The biochemistry and genetics of herbicide-induced changes in antibiotic resistance in *Salmonella enterica* and *Escherichia coli*

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Abstract

Antibiotic resistance is a leading global health concern and is no longer a threat of the future (World Health Organization, 2014). In order to combat resistance effectively, the different factors that influence its development need to be understood. Previous work from this lab showed that exposure of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium to three commercial herbicide formulations caused a change in the antibiotic response including resistance to antibiotics from different classes, with up to a 6-fold change in susceptibility being observed (Kurenbach *et al.*, 2015). Formulations have both active ingredients and adjuvants. To investigate which components of the herbicide formulations could be responsible for the observed effects, *S. Typhimurium* was separately exposed to either the pure active ingredients, dicamba, 2,4-dichlorophenoxyacetic acid (2,4-d) or glyphosate, or two surfactants, carboxymethyl cellulose (CMC) or Tween80.

The pattern of antibiotic resistance phenotypes caused by the active ingredients varied in direction and intensity, but was similar to the pattern observed for the formulations. Tween80 and CMC either increased or had no effect on resistance to the tested antibiotics.

These results suggested that the direction of the change in resistance to different antibiotics is determined by the active ingredient but may be modulated by other components in the formulation. The observed effects occurred within recommended application rate concentrations, and some were within the maximum residue limits (MRLs) set by the Codex Alimentarius Commission (Codex Alimentarius Commission, 2012). In addition, the surfactants tested are also found in processed food products and both induced increases in resistance to some antibiotics at concentrations within those recommended for food (Codex Alimentarius Commission, 2015). The effects of different chemicals that induce similar

changes in antibiotic resistance were also shown to be additive, which suggests that bacteria exposed to low concentrations of multiple compounds may still experience the observed changes in antibiotic susceptibility.

Two approaches were taken to determine the mechanism by which herbicides alter the antibiotic resistance phenotype of bacteria. Firstly, the broad-spectrum efflux pump inhibitor phenylalanine-arginine-β-naphthylamide (PAβN) (Lomovskaya & Bostian, 2006) was used to demonstrate that the effects were likely caused by changes in the levels of antibiotic efflux. Then, to test specific influx and efflux components, a selection of *E. coli* knockout strains from the Keio collection were used. The change in response of these strains to antibiotics upon exposure to the herbicides was determined and compared to the wild-type. Although the data was highly variable, it showed that the multidrug efflux pump AcrAB-TolC was likely involved in the phenomenon.

Abbreviations

2,4-d	2,4-dichlorophenoxyacetic acid
2,4-D	commercial formulation of 2,4-dichlorophenoxyacetic acid
ABC	ATP-binding cassette
ae	acid equivalent
Amp	ampicillin
AMPA	aminomethylphosphonic acid
ANOVA	Analysis of Variance
aph(3')-II	aminoglycoside-3'-phosphotransferase II
A site	aminoacyl site
ATP	adenosine triphosphate
C	centigrade
Cip	ciprofloxacin
Cm	chloramphenicol
CMC	carboxymethyl cellulose
DNA	Deoxyribonucleic acid
EOP	Efficiency of Plating
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FAO	Food and Agriculture Organisation of the United Nations
g	grams
GMP	Good Manufacturing Practice
GRAS	generally regarded as safe
HGT	horizontal gene transfer
IARC	International Agency for Research on Cancer
K	Kamba
Kan	kanamycin
L	Litres
LB	Luria broth
MATE	multidrug and toxic-compound extrusion
MFS	major facilitator superfamily

MIC	minimum inhibitory concentration
mg	milligrams
ml	millilitres
MRL	Maximum Residue Limit
mRNA	messenger ribonucleic acid
NZ	New Zealand
OD_{600}	Optical density measured at a wavelength of 600 nm
PAβN	phenylalanine-arginine β-naphthylamide
POEA	polyethoxylated tallow amine
ppm	parts per million
RNA	ribonucleic acid
RND	resistance nodulation division
rpm	revolutions per minute
RR	Roundup Ready
S	Salicylic acid
SMR	staphylococcal/small multidrug resistance
SEM	Standard Error of the Mean
TCA cycle	tricarboxylic acid cycle
Tet	tetracycline
tRNA	transfer ribonucleic acid
Tween80	polyoxyethylenesorbitanmonooleate
µg	micrograms
µl	microliters
UK	United Kingdom
USA	United States of America
WT	wild-type
WHO	World Health Organisation
YPD	yeast extract peptone dextrose
YPG	yeast extract peptone glycerol

