0.0806, Dunn's test). If the MIPC is defined by using statistical significance to only 95% confidence, then no MIPC was found. It does, however, suggest an effect is occurring at 10-fold lower than the LOEC determined by the same experiment (2,000 µg/L). This value is now only 2.09 times higher (as opposed to 20.9 times higher when using the LOEC) than the MEC_{max} of SMX on the UBA database (when pharmaceutical effluent data has been removed) (Umweltbundesamt 2016).

7.2.3 Effect of temperature on selective endpoints – work from Chapter 5

The patterns of persistence observed for *ermF* and *mphA* are presented in Table 10. *IntI1* is not shown as no persistence was observed. For *mphA* increased persistence was observed at a similar concentration when the experiment was undertaken at 20 and 28 °C as where positive selection was observed in the 37 °C experiment.

Table 10: Comparison of LOECs and MIPCs of *ermF* and *mphA* at different temperatures. 1 = PNECs derived by applying 10-fold assessment factor to the NOEC. 2 = PNECs calculated by applying 10-fold assessment factory to MIPC. 3 = Significant increase to 90% confidence is observed at 10,000 µg/L. Concentrations are in µg/L.

Gene	Temperature	LOEC	NOEC	PNEC[1]	MIPC	PNEC[2]
ermF	37 °C	N/A[3]	N/A	N/A	100	10
	28 °C	N/A	N/A	N/A	N/A	N/A
	20 °C	N/A	N/A	N/A	750	75
mphA	37 °C	750	500	50	N/A	N/A
	28 °C	1,000	750	75	750	50

	20 °C	10,000	1,000	100	500	50

The qPCR targets were chosen for this temperature experiment based on the genes that were positively selected for in Chapter 3. For gene targets where no positive selection, but increased persistence, was observed (Chapter 3), these genes were not targeted in the temperature experiment (Chapter 5). It would be interesting to see, therefore, whether firstly, these genes still persist at temperatures below 37 °C and secondly, whether significant positive selection occurs at similar concentrations at which persistence was observed at 37 °C. The different bacterial hosts in which specific resistance genes reside may be more competitive at lower temperatures and, therefore, positive selection may occur at lower temperatures at concentrations where only significant persistence was previously observed at higher temperatures. Using qPCR to quantify genes such as *ermB* and *mef* family in this experiment may be an important next step to understanding the link between persistence, environmental conditions and lab assays.

7.2.4 Summary of the persistence phenomenon

Having analysed the data presented in this thesis from a persistence perspective, it is clear that this phenomenon occurs for a range of antibiotics from a number of different classes and not just for TET. An increase in persistence was observed for a number of antibiotic resistance genes at a range of antibiotic concentrations, including those indicative of environmental concentrations. As when determining MSCs/LOECs, a case by case basis approach would need to be taken to determine the MIPC for each antibiotic for each gene.

Data produced from the temperature experiment indicates that persistence occurring at a low temperature (20 or 28 °C) could be indicative of positive selection occurring at a higher temperature (37 °C). It would be interesting to test other genes that do not see positive selection at 37 °C to see if the opposite is true.

In some cases (such as *ermB* in the presence of ERY) a linear relationship was not observed for increased persistence. At relatively low antibiotic concentrations, a significant increase in persistence occurs, which then disappears at concentrations immediately above, but a significant increase was, once again, observed at even higher concentrations. It might be expected that the relationship between antibiotic concentration and resistance gene prevalence should be linear, however, in a complex bacterial community it is entirely possible that a certain species or group of species that are unable to compete at low concentrations without harbouring resistance genes will see an increase in resistance prevalence. As concentrations of the antibiotic become toxic to this species, the resistance prevalence of this gene may drop back down. Species that are more tolerant to the low levels of antibiotics may only significantly increase their resistant mutants at significantly higher concentrations of antibiotic. “Environmental filtering” (where it is proposed that the environment (here the antibiotic concentration) that species reside in, selects for certain species and affect community structure (Cadotte & Tucker 2017)) could explain why a non-linear dose response in prevalence was observed across an antibiotic concentration gradient. There are a multitude of factors that need to be considered such as host/gene interaction and gene/mobile genetic element interaction and the associated fitness cost of such

interactions at various antibiotic concentrations to explain the non-linear response observed.

7.3 Future work

In light of the novel data presented here, it is evident that there is still a significant amount of work needed to fully elucidate aspects of selection and maintenance of resistance genes in the environment. In addition to future research suggested in individual chapters (investigating the effect of more complex mixtures and continuing work to fully determine the effect of temperature on selective endpoints), there are two other key areas where further work is essential to help improve environmental risk assessment:

1. Investigating the effect of inoculum on selective endpoints.

It is clear in the case of TRMP and SMX (Chapter 4), TET (Chapter 6 in comparison to Lundström *et al.*, 2016) and ciprofloxacin (experiment undertaken by Aimee Murray, presented in Stanton *et al.*, 2019 and in the Figure 87 and 88 (Page 325 - Appendix) in comparison to Kraupner *et al.*, 2018) that different inocula does not significantly affect selective endpoints. This does not appear to be the case, however, for the macrolides for which the concentration at which positive selection was observed varies with inoculum used. This variation was particularly evident for *ermF.* In Chapter 3, a LOEC of 10,000 µg/L was established whereas in Chapter 5, a LOEC could not be determined when repeating the experiment under the same experimental conditions but with new inoculum. Significance to 90% confidence was observed at 10,000 µg/L, which was 10-fold higher than the LOEC determined in Chapter 3. For ERY, in Chapter 3 the LOEC was 1,000 µg/L. In the verification experiment reported in Chapter 4 (Figure 38), the LOEC was 500

µg/L and was, therefore, reduced by a half. It is unclear whether this change in response is a result of the gene, the bacterial species where it is found, the antibiotic or a combination. It is also unclear if overall macrolide resistance would also be affected, as data from the metagenome analysis showing MLS resistance, correlate better with other macrolide resistance genes and *intI1*. Understanding more about the inoculum effect and whether selective endpoints are significantly affected will be important in understanding the robustness of the assay used in data presented in this thesis and whether it can be used for determining selective endpoints as part of risk assessment.

2. Investigating whether persistence at one temperature is an indicator of positive selection at another

Increased persistence and the MIPC have been observed for a number of resistance genes for three of the four antibiotic classes tested. Results from the temperature experiment presented in Chapter 5 showed that significant positive selection at 37 °C could potentially be an indicator of significant persistence at lower temperatures. Next steps would be to investigate whether this is the case for other antibiotics and other resistance genes or whether it just a phenomenon observed for *mphA*. If increased persistence is occurring regularly for the majority of antibiotics, it may justify use of the MIPC as the selective endpoint for environmental risk assessment.

7.4 Concluding remarks

Data produced and presented in this thesis shows that there are many variables to consider when assessing thresholds of antibiotic concentrations that will limit selection for resistance in the environment. Selection for resistance can occur by the introduction of antibiotic residues from anthropogenic sources, such as

WWTPs. By limiting the release of antibiotic residues, the maintenance and persistence of resistance genes in environmental microbial communities may be reduced. Developing a risk assessment that is able to encompass all of these considerations is vital for reducing selection and for minimising human exposure events. Limiting mobilisation of environmental resistance genes to clinical pathogens, which may occur in polluted environments, could prove to be one critical approach to reducing resistance load when treating clinical infections and is, therefore, highly important in the fight against antibiotic resistance.

Appendix

Figure 78: Map showing the two wastewater treatment plant sampling sites in Cornwall. Red indicates the Camborne and Redruth plant which serves a population of approximately 34,000 people. Blue indicates the Falmouth and Penryn plant that serves a population of approximately 43,000 people. Map was made using ArcGIS 10.5.1.

Table 11: Measured environmental concentrations of macrolide antibiotics. Table shows the measured environmental concentrations of azithromycin (AZ), clarithromycin (CLA), erythromycin (ERY) and erythromycin – H_2O (ERY-H_2O). The matrix, location and reference for each study can also be seen. All concentrations are in µg/L.

Location	AZ	CLA	ERY	ERY-H_2O	Reference
WWTPs					
Ebro River Basin – Vallas	0.068	-	-	-	(Gros et al. 2007)
Ebro River Basin – Ebro	0.016	-	0.034	-	(Gros et al. 2007)
Ebro River Basin – Iregua	0.009	-	0.029	-	(Gros et al. 2007)
Ebro River Basin – Arga	0.022	-	0.037	-	(Gros et al. 2007)
Ebro River Basin – Ebro	0.023	-	0.071	-	(Gros et al. 2007)
Ebro River Basin – Segre	0.017	-	0.021	-	(Gros et al. 2007)
Ebro River Basin – Ebro	0.014	-	0.044	-	(Gros et al. 2007)
Tone River, Japan	0.165	0.568	-	-	(Nakada et al. 2007)
Grand Island, Nebraska	0.6695	-	-	-	(Bartelt-Hunt et al. 2009)
Columbus, Nebraska	0.0635	-	-	-	(Bartelt-Hunt et al. 2009)

Lincoln, Nebraska	1.5467	-	-	-	(Bartelt-Hunt et al. 2009)
Hastings, Nebraska	0.2835	-	-	-	(Bartelt-Hunt et al. 2009)
Omaha, Nebraska	0.6904	-	-	-	(Bartelt-Hunt et al. 2009)
River Sava, Serbia	0.150	-	-	-	(Grujić et al. 2009)
Milan, Italy	-	0.104	0.034	-	(Zuccato et al. 2010)
Varese, Italy	-	0.052	0.027	-	(Zuccato et al. 2010)
Lugano, Italy	-	0.437	0.059	-	(Zuccato et al. 2010)
Como, Italy	-	0.5	0.0065	0.017	(Zuccato et al. 2010)
Northwest Ohio (1)	-	0.702	-	-	(Spongberg & Witter 2008)
Northwest Ohio (2)	-	0.6106	-	-	(Spongberg & Witter 2008)
Lausanne, Switzerland	0.960	1	-	-	(Morasch et al. 2010)
Not stated	-	0.52	0.173	-	(Nödler et al. 2010)
Italy (urban wastewater)	-	0.0181	0.0474	-	(Zuccato et al. 2010)
Colorado (1)	-	0.01	-	-	(Ferrer et al. 2010)
Colorado (2)	-	0.04	0.021	-	(Ferrer et

					al. 2010)
Colorado (3)	-	0.172	1.2	-	(Ferrer et al. 2010)
Germany	-	0.24	-	2.5	(Hirsch et al. 1999)
Cilfynydd, Wales	-	-	-	1.152	(Kasprzyk-Hordern et al. 2009)
Coslech	-	-	-	0.019	(Kasprzyk-Hordern et al. 2009)
Iowa (1)	-	-	-	0.22	(Kolpin et al. 2004)
Iowa (2)	-	-	-	0.02	(Kolpin et al. 2004)
Pearl River Delta, S China (1)	-	-	0.43	-	(Weihai Xu et al. 2007)
Pearl River Delta, S China (2)	-	-	2.054	-	(Weihai Xu et al. 2007)
Pearl River Delta, S China (3)	-	-	0.216	-	(Weihai Xu et al. 2007)
Pearl River Delta, S China (4)	-	-	0.259	-	(Weihai Xu et al. 2007)
Surface Water					
Tone River, Japan – mainstream	0.008	0.012	-	-	(Nakada et al. 2007)
Tone River, Japan –	0.0065	0.013	-	-	(Nakada et al. 2007)

tributary					
River Danube, Serbia	0.055	-	-	-	(Grujić et al. 2009)
River Tamis, Serbia	0.036	-	-	-	(Grujić et al. 2009)
Lake Ocaga, Serbia	0.081	-	-	-	(Grujić et al. 2009)
River Lein, Germany	-	0.077	0.022	-	(Nödler et al. 2010)
Baltic Sea, Usedom	-	0.014	-	-	(Nödler et al. 2010)
River Po, Italy	-	0.0016	0.0032	-	(Zuccato et al. 2010)
River Lambro, Italy	-	0.0083	0.0045	-	(Zuccato et al. 2010)
Colorado (1)	-	-	0.052	-	(Ferrer et al. 2010)
Colorado (2)	0.005	0.005	0.007	-	(Ferrer et al. 2010)
Lake Wannsee, Germany	-	0.0089	-	-	(Heberer et al. 2008)
Germany	-	-	-	0.15	(Hirsch et al. 1999)
River Taff, Trefforest Estate	-	-	-	0.0195	(Kasprzyk-Hordern et al. 2007)
River Taff, Cardiff	-	-	-	0.0075	(Kasprzyk-Hordern et al. 2007)
River Taff, Brecon Beacons	-	-	-	0.003	(Kasprzyk-Hordern et al. 2008)