Chapter One

Introduction

The discovery of penicillin in 1928 resulted in a revolution in both medicine and scientific research. What followed was a golden age of lives saved and new drugs discovered (Bérdy, 2012). The benefits came with a note of warning, however, when in his Nobel Lecture Sir Alexander Fleming noted the dangers of under dosage and the ease by which bacteria could become resistant to penicillin (Fleming, 1947). Eventually his prediction came true, the widespread use of antibiotics caused a strong selection pressure for resistance in bacteria (Kunin, 1993). This soon became apparent as different antibiotics rapidly became obsolete (Kunin, 1993). Bérdy (2012) notes that of pathogenic bacteria, more than 70% are considered resistant to most commercially available antibiotics. In addition, development of novel drugs for use in treatment also reduced significantly. Today, antibiotic resistance is a leading global health concern (Piddock, 2006).

Herbicides have become ubiquitous in modern society. They are widely used not just in the agricultural industry but also in urban and household settings. Herbicides are generally considered safe, if used as directed (Dill, 2005; Munro *et al.*, 1992), however, they can have unintended effects on off-target organisms. Formulations of the herbicides dicamba, 2,4-dichlorophenoxyacetic acid (2,4-d) and glyphosate have been shown to induce changes in the response of *Salmonella enterica* serovar Typhimurium and *Escherichia coli* to antibiotics (Kurenbach *et al.*, 2015). Further understanding of this phenomenon and the mechanism by which it occurs is imperative.

1.1 Antibiotics and Resistance

Antimicrobial agents are defined as molecules with the ability to kill or suppress the growth of microorganisms (Milić *et al.*, 2013). Strictly speaking, antibiotics are a class of antimicrobial agents that are produced by one microorganism to inhibit the growth of another. However, the distinction between these two terms is not relevant to this thesis and thus the term antibiotic will be used throughout to refer to antibacterial agents that are either derived from natural sources or are entirely synthetic.

Antibiotics are widely used not just to treat infections in humans but also in veterinary medicine and the agricultural industry (Kemper, 2008). It has been estimated that in the USA agriculture uses between 9 and 13 million kg of antibiotics annually (Dolliver *et al.*, 2008; Shea, 2003). They are predominantly used at sub-therapeutic levels for growth enhancement and prophylaxis (Diarra & Malouin, 2014; Kemper, 2008). Their use as feed additives to increase physiological performance and weight gain was banned from commercial agriculture in Europe in 2006 (Kemper, 2008), however, the practice still persists in other countries, particularly the USA and China (Milić *et al.*, 2013). Antibiotics are also used to prevent and control disease within the population, herd or flock (Diarra & Malouin, 2014; Kemper, 2008). The antibiotics ceftiofur, bacitracin and virginiamycin are approved for this use in Canadian poultry farming (Diarra & Malouin, 2014). In USA honeybee colonies, oxytetracycline has been used since the 1950s to control infections caused by *Meissococcus pluton* and *Paenibacillus larvae*, and in 2005 tylosin was also approved for this use (Tian *et al.*, 2012). In addition, pig farming in the USA relies heavily on the prophylactic and growth enhancing abilities of antibiotics (Key & McBride, 2014; Mackie *et al.*, 2006). Many of the

antibiotics used in agriculture are similar or identical to those used for the treatment of infections in humans (Dolliver *et al.*, 2008; Shea, 2003).

When a dose of antibiotic is administered, most of it is not metabolised. It is estimated that around 70-75% of an antibiotic dose for an animal is excreted in the faeces or urine (Dolliver *et al.*, 2008; Mackie *et al.*, 2006). In the USA, effluent from pig farms is most often disposed of through land application (Mackie *et al.*, 2006). Indeed, addition of manure to soil as fertiliser is common. This provides one mechanism by which antibiotics may enter the environment as contaminants. Movement of water through contaminated soil can then spread antibiotics into the waterways (Kemper, 2008). More than 30 different antibiotics have been found in samples taken from surface and drinking water as well as effluent (Kemper, 2008). Antibiotics can also enter the waterways and the environment from urban areas through effluent from private households and hospitals as well as waste disposal from pharmaceutical companies (Manzetti & Ghisi, 2014).

Some of the most commonly prescribed antibiotics in humans are the fluoroquinolones and aminoglycosides (Milić *et al.*, 2013). Chloramphenicol, tetracycline, and ampicillin are also important in clinical practice (Kemper, 2008). In veterinary medicine, penicillins and tetracyclines are commonly used (Milić *et al.*, 2013). All of these antibiotics have different modes of action.

Ampicillin is a member of the β-lactam family of antibiotics. These molecules enter the outer-membrane of Gram negative bacteria through porin channels (Pagès *et al.*, 2008) and are bacteriocidal through the inhibition of cell-wall synthesis, specifically of the peptidoglycan layer (Yao *et al.*, 2012). This layer is crucial for maintaining cellular shape and turgor pressure and is made of glycan chains crosslinked with peptides (Yao *et al.*, 2012).

Penicillin-binding-proteins (PBPs) form these cross-links using their transpeptidase activity. Ampicillin acts as a substrate for these enzymes then inhibits their activity through penicilloyation of the active site (Kohanski *et al.*, 2010) which eventually results in cell lysis (Yao *et al.*, 2012).

Chloramphenicol is a bacteriostatic antibiotic which reversibly inhibits protein biosynthesis. This occurs through binding with the 50S subunit of the prokaryotic ribosome at the peptidyltransferase site which results in inhibition of peptide elongation (Schwarz *et al.*, 2004). The porin OmpF is required for the entry of chloramphenicol into the cell (Toro *et al.*, 1990)

Ciprofloxacin is a member of the fluoroquinolone antibiotic family. It enters Gram negative bacteria through porins in the outer membrane (Pagès *et al.*, 2008), and then targets the bacterial type II topoisomerases, DNA gyrase and topoisomerase IV (Aldred *et al.*, 2014). DNA gyrase is responsible for introducing negative supercoiling and for releasing torsional strain from replication forks (Aldred *et al.*, 2014; Hawkey, 2003), whereas the main function of topoisomerase IV is to untangle knots in the bacterial chromosome (Aldred *et al.*, 2014). The enzymes act by forming a complex with DNA and making a staggered double-stranded break with covalent bonds to the DNA on either side (Hawkey, 2003). Another DNA duplex is then guided through and the break is re-ligated (Aldred *et al.*, 2014). Fluoroquinolones bind to the enzyme-DNA complex and inhibit re-ligation of the double-stranded break, creating a block. This both causes DNA damage and stalls DNA replication machinery (Cheng *et al.*, 2013; Li & Liu, 1998). DNA cannot be synthesised and bacteriostasis occurs immediately (Cheng *et al.*, 2013). At high enough concentrations ciprofloxacin is bacteriocidal (Hawkey, 2003).

The aminoglycosides are a broad-spectrum and bacteriocidal class of antibiotics that include kanamycin and streptomycin (Kotra *et al.*, 2000). They enter the cell by permeating through the outer membrane lipid bilayer (Nikaido, 2003). These molecules interact with the 16S rRNA of the 30S ribosomal subunit at the A (aminoacyl) site and alter the conformation of the complex that forms between an mRNA codon and its associated aminoacylated-tRNA (Kohanski *et al.*, 2010). This interferes with tRNA recognition and results in mismatches and ultimately protein mistranslation (Kotra *et al.*, 2000).

Tetracycline is a bacteriostatic antibiotic that inhibits bacterial protein synthesis. Like the aminoglycosides it binds to the 30S subunit of the ribosome (Chopra & Roberts, 2001). This bond is reversible, and blocks aminoacylated-tRNAs from associating with the ribosome, thus preventing the elongation of peptides (Brodersen *et al.*, 2000; Chopra & Roberts, 2001). Tetracyclines enter the outer-membrane of gram negative bacteria through the OmpF and OmpC porins as positively charged metal ion complexes. In the periplasm, the antibiotic dissociates from the metal ion and becomes a small, weakly lipophilic molecule that is then able to diffuse through the inner-membrane into the cytoplasm (Chopra & Roberts, 2001).