River Taff, Merthyr Tydfil	-	-	-	0.001	(Kasprzyk-Hordern et al. 2008)
River Taff, Abercynon	-	-	-	0.007	(Kasprzyk-Hordern et al. 2008)
River Taff, Pontypridd	-	-	-	0.091	(Kasprzyk-Hordern et al. 2008)
River Taff, Tefforest Estate	-	-	-	0.061	(Kasprzyk-Hordern et al. 2008)
River Taff, Cardiff	-	-	-	0.08	(Kasprzyk-Hordern et al. 2008)
River Taff, Abercynon	-	-	-	0.004	(Kasprzyk-Hordern et al. 2009)
River Taff, Pontypridd	-	-	-	0.052	(Kasprzyk-Hordern et al. 2009)
River Ely, Peterson-super	-	-	-	0.005	(Kasprzyk-Hordern et al. 2009)
Llobregat River Basin	-	-	0.030	-	(Muñoz et al. 2009)
Southern Ontario	-	-	0.0056	-	(Lissemore et al. 2006)
Victoria Harbour, Hong Kong	-	-	-	0.00335	(Wei-hai Xu et al. 2007)
Pearl River (high water season)	-	-	-	0.03	(Wei-hai Xu et al. 2007)

Pearl River (low water season)	-	-	-	0.46	(Wei-hai Xu et al. 2007)
Thames, Oxford	0.03	0.092	0.236	-	(Singer et al. 2014)
Thames, Benson	0.034	0.05	0.244	-	(Singer et al. 2014)
Yangtze Estuary, China	-	-	0.00757	-	(Zhao et al. 2015)
Jianghan Plain, China (winter)	-	-	0.510	-	(Yao et al. 2015)
Jianghan Plain, China (spring)	-	-	2.42	-	(Yao et al. 2015)
Umgeni River, S Africa (North outlet)	-	-	0.24	-	(Matongo et al. 2015)
Jianhan Plain, China	-	-	-	4	(Tong et al. 2014)
Wangyan River, China	-	-	-	0.0678	(Y. Jiang et al. 2014)
Yangtze Estuary, China	-	-	-	0.0896	(Shi et al. 2014)
Llobregat, Spain	-	-	0.3625	-	(Osorio et al. 2012)
Llobregat, Spain			0.107		(Osorio et al. 2012)
Ebro, Spain	-	-	0.071	-	(Osorio et al. 2012)
Ebro, Spain	0.00457	0.0121	-	-	(López-

					Serna et al. 2012)
Ebro tributaries, Spain	0.0044	0.022	-	0.0007	(López-Serna et al. 2012)
River Po, Mezzana Corti, Italy	-	0.00178	0.00078	0.00166	(Zuccato et al. 2010)
River Po, Monticelli Pv, Italy	-	0.00089	0.00351	0.00427	(Zuccato et al. 2010)
River Po, Piacenza, Italy	-	0.00219	0.00462	0.00531	(Zuccato et al. 2010)
River Po, Cremona, Italy	-	0.00189	0.00282	0.00363	(Zuccato et al. 2010)
River Arno, Rignano sull'Arno, Italy		0.0067	0.00391	0.01396	(Zuccato et al. 2010)
River Arno, Limite sull'Arno, Italy		0.01655	0.00288	0.00968	(Zuccato et al. 2010)
River Arno, Castelfranco, Italy		0.03359	0.00681	0.01729	(Zuccato et al. 2010)
River Arno, Pisa, Italy		0.04479	0.00812	0.03052	(Zuccato et al. 2010)
Ground Water					
Belgrade, Serbia	0.025	-	-	-	(Grujić et al. 2009)

Belgrade, Serbia	0.14	-	-	-	(Grujić et al. 2009)
Jianghan Plain, China	-	-	-	2.3	(Tong et al. 2014)
Downstream of WWTPs					
Chivasso, N. Italy	-	0.0203	0.0159	-	(Calamari et al. 2003)
Mezzano, N. Italy	-	0.00124	0.00392	-	(Calamari et al. 2003)
Boscone, N. Italy	-	0.00156	0.00324	-	(Calamari et al. 2003)
Pacenza, M. Italy	-	0.00338	0.00456	-	(Calamari et al. 2003)
Cremona, N. Italy	-	0.00049	0.00141	-	(Calamari et al. 2003)
Casalmaggiore, N. Italy	-	0.0008	0.0014	-	(Calamari et al. 2003)
Pieve Saliceto, N. Italy	-	0.00167	0.00275	-	(Calamari et al. 2003)
Parco Lambro, N. Italy	-	0.00831	0.0045	-	(Calamari et al. 2003)

Figure 79: *mef* family as a function of low azithromycin concentrations (1 high outlier replicate removed). Standard error is represented by the error bars.

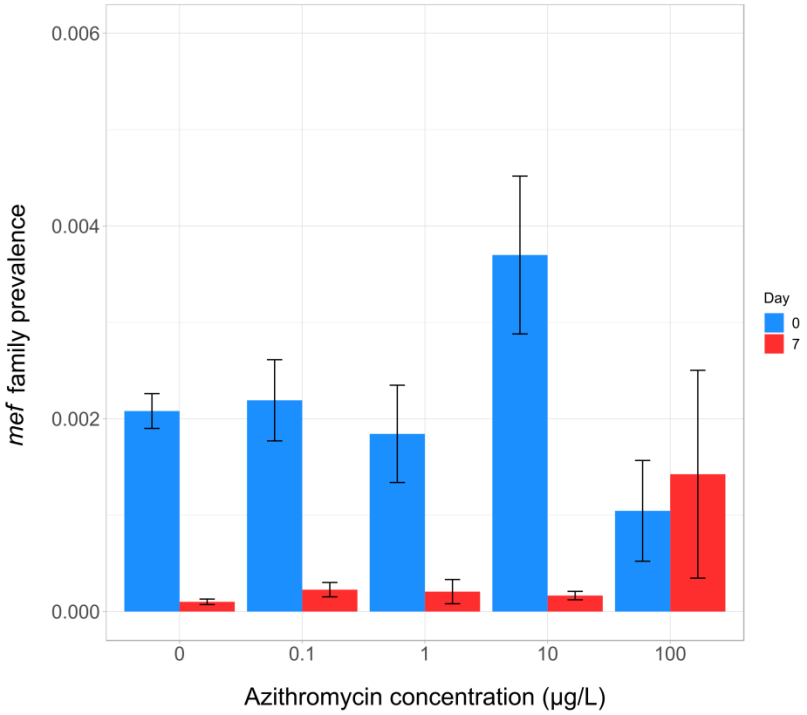


Figure 80: Scatter plot showing the variation in *mphA* prevalence at day 7 in samples selected for by increasing concentrations of AZ.

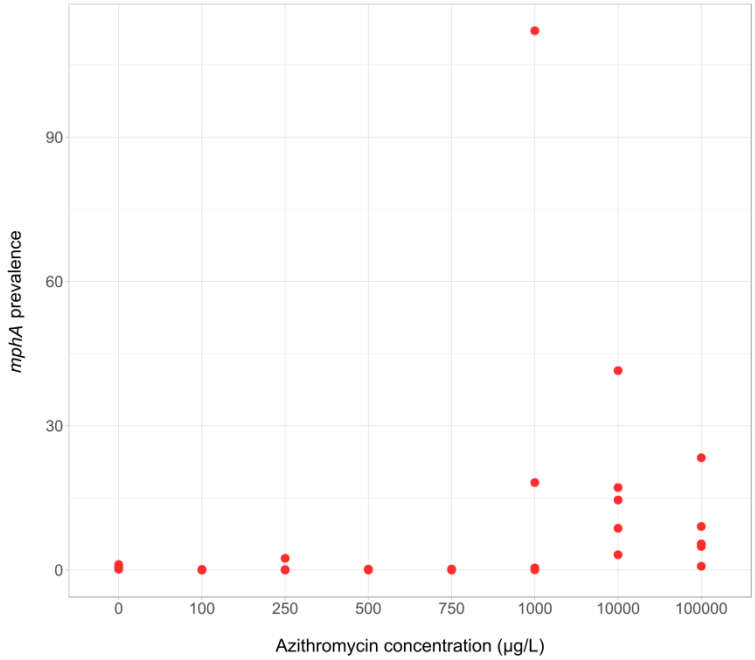


Figure 81: Graph showing the selection coefficient of *ermF* by AZ. The line of best fit (polynomial order 3, $y = 0.2305 + 0.001955x - 4.514e^{-06}x^2 + 2.9e^{-09}x^3$, $R^2 = 0.3268$, standard error = 0.165) lies above and never crosses the x axis. A MSC can never, therefore, be determined.

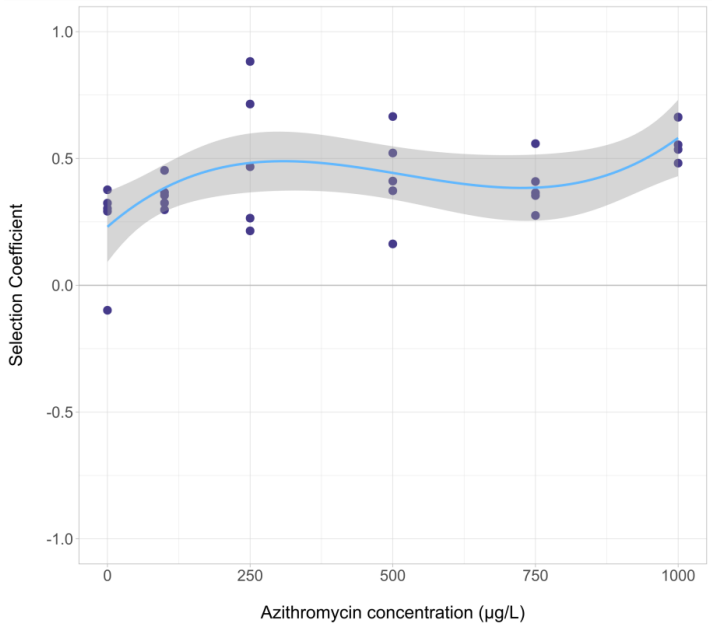


Figure 82: Graph showing the selection coefficient of *ermF* by CLA. The line of best fit (polynomial order 3, $y = 0.05846 + 0.0006282x - 2.2e^{-06}x^2 + 1.752e^{-09}x^3$, $R^2 = 0.07934$, standard error = 0.2595) never crosses the x axis and no MSC can, therefore, be determined for this dataset.

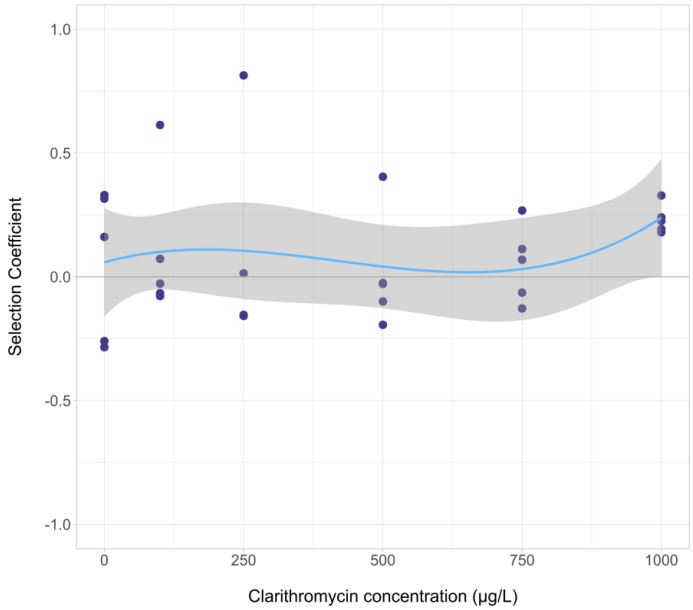


Figure 83: Bar chart showing the change in *ermB* with increasing concentrations of ERY from the metagenome dataset. A biological effect is observed at every concentration of ERY but this is not reflected by the statistical analyses. Standard error is represented by the error bars.

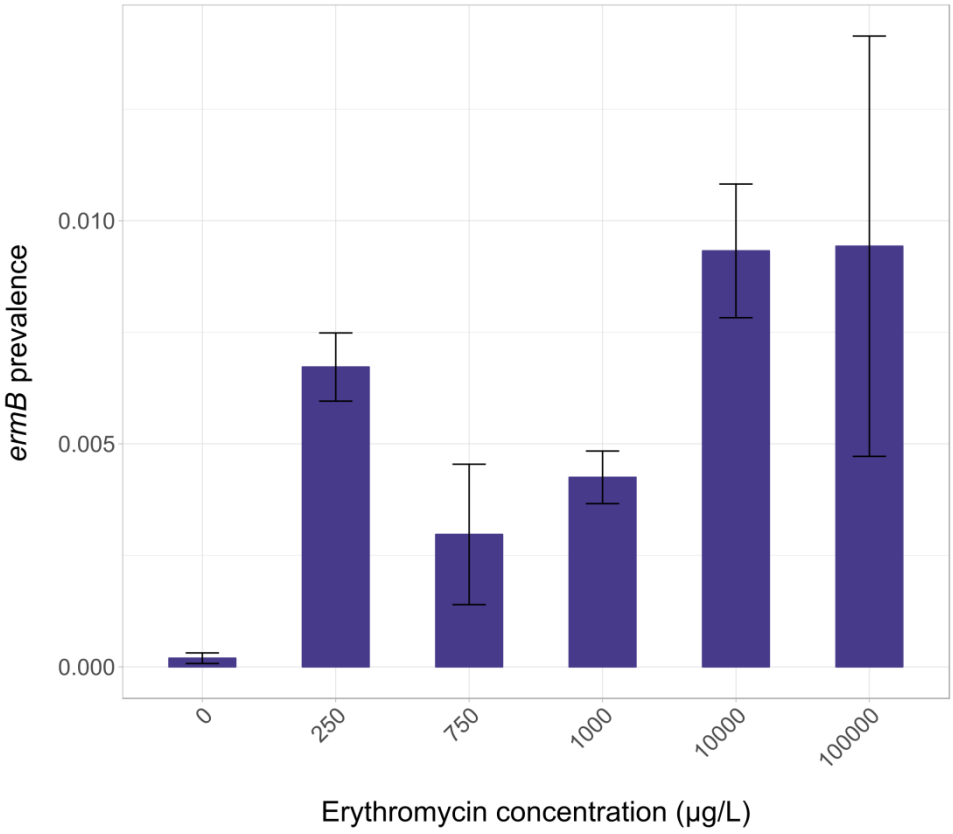


Table 12: Table showing the macrolide resistance genes prevalence data as a function of AZ concentration. This corresponds to the heat map in Figure 24.

AZ	Concentration (µg/L)					
Gene	**0**	**250**	**750**	**1,000**	**10,000**	**100,000**
ereA	3.54103E-05	0	0	0	0	0
erm(33)		4.6E-05		2.81E-05	3.31E-05	0.000474
erm(35)	0	0	0	1.28E-05	0	0
erm(TR)	0.00746485	0.006062	0	7.02E-05	0	0
ermA	0.000194058	8.49E-05	1.53E-05	7.38E-05	0.000248	0.000706
ermB	0.023137095	0.009235	0.002392	0.002942	0.016567	0.01461
ermC	0.031146696	0.018534	0.003893	0.003684	0.027257	0.654675
ermF	0.000277894	0.000993	0.005862	0.001605	0.00387	0
ermG	1.77022E-05	0.000232	0.000164	7.89E-05	0.000231	0
ermT	1.77022E-05	0	2.7E-05	0	0	0.000184
ermX	0	8.84E-05	0.000105	0.000273	0.001221	0
macA	0.044334584	0.05598	0.058576	0.061876	0.056861	0.018053
macB	0.037428599	0.047557	0.041486	0.049973	0.05255	0.017585
mefA	0.000249631	0.001168	0.000658	0.000296	0.000241	0
mphA	0.034428077	0.073797	0.005827	0.329763	0.773598	0.811059
mphC	0	0	0	0	4.08E-05	0
msrA	0	0	0	0	3.09E-05	0
msrC	0	0.000301	0.00033	4E-05	0.000892	0.000914

Table 13: Table showing the macrolide resistance genes prevalence data as a function of CLA concentration. This corresponds to the heat map in Figure 25.

CLA	Concentration (µg/L)					
Gene	**0**	**250**	**750**	**1,000**	**10,000**	**100,000**
ereA	0	0	3.28E-05	0	0	0
erm(33)	3.6E-05	2.53539E-05	0.000105412	2.71E-05	8.08E-05	0.000263
erm(35)	1.62E-05	0	8.88741E-05	0	0	0
erm(TR)	0.004957	0.002411652	0.003208841	0	0	0
ermA	0.000779	0.000749452	0.001184186	0.000166	0.000965	0.002859
ermB	0.000569	0.009769525	0.008855561	0.005629	0.002608	0.006882
ermC	0.000942	0.015195154	0.017274193	0.011173	0.0078	0.049227
ermF	0.000361	0.002727658	0.002717686	0.004233	0.007371	0
ermG	5.28E-05	5.05E-05	0.000560938	0.001114	0.000998	0
ermT	1.77E-05	0	0.000775099	0	0	5.43E-05
ermX	0.000411	0.000283887	0.000578388	0.000643	0	0
macA	0.051843	0.058223806	0.05135268	0.060243	0.058272	0.054447
macB	0.038174	0.046608147	0.039035005	0.048187	0.055031	0.053273
mefA	4.02E-05	0.0003612	0.000234183	0.000974	0.002772	9.76E-05
mphA	0.061343	0.01591802	0.017642728	0.025576	0.177631	0.625464
mphC	0	8.24215E-05	0	0	0	8.56E-05

msrA	0	5.95362E-05	0	0	0	8.16E-05
msrC	0.000152	0.000247554	0.000597686	0.000342	0.000374	0.000309

Table 14: Table showing the macrolide resistance genes prevalence data as a function of ERY concentration. This corresponds to the heat map in Figure 26.

ERY	**Concentration (µg/L)**					
Gene	**0**	**250**	**750**	**1,000**	**10,000**	**100,000**
ereA	0	3.598E-05	0	0	0	1.43E-05
ereB	0	0	0	0	0	3.2991E-05
erm(33)	5.92E-05	0	0	0	6.9E-05	0
erm(TR)	0.00826	0	0	0	0	0
ermA	0.0012979	0.00118248	0.00022	0.00013	0.00092	0.000443
ermB	0.0001945	0.0067202	0.00297	0.00425	0.00932	0.009428
ermC	0.0010816	0.0129639	0.00515	0.00375	0.009	0.0159287
ermF	0.0003654	0.00062207	0.000905	0.00362	0.00754	0.00033338
ermG	0	5.538E-05	6.14E-05	0.00025	0.00146	0.000643
ermT	2.95E-05	0	0	0	0.00152	0
ermX	0.000103	0.000225	0.001027	0.00066	0.0002	0
macA	0.049802	0.0514219	0.058285	0.0558	0.05732	0.05303
macB	0.03324223	0.051526516	0.05309	0.05211	0.050329	0.057072269
mefA	1.815E-05	0.000455	4.98E-05	0.00093	0.00013	5.066E-05
mphA	0.0974475	0.00816996	0.00022	0.027	0.1371	0.68416351
msrA	0	0	1.02E-05	0	0	0
msrC	0.000157	0.0003775	6.84E-05	0.00025	0.00035	0.0006817

Table 15: Table showing the prevalence data of antibiotic resistance gene classes as a function of AZ concentration. This corresponds to the heat map in Figure 29.

AZ	**Concentration (µg/L)**					
Class	**0**	**250**	**750**	**1,000**	**10,000**	**100,000**
Aminoglycoside	0.064836	0.134292	0.035647	0.508072	1.249978	1.122161
Bacitracin	0.046971	0.055234	0.043477	0.051003	0.05991	0.076859
Beta-lactam	0.173268	0.130162	0.09282	0.29972	0.578996	0.734592
Bleomycin	0	0	4.52E-05	2.23E-05	2.63E-05	3.17E-05
Chloramphenicol	0.027909	0.0118	0.028812	0.046269	0.063851	0.018922
Fosfomycin	0.008343	0.002661	0.005452	0.001697	0.000618	0.001479
Fosmidomycin	0.04222	0.05944	0.04929	0.05864	0.05855	0.02023

	8	6	5	5	5	9
Fusaric-acid	0	0	0	0	0	2.28E-05
Kasugamycin	0.047015	0.062697	0.058164	0.061873	0.06412	0.02292
Multidrug	1.309957	1.548926	1.455131	1.647193	1.682531	0.810372
Polymyxin	0.042324	0.050711	0.045189	0.055235	0.056828	0.01818
Quinolone	0.007438	0.000961	0.001823	0.000292	0.003098	0.001258
Sulfonamide	0.025742	0.110017	0.030502	0.468063	1.1179	1.042225
Tetracycline	0.141378	0.110806	0.084336	0.272187	0.527342	0.320601
Trimethoprim	0.025866	0.095662	0.011893	0.434083	1.130699	1.002218
Unclassified	0.337331	0.405545	0.372879	0.438595	0.464	0.239836
Vancomycin	0.001681	0.001713	0.002638	0.001664	0.015323	0.009134

Table 16: Table showing the prevalence data of antibiotic resistance gene classes as a function of CLA concentration. This corresponds to the heat map in Figure 30.

CLA	**Concentration (µg/L)**					
Class	**0**	**250**	**750**	**1,000**	**10,000**	**100,000**
Aminoglycoside	0.121343	0.313793	0.107446	0.075559	0.289735	1.009885
Bacitracin	0.046778	0.045918	0.047371	0.052279	0.059788	0.069938
Beta-lactam	0.157605	0.173737	0.105326	0.156915	0.204208	0.483602
Bleomycin	2.79E-05	0	0	0	4.14E-05	0
Chloramphenicol	0.027571	0.069731	0.028857	0.019905	0.022062	0.058845
Fosfomycin	0.006413	0.003342	0.001969	0.003083	0.001298	0
Fosmidomycin	0.049008	0.054552	0.048682	0.055745	0.060696	0.060355
Fusaric-acid	0	0	0	0	0	0.00033
Kasugamycin	0.049805	0.056467	0.050712	0.054822	0.065085	0.063413
Multidrug	1.428198	1.560356	1.373171	1.55341	1.760276	1.713875
Polymyxin	0.044932	0.047489	0.041873	0.047118	0.05729	0.0576
Quinolone	0.000863	0.000304	0.000178	0.000682	2.96E-05	7.02E-05

Sulfonamide	0.090819	0.07167	0.036275	0.051644	0.254319	0.847377
Tetracycline	0.125086	0.338908	0.127565	0.104171	0.167268	0.478799
Trimethoprim	0.09168	0.079996	0.037526	0.037294	0.237213	0.826292
Unclassified	0.379879	0.404837	0.354555	0.397953	0.452961	0.467602
Vancomycin	0.006402	0.005937	0.005986	0.008493	0.003622	0.008511

Table 17: Table showing the prevalence data of antibiotic resistance gene classes as a function of ERY concentration. This corresponds to the heat map in Figure 31.

ERY	**Concentration (µg/L)**					
Class	**0**	**250**	**750**	**1,000**	**10,000**	**100,000**
Aminoglycoside	0.121343	0.13118	0.24099	0.113331	0.235498	1.088504
Bacitracin	0.046778	0.059976	0.060405	0.057147	0.054702	0.06338
Beta-lactam	0.157605	0.141154	0.145167	0.107974	0.164816	0.502178
Bleomycin	2.79E-05	0	0	0	0.000197	0.00018
Chloramphenicol	0.027571	0.01972	0.042148	0.02304	0.023455	0.061199
Fosfomycin	0.006413	0.001043	0.001496	0.002063	0.000856	5.2E-05
Fosmidomycin	0.049008	0.055361	0.061701	0.059681	0.057967	0.064057
Kasugamycin	0.049805	0.059564	0.064642	0.059278	0.061201	0.069634
Multidrug	1.428198	1.676908	1.784189	1.739626	1.691372	1.737235
Polymyxin	0.044932	0.051423	0.056288	0.054263	0.052438	0.058516
Quinolone	0.000863	3.1E-05	9.41E-05	0.000173	3.08E-05	0
Sulfonamide	0.090819	0.057553	0.050911	0.058768	0.182493	0.929188
Tetracycline	0.125086	0.175906	0.212917	0.129694	0.151408	0.495229
Trimethoprim	0.09168	0.037514	0.046453	0.045487	0.172099	0.880979
Unclassified	0.379879	0.422671	0.466709	0.442494	0.439275	0.467317
Vancomycin	0.0064	0.0056	0.00318	0.00669	0.0067	0.0127

Figure 84: Heatmap showing the total community structure of samples passaged in the presence of azithromycin.

Table 18: Table showing the data that corresponds to the heatmap shown in Figure 34. Data is the output from MetaPhlAn2 is relative abundance of species found in the day 7 sample of the AZ evolution experiment. Relative abundance is calculated by normalising the total reads per clade to the nucleotide length of the clade's key markers (Segata et al. 2012). E. unclassified = Escherichia unclassified.