The continued development and use of a wide range of antimicrobial agents has provided strong selective pressure for different antibiotic resistance mechanisms and the discovery of pathogens with resistance to more than one or two antibiotics is becoming more common (Alekshun & Levy, 2007). In a collection of *Streptomyces* isolates taken from a sample of soil, every one showed resistance to multiple antibiotics, including both recently approved and entirely synthetic drugs (D'Costa *et al.*, 2006). Bacteria can become resistant via a number of different mechanisms that can be sorted into three main groups; target site alterations,

direct attack at the antibiotic molecule and reduced intracellular concentrations (Heinemann, 1999).

The target site can be modified so that the antibiotic molecule cannot bind. Mutations in the *gyrA*, *gyrB*, *parC* and *parE* genes that change DNA gyrase or topoisomerase IV sufficiently to provide some resistance to fluoroquinolones are an example of this (Aldred *et al.*, 2014). Overproduction of the target to outcompete drug concentrations, production of protector proteins that block the binding site, or acquisition of alternate metabolic pathways are other methods by which access of the antibiotic molecule to the target can be reduced (van Hoek *et al.*, 2011).

The antibiotic molecule can be directly targeted by enzymes that are capable of inactivation or complete degradation (van Hoek *et al.*, 2011). For example, β-lactamases that deactivate the β-lactam ring of penicillin and derivatives, or chloramphenicol acetyltransferases which acetylate chloramphenicol, rendering it inactive (Alekshun & Levy, 2007).

Resistance to antibiotics can also be increased by reducing the concentration of antibiotics allowed to accumulate within the cell. This can occur through either decreased influx or increased efflux of the antibiotic molecule, although increased resistance is frequently due to the combinatorial effects of both of these mechanisms (Delcour, 2009).

Antibiotic influx can occur through two main mechanisms. Large, hydrophobic molecules are able to diffuse through the lipid bilayer (Delcour, 2009). The permeability of the outer membrane is reduced through changes in the lipid and protein composition. This can result in increased resistance to some antibiotics such as novobiocin, fusidic acid, and members of the aminoglycoside family (Nikaido, 2003; Vaara, 1992). Small, hydrophilic antibiotics are

able to diffuse through non-specific outer-membrane porins (Delcour, 2009). In Gram negative bacteria, such as *Salmonella* and *E. coli*, two of the most common porins are OmpF and OmpC (Chubiz & Rao, 2011). Several different families of antibiotics have been found to utilise these proteins to enter the cell. The β-lactams ampicillin and amoxicillin enter through OmpF (Kobayashi *et al.*, 1982; Yoshimura & Nikaido, 1985) and reduced expression of OmpC has been found to limit the entry of kanamycin (Sánchez-Romero & Casadesús, 2014). In addition, susceptibility to both quinolones and tetracycline has been shown to be dependent on the presence of OmpF, although in both cases membrane permeability appeared to also play a role (Delcour, 2009). Influx of antibiotics can be reduced by a loss of function mutation in, or complete loss of, outer membrane porin genes. Changes in porin-mediated influx in *E. coli* and related organisms can occur, at least in part, through changes in the expression of these proteins (Chubiz & Rao, 2011). For OmpF and OmpC this can occur at the transcriptional level, in response to changes in environmental conditions, as well as at the translational level (Chubiz & Rao, 2011). OmpR binds specifically to the promoter regions of *ompF* and *ompC*, activating transcription (Norioka *et al.*, 1986). The antisense RNA product of *micF* binds to *ompF* mRNA, inhibiting the binding of the ribosome and preventing translation (Chubiz & Rao, 2011). Transcription of *micF* is regulated by a number of activators including MarA, SoxS, and Rob (Li & Nikaido, 2004).