Azithromycin (µg/L)	**0**			**250**			**750**			**1000**			**10000**			**100000**		
V. ratti	8.64	0	21.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. infantarius	3.30	0.95	2.67	0	0	0	0.01	0.03	0	0	0.005	0	0.02	0	0.01	0.33	0.19	0.03
A. baumannii	0.54	0.72	0.00	0	0	0	0	0	0	0	0	0	0	0.22	0	0	0	0
P. harei	1.40	16.85	0.00	0	11.44	0.03	0	0	0	0	0	0	0	0	0	0	0	0
S. aureus	0.77	16.57	1.13	0	0.00	0.00	0	0	0	0	0	0	0	0	0	0	0	0
E. limosum	0.01	0.22	0.00	0.21	1.98	0.56	0	0	0	0	0	0	0	0	0	0	0	0
B. dorei	0.22	0.28	0.09	0.03	1.32	0.33	2.36	0	0.06	0.85	0.96	0.19	0	0	0	0	0	0
F. ulcerans	3.61	0.15	0.00	0.00	1.62	3.95	6.79	0	0	0.02	0	0.02	0	0	0	0	0	0
M. morganii	0.68	0.44	0.54	1.83	0.99	1.39	0.43	1.30	0.39	1.05	0.49	0.53	3.17	1.08	1.54	0	0	0
B. uniformis	1.17	0	0.53	0.90	1.48	1.12	0.47	1.47	0.56	0.58	0.87	0.45	1.08	8.30	0.54	0	0	0
C. freundii	0.30	0.19	0.11	0.20	0.07	0.02	0.25	0.12	0.07	0	0.21	0.30	0.01	0	0	0	0	0
B. thetaiotaomicron	0.42	0.29	0.11	0.32	0.33	0.32	0.23	0	0.60	0	0	0.61	0	0	0	0	0	0
B. fragilis	0.17	0.88	0.30	0.25	0.18	0.10	0.94	0.41	0.10	0	0	0.22	0.26	0	0	0	0	0
P. alcalifaciens	0.00	0	0.00	0.03	0.00	0.06	0.48	0.39	0.08	0	0.22	0.06	0	0	0	0	0	0
E. cloacae	0.13	0	0.03	0.62	0.05	0.15	0.35	1.10	0.05	0.05	0.18	0	0	0	0	0	0	0
E. faecium	0.00	0	0.00	0.76	0.00	0.00	0.62	0	0.29	0.18	0	0.03	1.18	1.19	0.78	0.97	0.65	1.46

E. avium	0.14	0.17	0.13	0.15	0.50	0.44	0.31	0.07	0.09	0	0.21	0	0.45	0	0.42	0.19	1.21	1.35
E. faecalis	0.43	0.10	1.84	0.74	0.10	1.21	0.00	0.02	0	0.06	0	0	0	0.05	0	2.00	0.60	1.73
S. lutetiensis	0.00	0	0.00	0.04	0.11	0.12	0.06	0.24	0	0	0.10	0.05	0.06	0	0.03	0.98	0.51	0.17
P. mirabilis	6.45	13.6	21.26	4.78	2.61	12.53	48.23	24.66	20.41	5.11	45.98	13.42	16.56	7.64	0.15	0.00	0	0
E. unclassified	17.36	9.1	10.52	24.82	21.74	24.63	6.77	13.58	15.12	22.25	14.76	8.97	18.33	11.47	16.59	4.78	8.10	14.45
K. pneumoniae	3.40	0.8	1.07	5.70	5.62	10.29	8.74	25.34	8.53	4.29	10.56	2.32	0	0.17	9.08	0.00	0	0
E. coli	49.51	34.1	38.11	57.31	46.27	42.28	19.76	16.24	50.58	65.19	24.58	57.46	55.15	69.61	70.01	9.97	20.47	38.82
C. phage CR5	0.00	0	0.00	0	0	0	0	1.15	2.68	0	0	14.93	0	0	0	0.00	0	0
S. haemolyticus	0.00	0	0.00	0	0	0	0	0	0	0	0	0	0	0	0	80.47	68.26	0

Figure 85: Heatmap showing the total community structure of samples passaged in the presence of clarithromycin.

Table 19: Table showing the data that corresponds to the heatmap shown in Figure 35. Data is the output from MetaPhlAn2 is relative abundance of species found in the day 7 sample of the CLA evolution experiment. Relative abundance is calculated by normalising the total reads per clade to the nucleotide length of the clade's key markers (Segata et al. 2012). E. unclassified = Escherichia unclassified. K. unclassified = Klebsiella unclassified.

Clarithromycin (µg/L)	**0**			**250**			**750**			**1000**			**10,000**			**100,000**		
S. simulans	6.45	0.23	2.21	0	0	0	4.23	0	0	0	0	0	0	0	0	0	4.50	0
S. haemolyticus	0	0.02	0	0	0	0	0	0	0.27	0	0	0	0.13	0	0	2.28	3.50	1.40
E. faecalis	0	0.31	0.09	2.80	0	0	12.22	0	0	1.96	0	0	1.28	1.10	0	2.93	1.18	0.14
P. harei	0.05	0	10.93	0	0	0.07	0.04	2.04	0	0	0	0	0	0	0	0	0	0
S. aureus	0	6.20	12.63	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. infantarius	4.33	0.14	8.20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. unclassified	6.69	22.18	9.53	11.82	17.55	9.00	8.90	14.00	21.86	26.63	16.80	14.08	26.66	15.32	15.95	22.12	20.01	28.07
E. coli	40.24	36.44	9.40	40.81	44.30	35.98	27.62	37.79	42.51	49.01	53.21	30.30	60.51	53.06	67.32	72.10	68.21	67.21
P. mirabilis	33.33	21.09	40.61	33.68	21.51	25.18	34.55	23.60	9.49	4.08	2.01	37.73	0	23.82	0.62	0	0	0
K. pneumoniae	4.33	2.24	3.51	6.13	5.23	22.42	7.40	12.95	7.37	11.90	12.62	8.93	3.27	3.61	8.12	0	0	0.07
V. ratti	0	0	0	0	0	0	0	1.93	14.49	0	0	0	0	0	0	0	0	0
E. faecium	0.09	0.20	0.04	0.25	0.25	0.33	0	1.77	0.55	0.02	0	1.29	0	0	1.67	0	0	1.01
B. dorei	0.29	0.36	0.15	0.29	0	0	0	0.22	0	0	2.67	0.83	0	0	2.20	0	0	0
M. morganii	0.78	0.03	0.32	0.69	0.92	0.86	0.59	0.56	0.83	0.94	2.60	0.07	0.15	0	0	0	0	0
B. uniformis	0.83	0.53	1.00	1.87	3.96	1.03	1.56	3.41	0.82	3.54	1.74	2.01	0.20	0.00	3.30	0	0	0
K. unclassified	0	0.22	0	0	0	0	0	0.51	0	0	1.17	0.97	0	0.68	0	0	0	0
B. ovatus	0.44	0.09	0.15	0	0	0	0.51	0	0.10	0.01	1.45	0.03	0	1.70	0	0	0	0

E. cloacae	0.12	0.14	0	0.11	0.18	0.48	0.27	0.35	0.07	0.06	0.10	0.24	0.04	0	0	0	0	0
C. freundii	0.33	0.01	0.03	0.14	0.06	0.47	0.14	0.01	0.15	0.13	0	0.19	0.04	0	0	0	0	0
B. pseudolongum	0	0	0	0.26	0	0.43	0.50	0.29	0.30	0	0	1.77	0	0	0	0.02	0	0
E. avium	0.59	0.62	0.22	0.08	0.62	1.69	0.16	0	0.46	0.01	0.03	1.07	0.18	0	0	0.21	0.58	1.60
B. thetaiotaomicron	0.37	0.58	0.17	0	0.69	0.49	0	0	0.46	0	0	0	0	0	0	0	0	0
E. raffinosus	0	0.13	0	0.46	0	0	0	0	0	0	0	0	0	0.17	0.58	0	0	0
E. asini	0	0	0	0.34	0	0	0.66	0	0	0.37	0	0	0	0	0	0	0	0
B. fragilis	0.21	0.63	0.42	0	0	0	0.18	0	0	0	0	0	6.75	0	0	0	0	0

Figure 86: Heatmap showing the total community structure of samples passaged in the presence of erythromycin.

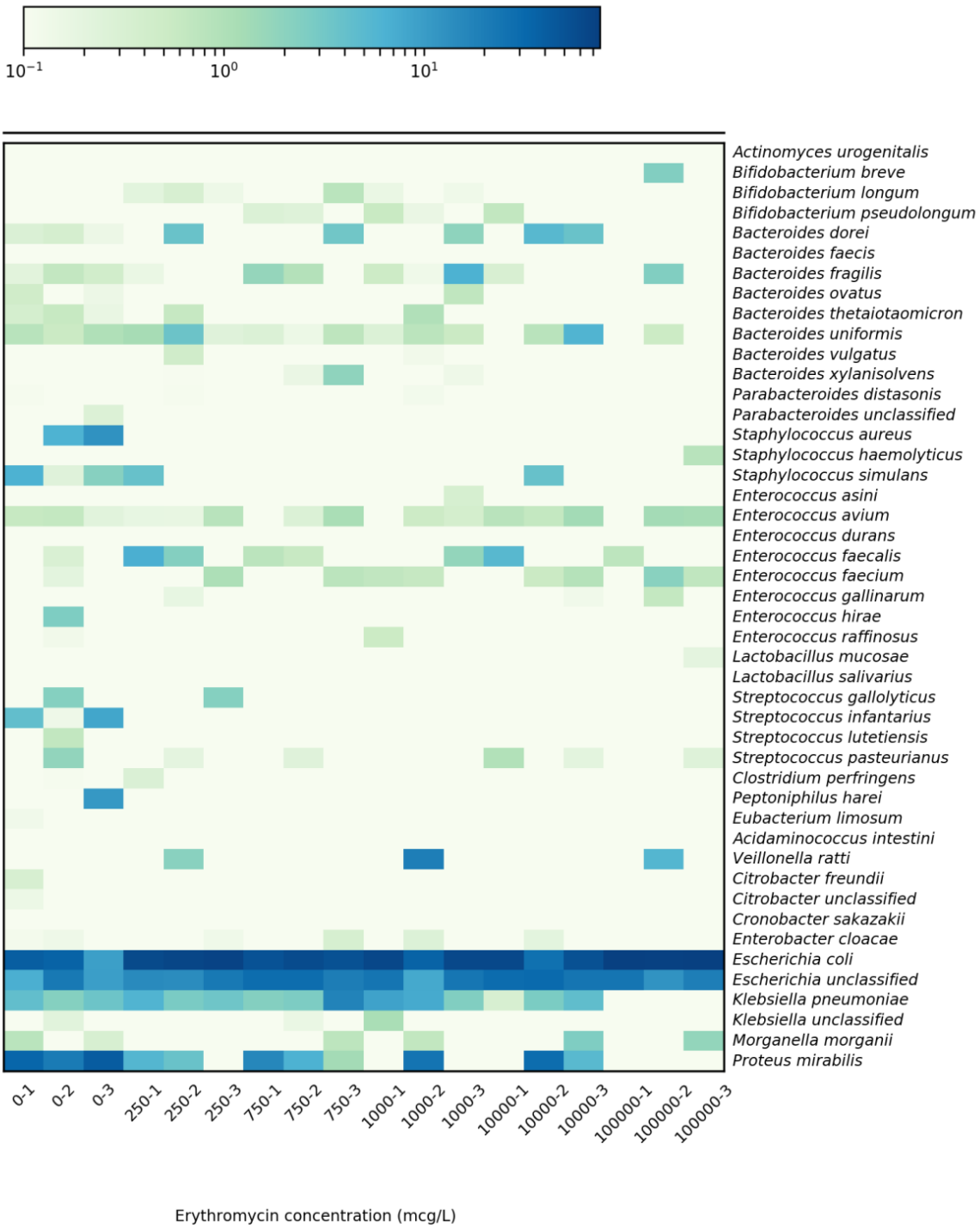


Table 20: Table showing the data that corresponds to the heatmap shown in Figure 36. Data is the output from MetaPhlAn2 is relative abundance of species found in the day 7 sample of the ERY evolution experiment. Relative abundance is calculated by normalising the total reads per clade to the nucleotide length of the clade's key markers (Segata et al. 2012). E. unclassified = Escherichia unclassified. K. unclassified = Klebsiella unclassified.

Erythromycin (µg/L)	**0**			**250**			**750**			**1000**			**10,000**			**100,000**		
K. pneumoniae	4.33	2.24	3.51	5.94	2.80	3.46	2.32	2.66	17.64	9.00	7.49	2.45	0.33	2.68	4.40	0.00	0	0
S. simulans	6.45	0.23	2.21	3.87	0	0	0	0.03	0	0	0	0	0	3.89	0	0	0	0
S. infantarius	4.33	0.14	8.20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. aureus	0	6.20	12.63	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. unclassified	6.69	22.18	9.53	14.94	14.21	21.98	28.60	28.42	19.76	23.59	7.70	23.02	29.67	30.63	24.08	23.62	12.05	19.50
E. coli	40.24	36.44	9.40	60.81	65.14	69.37	50.39	59.60	51.41	63.39	36.42	62.22	61.98	26.68	51.73	75.55	72.99	75.50
P. mirabilis	33.33	21.09	40.61	5.54	3.80	0	15.66	6.31	1.28	0	24.32	0	0	28.70	4.99	0	0	0.05
V. ratti	0	0	0	0	2.10	0	0	0	0	0	19.43	0	0	0	0	0	5.56	0
B. thetaiotaomicron	0.37	0.58	0.17	0	0.61	0	0	0	0	0	0.98	0	0	0	0	0	0	0
B. ovatus	0.44	0.09	0.15	0	0	0.07	0	0	0.09	0	0	0.69	0	0	0	0	0	0
E. cloacae	0.12	0.14	0	0	0	0.13	0	0	0.34	0.05	0.26	0	0	0.20	0	0	0	0
B. longum	0	0.06	0	0.21	0.33	0.14	0	0.07	0.79	0.16	0.07	0.13	0	0	0	0	0.00	0
E. faecium	0.09	0.20	0.04	0	0.04	1.08	0	0.03	0.76	0.67	0.61	0.04	0	0.54	0.89	0	2.09	0.70
E. avium	0.59	0.62	0.22	0.17	0.17	0.82	0	0.28	1.12	0.02	0.50	0.38	0.81	0.62	1.30	0.08	1.27	1.21

M. morganii	0.78	0.03	0.32	0	0	0.08	0	0	0.75	0.03	0.66	0.02	0	0.07	2.49	0	0	1.75
B. uniformis	0.83	0.53	1.00	1.23	3.57	0.24	0.28	0.15	0.72	0.28	0.77	0.52	0.10	0.79	5.86	0	0.52	0
B. dorei	0.29	0.36	0.15	0.04	3.85	0	0	0.01	3.21	0	0	1.94	0	5.12	3.77	0	0	0
E. faecalis	0	0.31	0.09	6.67	2.31	0	0.76	0.58	0	0	0	1.75	5.22	0	0	0.74	0	0
B. fragilis	0.21	0.63	0.42	0.16	0	0.07	1.70	0.93	0	0.49	0.13	6.36	0.32	0	0	0	2.44	0
K. unclassified	0	0.22	0	0	0	0	0.00	0.16	0	1.13	0	0	0	0	0	0	0	0
E. raffinosus	0	0.13	0	0	0	0	0	0.06	0	0.52	0	0	0	0.01	0	0	0.09	0
B. pseudolongum	0	0	0	0.02	0	0	0.28	0.24	0	0.56	0.16	0	0.65	0	0	0	0	0
S. gallolyticus	0	2.26	0	0	0	2.26	0	0	0	0	0	0	0.00	0	0	0	0	0
E. hirae	0	2.56	0	0	0	0	0	0	0	0	0	0	0	0	0.04	0	0	0
S. pasteurianus	0	1.78	0	0	0.19	0.06	0	0.24	0.08	0	0	0	0.94	0.06	0.21	0	0	0.24

Figure 87: Selection of *intI1* by ciprofloxacin. Significant positive selection was observed to 90% confidence at 15.625 (p = 0.0634, GLM Gamma (identity)) and 31.25 (p = 0.0553, GLM Gamma(identity)) and to 95% confidence at 62.5 µg/L (p = 0.0491, GML, Gamma (identity)) and at every subsequent higher concentration. Standard error is represented by the error bars. * = significant positive selection to 90% confidence. ** = significant positive selection to 95% confidence.

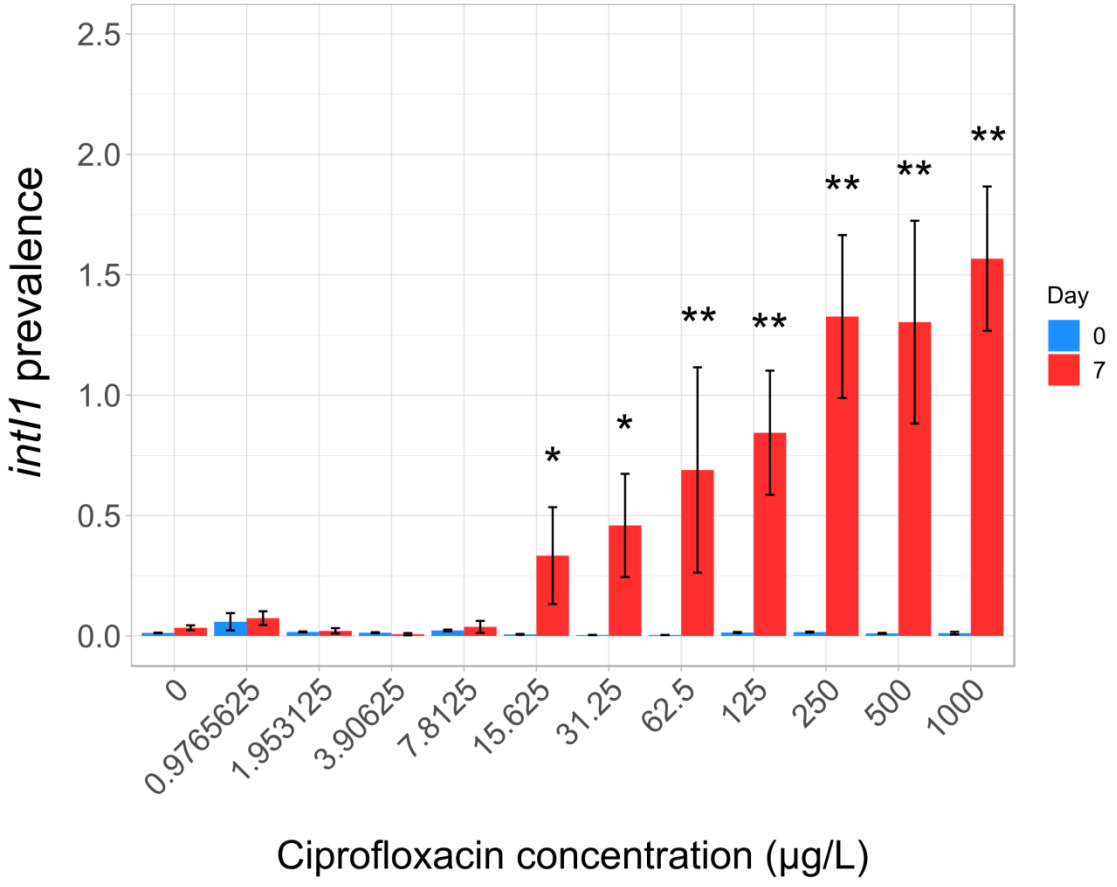


Figure 88: Selection coefficient of *intI1* in the presence of ciprofloxacin. The line of best fit (polynomial, order 4 – $y = 0.1093 + 0.293x – 0.5274x^2 + 0.1921x^3 – 0.0188x^4$, $R^2 = 0.4397$, standard error = 0.2645, x intercept = 10.77) determines a MSC of approximately 11 µg/L of ciprofloxacin. The square root of ciprofloxacin concentration used to better visualise the data.