Intracellular concentrations of antibiotics can also be reduced by active efflux of the drug through specialised transport pumps. Efflux pumps are commonly linked to tetracycline resistance (van Hoek *et al.*, 2011) and are becoming increasingly associated with multidrug resistance (Corona & Martinez, 2013). Multidrug efflux pumps generally belong to one of five protein families. Those in the resistance nodulation division (RND), staphylococcal/small

multidrug resistance (SMR), multidrug and toxic-compound extrusion (MATE) families and the major facilitator superfamily (MFS) are secondary transporters (Alekshun & Levy, 2007). They move molecules down an electrochemical gradient (Alekshun & Levy, 2007). Pumps belonging to the ATP-binding cassette (ABC) superfamily are active transporters that get energy from ATP hydrolysis (Li & Nikaido, 2004; Nikaido, 1998). In Gram-negative bacteria efflux pumps in the RND family are most commonly associated with clinically significant changes in minimum inhibitory concentration (MIC) whereas in Gram-positive bacteria this is more commonly the role of MFS transporters (Piddock, 2006).

At least 37 different transporters have been identified through genomic scans in *E. coli*, with 7 of these being of the RND family (Nishino & Yamaguchi, 2001). Of these, AcrAB-TolC is the best characterised thus far and has been associated with efflux of the widest range of antimicrobial agents (Li & Nikaido, 2004). AcrB is a transporter protein that spans the inner membrane. It pumps molecules down a proton gradient from the cytoplasm to the periplasm where they then move through the outer membrane channel TolC to the external medium (Tikhonova & Zgurskaya, 2004). Although it is not the only outer membrane channel, TolC is a component of many efflux systems in *E. coli* (Li & Nikaido, 2004) and is critical to the function of the AcrAB-TolC complex (Fralick, 1996). The periplasmic membrane fusion protein AcrA is involved in the interaction between AcrB and TolC (Zgurskaya *et al.*, 2011). This efflux pump system has been found to be important for the resistance of *E. coli* to a number of antibiotics including tetracycline, chloramphenicol, fluoroquinolones, rifampicin and β-lactams (Li & Nikaido, 2004; Nikaido, 1996). In addition, it has also been demonstrated to play an important role in the resistance of a strain of

Salmonella enterica serovar Typhimurium to fluoroquinolones, chloramphenicol, florfenicol and tetracycline (Baucheron *et al.*, 2004).

Expression of the *acrABR* operon is both locally and globally regulated. Local regulation occurs predominantly through transcriptional repression by AcrR which is divergently transcribed from the same promoter region as *acrAB*. As such it down-regulates but does not completely stop transcription. (Li & Nikaido, 2004). Transcriptional activation of the *acr* operon occurs through global regulation by MarA. The *marRAB* (multiple antibiotic resistance) operon has regulatory effects on at least 10 different genes (Sulavik *et al.*, 1997). The repressor, *marR*, as well as *marA*, *marB* and *marC* are all transcribed from the operator region (*marO*). Repression of these genes occurs through the binding of MarR to *marO*.

In addition to MarA the *acrABR* operon is also transcriptionally activated by the global regulators SoxS and Rob. SoxS is a global activator that regulates transcription of genes involved in the defence and repair response to superoxide stress as well as number of operons involved in nonspecific antibiotic resistance (Amábile-Cuevas & Arredondo-García, 2013). Upon activation, SoxR becomes a transcriptional activator of SoxS, which in turn activates *tolC*, *micF* and *acrABR* (Amábile-Cuevas & Arredondo-García, 2013). Rob is constitutively expressed and upon activation by an inducer is involved in the regulation of a number of genes important for antibiotic resistance including *tolC*, *micF* and *acrABR* (Li & Nikaido, 2004).

E. coli also has a number of other RND transporters that are closely related to AcrB and require the function of TolC (Li & Nikaido, 2004). AcrF has a high similarity to AcrB and forms a complex with the membrane fusion protein AcrE. It is expected to transport a similarly large range of substrates; however, deletion of the *acrF* gene does not induce