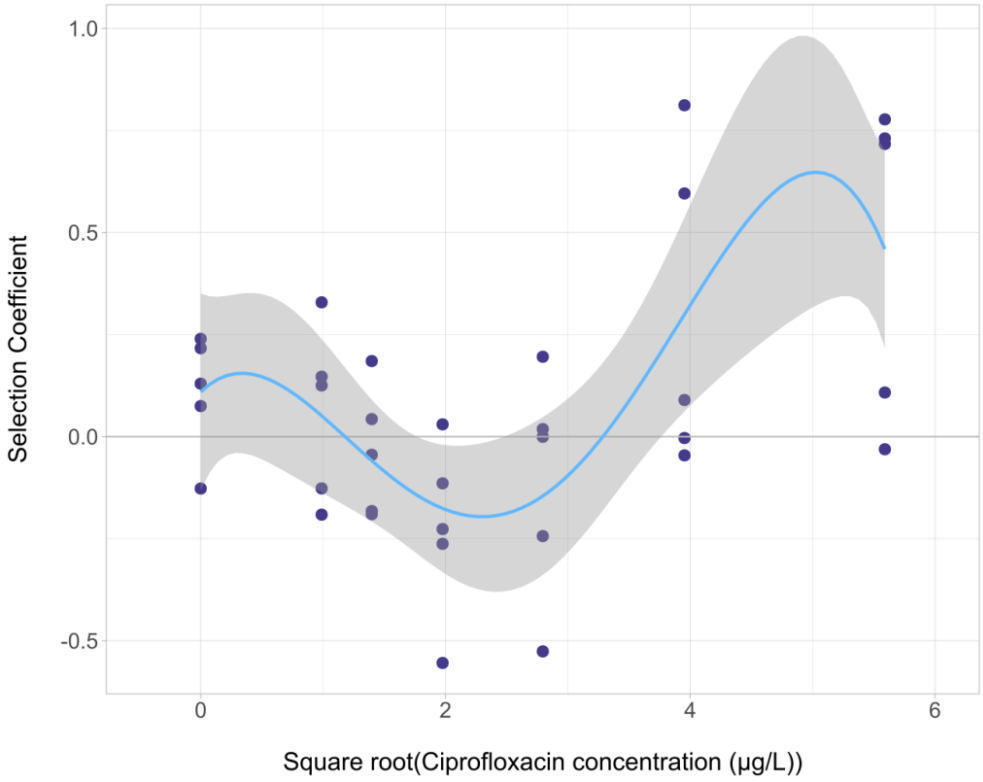


Figure 89: Graph showing full 24 hour growth rate for SMX experiment 1

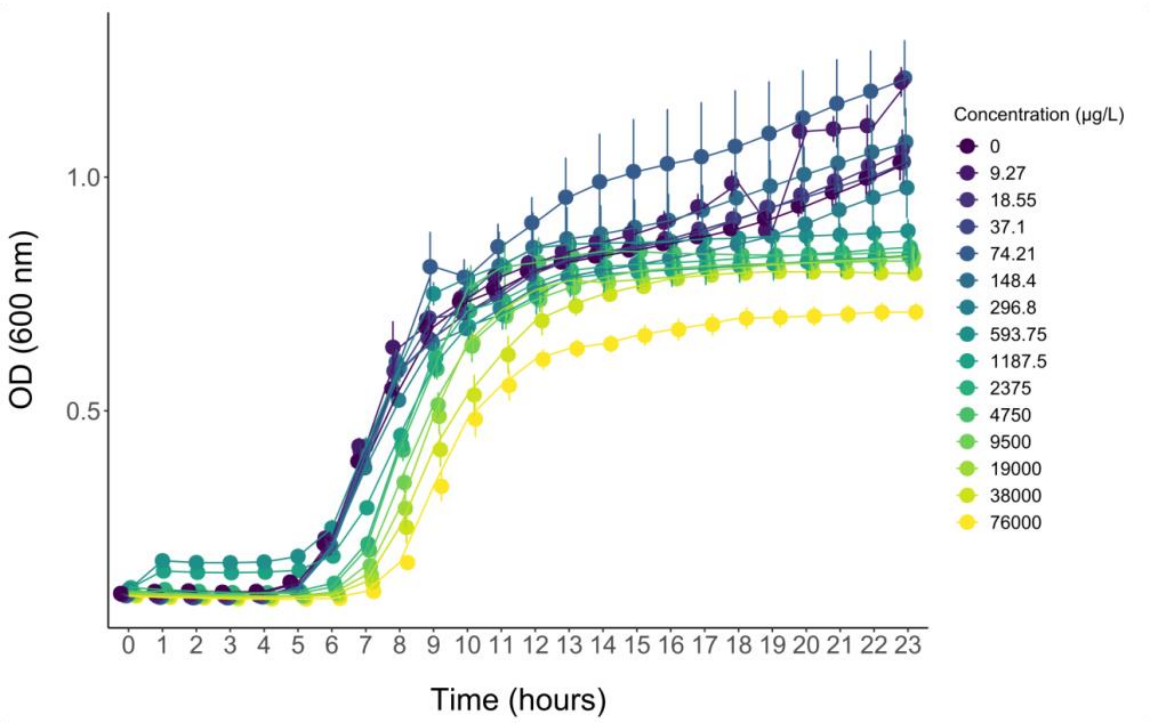


Figure 90: Graph showing full 24 hour growth rate for TRMP experiment 2

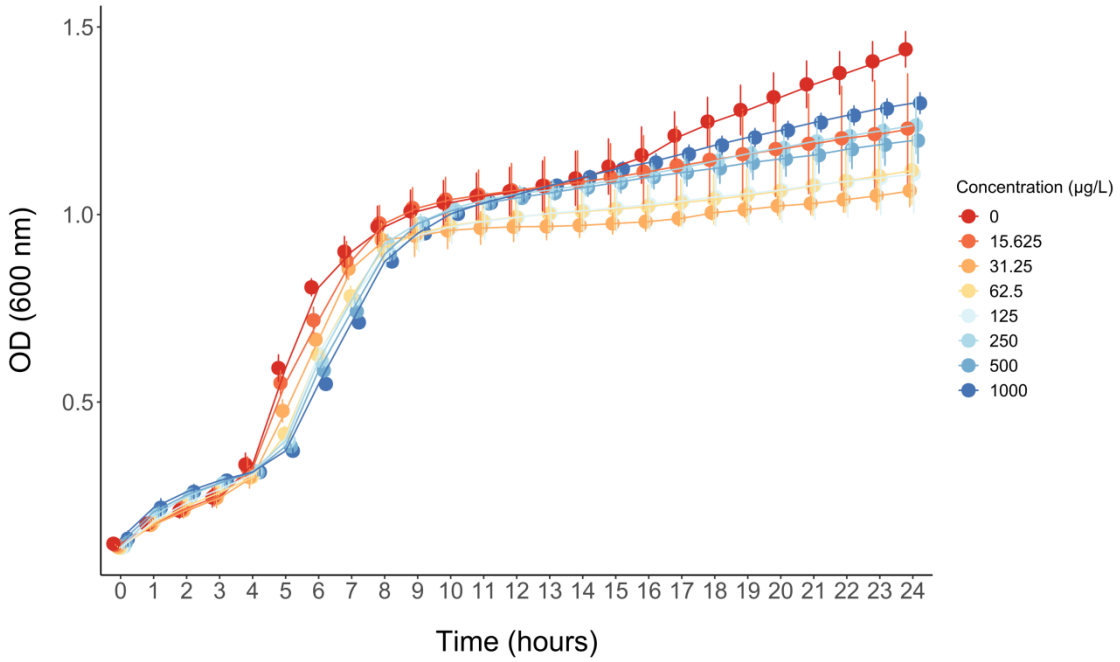


Figure 91: Graph showing full 24 hour growth rate for SMX experiment 2

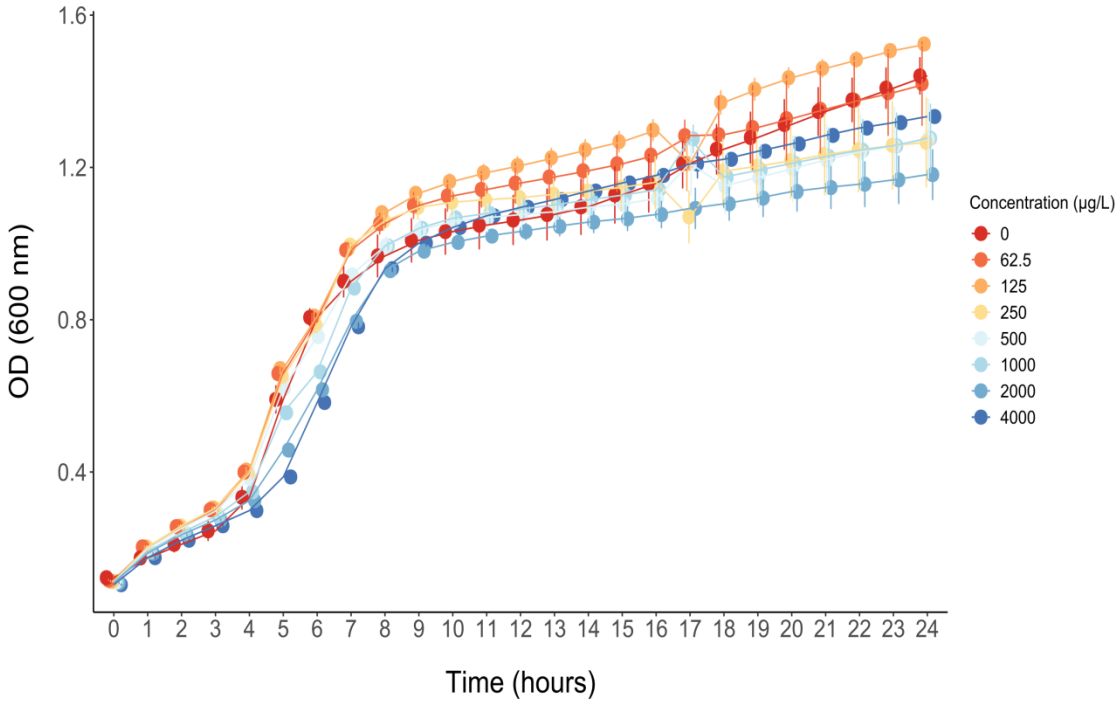


Figure 92: Graph showing full 24 hour growth rate for TRMP-SMX in combination

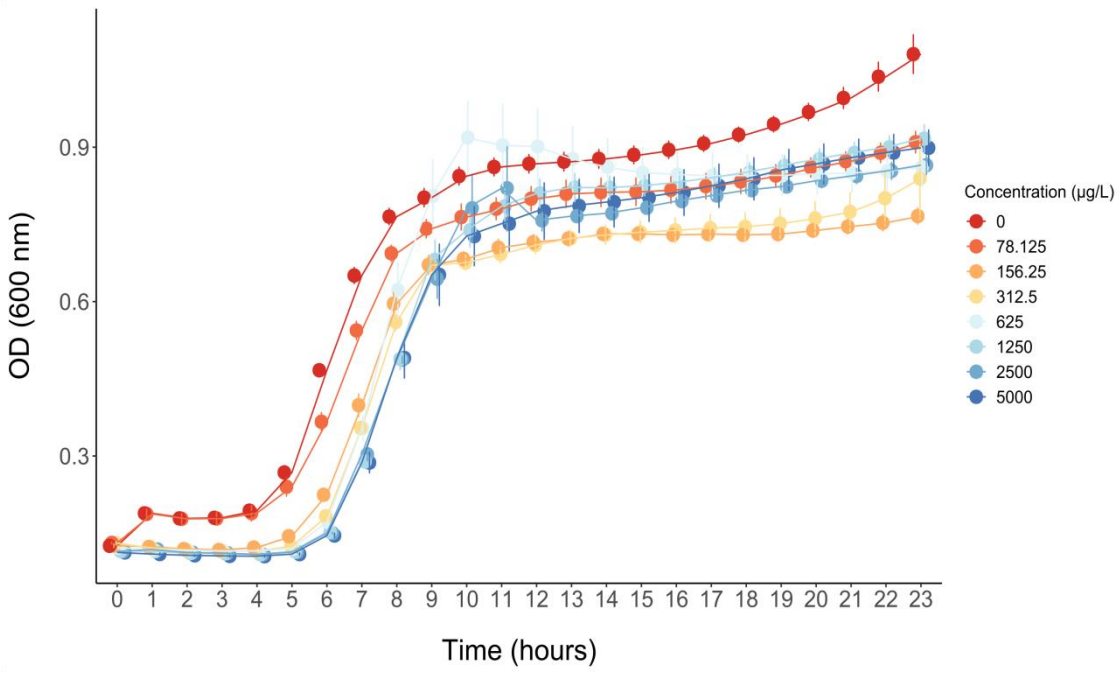


Figure 93: Selection coefficient graph for *intI1* in the presence of TRMP in isolation. The MSC is determined to be approximately 38 µg/L.

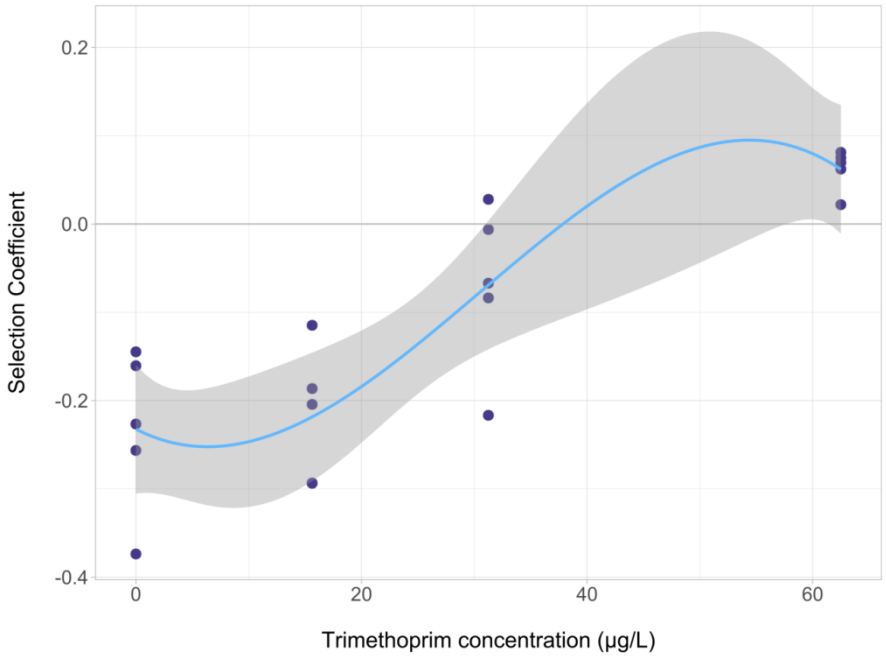


Figure 94: Selection coefficient graph for *intI1* in the presence of TRMP in when mixed with SMX. The MSC is determined to be approximately 19 µg/L.

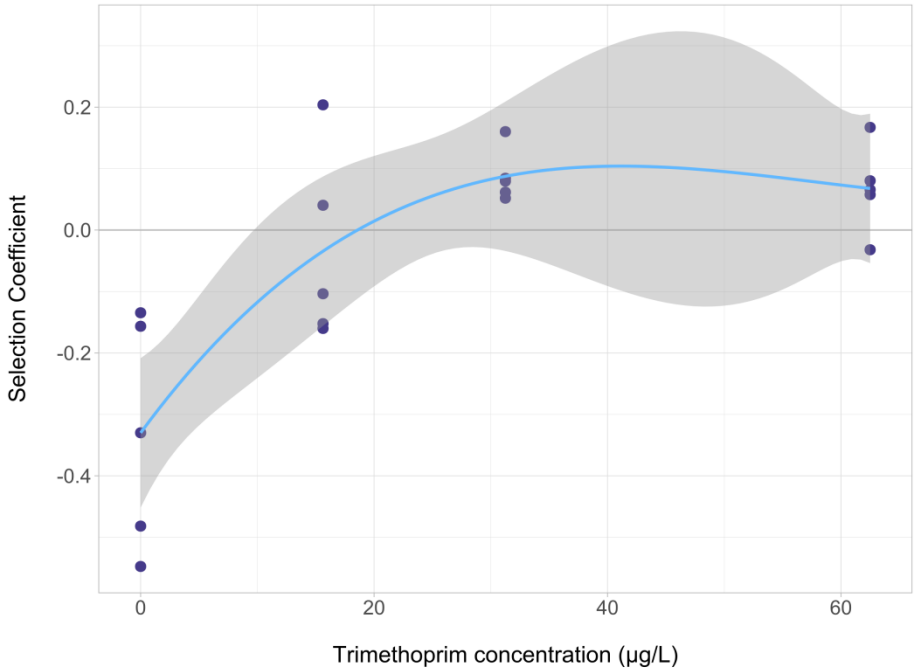


Figure 95: Selection coefficient graph for *intI1* in the presence of SMX in isolation. The MSC is determined to be approximately 841 µg/L. The square root of SMX concentration has been plotted. The data values plotted equate to the absolute concentration values of 0, 62.5, 125, 250, 500, 1000 and 2000 µg/L.

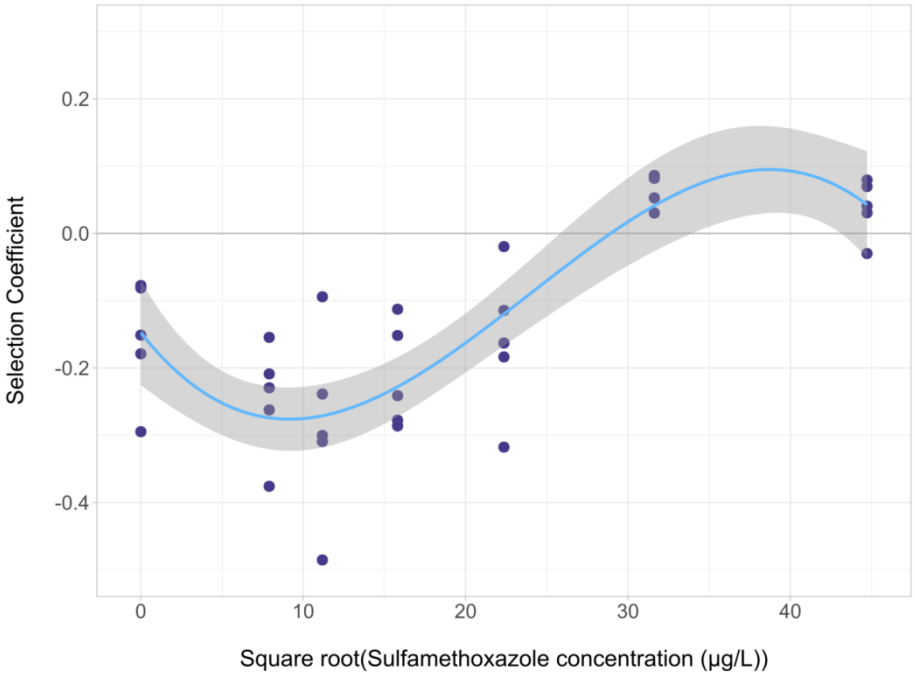


Figure 96: Selection coefficient graph for *intI1* in the presence of SMX in when mixed with TRMP. The MSC is determined to be approximately 69 µg/L. The square root of SMX concentration has been plotted. The data values plotted equate to the absolute concentration values of 0, 62.5, 125 and 250 µg/L of SMX in the experiment.

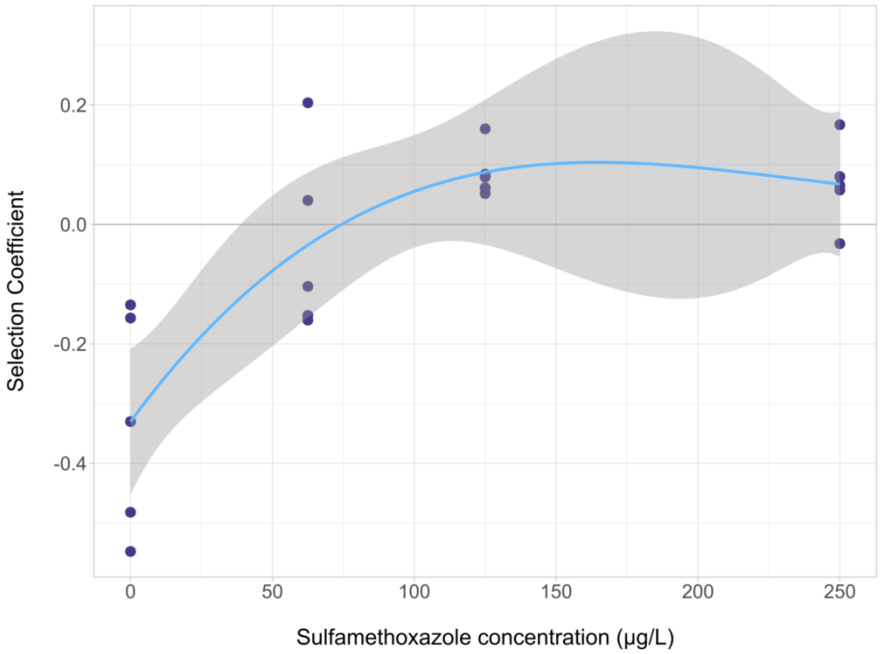


Figure 97: Graph showing the growth rate of a mixed community at 37 °C during a 24 hour period.

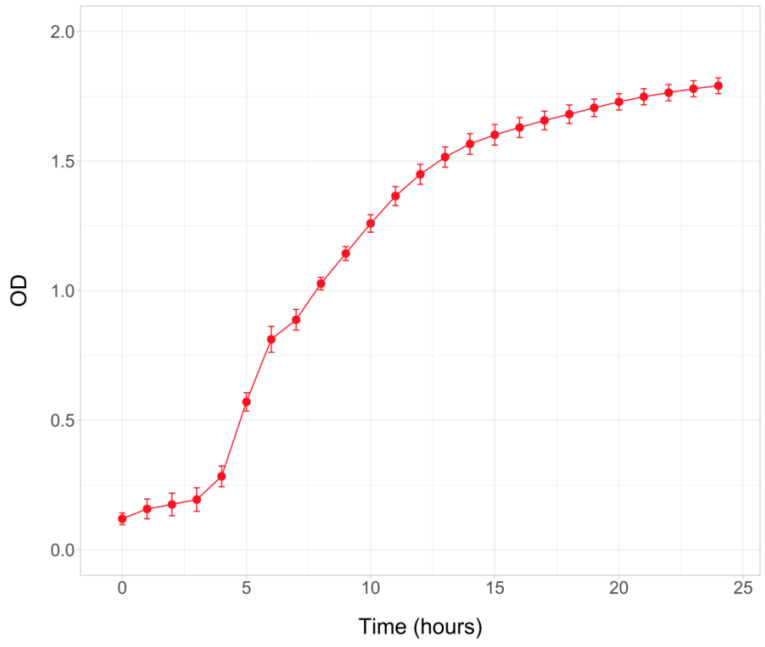


Figure 98**:** Graph showing the growth rate of a mixed community at 28 °C during a 24 hour period.

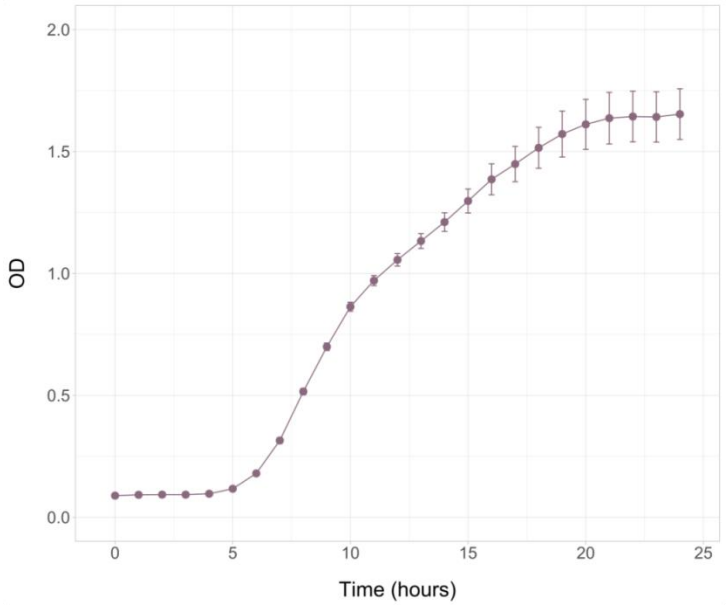


Figure 99: Graph showing the growth rate of a mixed community at 20 °C. A starting and final OD was quantified. Hour readings were taken between hour 14 and 23 (exponential phase) so a growth rate could be calculated.

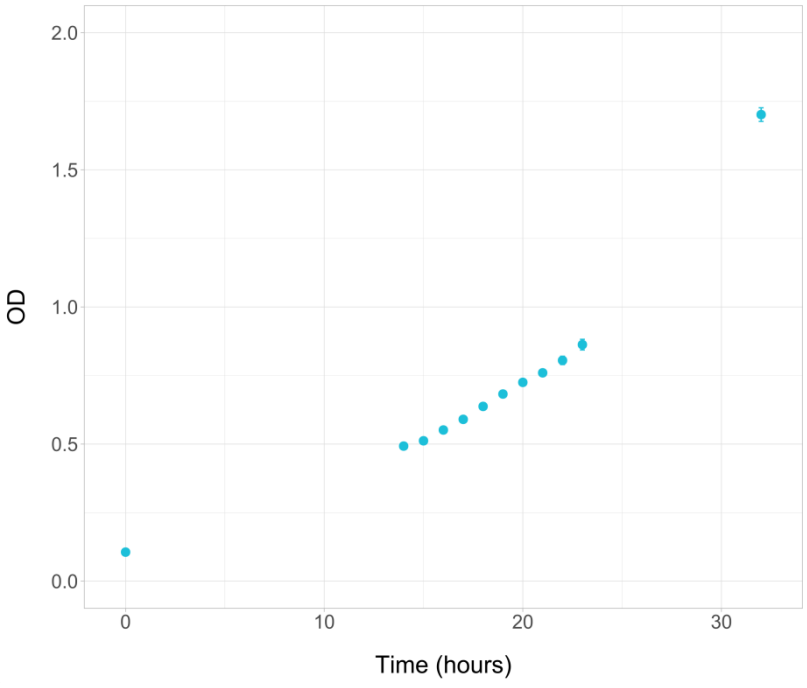
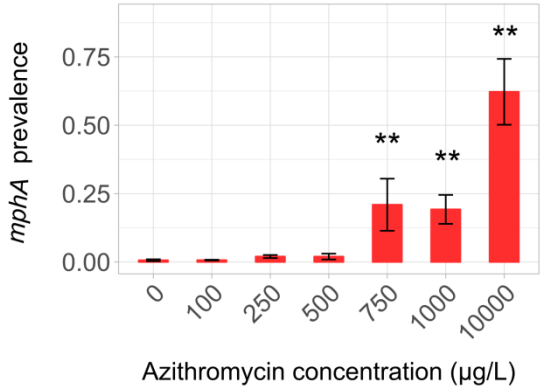
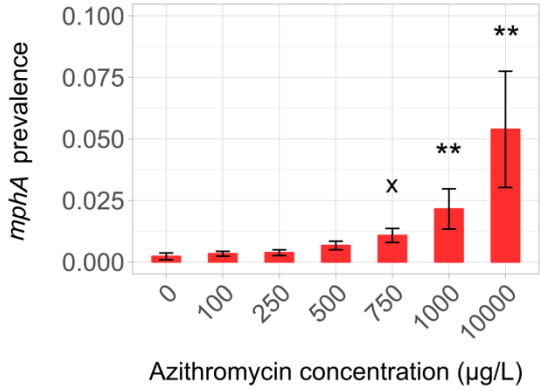


Figure 100: *mphA* as a function of AZ at 37 °C at day 7. B: *mphA* as a function of AZ at 28 °C at day 7. C: *mphA* as a function of AZ at 20 °C at day 14. x = significant increase in comparison to the control to 90% confidence. xx = significant increase in comparison to the control to 95% confidence. ** = significant positive selection to 95% confidence. Standard error is represented by the error bars.





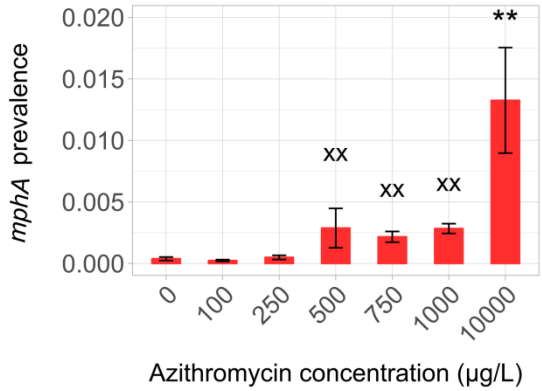


Figure 101: Selection coefficient of *tetG* by TET. A MSC is not determined as the line of best fit (polynomial order 4, y = -1.357 – 0.01895x + 0.0003157x^2 – 1.439e-06x^3 + 1.994e-09x^4, R^2 = 0.239, standard error = 0.4172) never crosses the x axis. The square root of all tetracycline concentrations from both the low and high experiments were plotted.

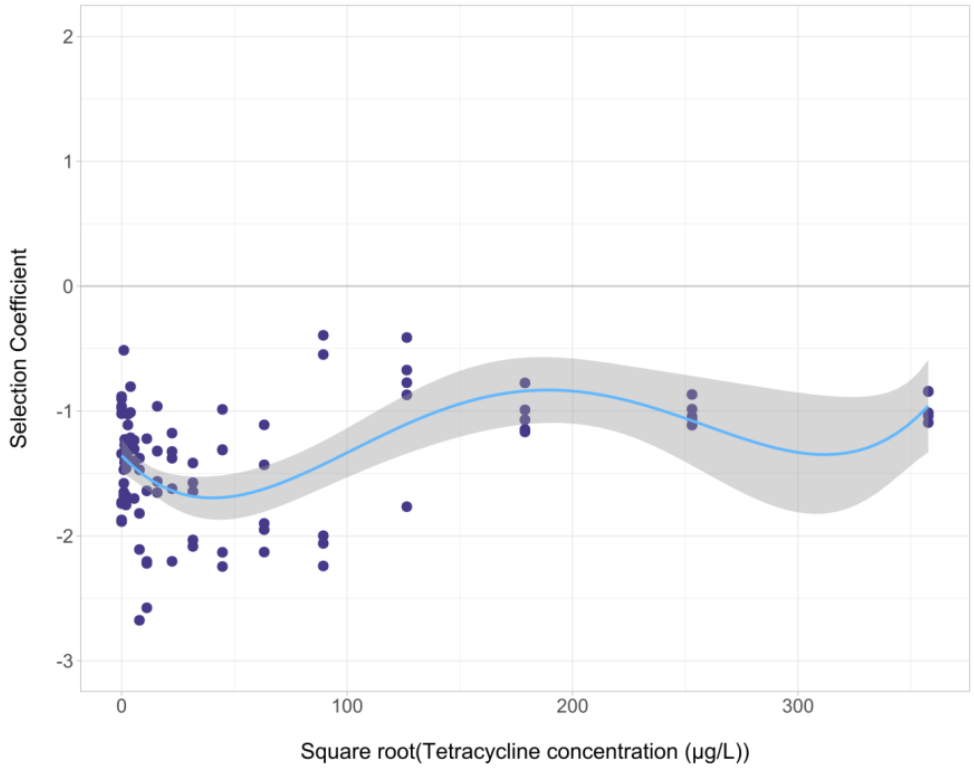


Figure 102: Selection coefficient of *tetM* by TET. A MSC is not determined here as the line of best fit (polynomial order 2, y = 0.41 – 0.0024x + 7.5e-6x^2, R^2 = 0.119, standard error = 0.119) never crosses the x axis. The square root of tetracycline concentration was plotted. All of the concentrations from both the low and high concentration experiment are represented here.

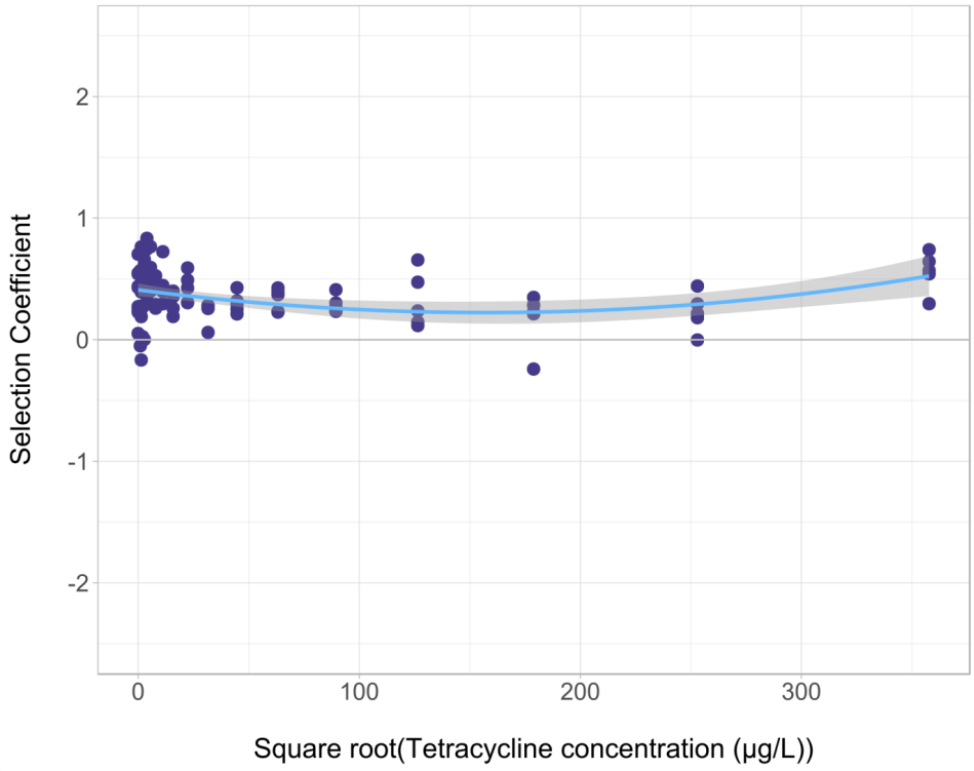


Figure 103: Heatmap showing all detectable tetracycline resistance genes as a function of TET concentration. If the prevalence is displayed the genes with high prevalences skew the data and it appears that genes at lower prevalences are undetectable. The log(prevalence) is, therefore, represented in this graph. Genes that were undetected in all samples were excluded; these were *tcr3, tet31, tet36, tet39, tet41, tet43, tetG, tetT, tetV, tetY* and *tetZ.* White areas represent where the gene was below the limit of detection. * = significance to 90% confidence, ** = significance to 95% confidence.

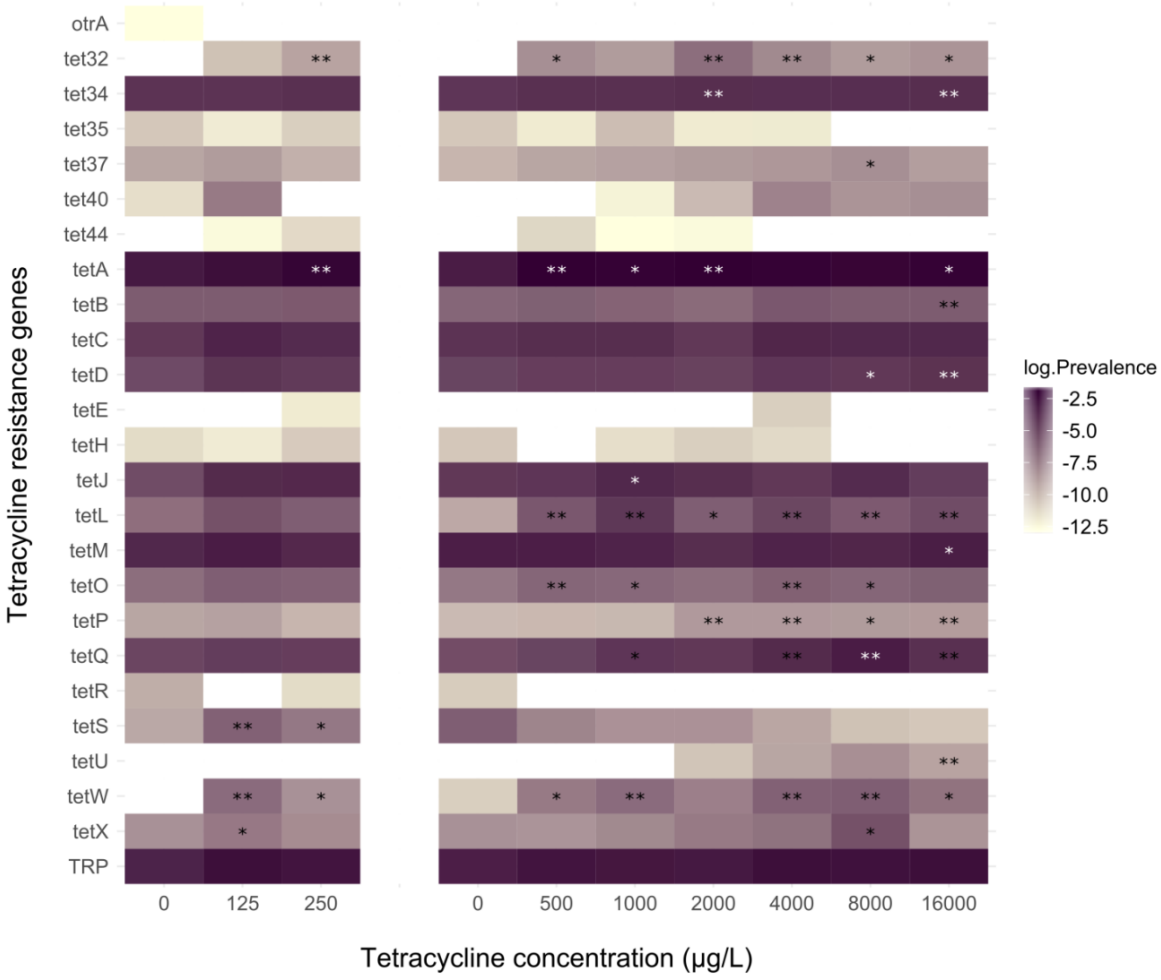
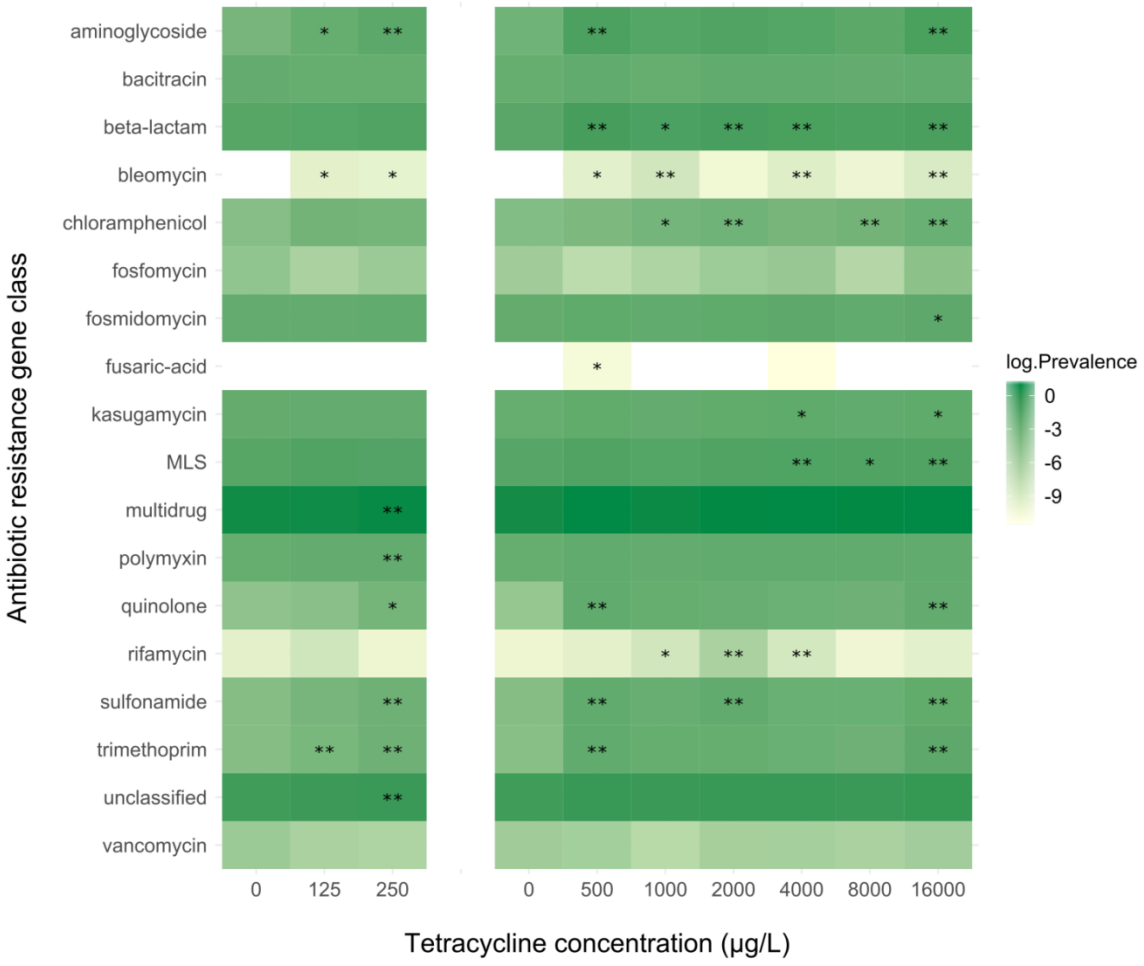


Figure 104: Co-selection of antibiotic resistance gene classes by tetracycline. Co-selection is observed at the lowest concentration of TET sequenced for the classes aminoglycoside, bleomycin and trimethoprim. Other classes displayed here see an increase in their resistance genes at higher concentrations. White represents where the prevalence of a class is below the limit of detection. Classes that were below the limit of detection in every sample sequenced are not represented here. These were carbomycin, fusidic-acid, puromycin, spectinomycin and tetracenomycin-C. * = significance to 90% confidence, ** = significance to 95% confidence.



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