hypersensitivity, suggesting that it is not highly expressed in wild-type *E. coli* (Li & Nikaido, 2004). AcrD is more distantly related to AcrB however it also forms a complex with AcrA and TolC (Li & Nikaido, 2004). The AcrAD-TolC efflux complex has been implicated in the removal of aminoglycosides from *E. coli* (Rosenberg *et al.*, 2000). The increased expression of *acrD* caused the MICs of amikacin, gentamicin, neomycin, kanamycin and tobramycin to decrease and in addition the removal of this gene caused increased accumulation of [^{3}H]di-hydrostreptomycin and [^{3}H]gentamicin (Rosenberg *et al.*, 2000). Nishino and Yamaguchi (2001) also found that an increased copy number of *acrD* in *E. coli* resulted in an increase in the MIC for both kanamycin and novobiocin. Two two-component signal transduction systems have been linked to the regulation of *acrD* and both have been identified in *E. coli* and *S. Typhimurium* (Nishino *et al.*, 2009). BaeS and CpxA are the sensor kinases for these systems. They regulate the phosphorylation state of the response regulators, which are BaeR and CpxR, respectively (Hirakawa *et al.*, 2003). When in the active state, the response regulator binds upstream of the *acrD* gene causing upregulated expression (Nishino *et al.*, 2005). In addition, BaeR has also been found to affect the expression of TolC (Nishino *et al.*, 2005).

The level of resistance possessed by a bacterium can come about in a number of ways. Intrinsic and acquired resistance are characterised by being irreversible and independent of the environmental conditions of the bacterium (Fernández *et al.*, 2011). Intrinsic resistance is caused by genes found ubiquitously within the genome of a bacterial species (Cox & Wright, 2013). Resistance is acquired when it is obtained through the exchange of genetic material between organisms or arises through mutation. This can occur through mechanisms such as conjugation, transduction and transformation (van Hoek *et al.*, 2011).

The phenomenon of horizontal gene transfer (HGT) has been a large contributing factor to the spread of resistance since the beginning of the antibiotic era (van Hoek *et al.*, 2011).

Adaptive resistance is induced by an environmental signal (Fernández *et al.*, 2011). It can occur in response to changes in pH, oxygen level or the presence of ions (Fernández *et al.*, 2011), as well as to social cues such as biofilm formation (Levin & Rozen, 2006) or exposure to sub-lethal concentrations of toxic chemicals (Fernández *et al.*, 2011; Sidhu *et al.*, 2012). Adaptive resistance can be reversible, although this depends on the mechanism by which it occurs, and the baseline resistance phenotype may not be the same level as before (Fernández *et al.*, 2011).

One mechanism by which drugs can induce resistance is through changes in the expression of relevant genes (Heinemann *et al.*, 2000). Rosner (1985) found that susceptible *E. coli* cultured in the presence of either salicylic acid or acetylsalicylic acid and an antibiotic displayed increased resistance to ampicillin, chloramphenicol, tetracycline and nalidixic acid. However, after the bacteria were grown in the absence of the inducer, the phenotype reverted to susceptible. It was later shown that salicylic acid upregulates the expression of the *marRAB* operon, which has downstream effects on influx and efflux of antibiotics and other toxic chemicals (Cohen *et al.*, 1993). In addition, salicylic acid has been shown to induce the expression of a multidrug efflux pump in *Campylobacter jejuni*, thus increasing resistance to fluoroquinolones (Shen *et al.*, 2011). Other chemicals that bacteria might be exposed to in their environment could have similar inducing effects on antibiotic resistance (Kurenbach *et al.*, 2015).

1.2 Herbicides

Herbicides are compounds that are used to kill weeds. They are used in urban settings to control weeds in public areas and by homeowners on their gardens and lawns. In the last century, they have replaced other forms of weed control in many commercial cropping systems. After herbicide resistant crops were commercialised in 1996, patterns of herbicide usage changed significantly (Benbrook, 2012a). Roundup Ready (RR) crops quickly took over the market as they allowed farmers to replace more labour intensive weed management strategies with a single, broad-spectrum, non-selective, glyphosate-based herbicide that could be used throughout the growing season (Service, 2007).

Under the RR crop system, herbicide types reduced and volume of active ingredient increased, concentrating a selection pressure on weeds. Following the emergence of glyphosate-resistant weeds, concentrations and application rates have increased even further (Benbrook, 2012a). This forced a reversion back to older, less-desirable herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-d) and dicamba, and crops resistant to these two herbicides have been approved for use in some countries (Behrens *et al.*, 2007; Bunge, 2014).

Due to their high solubility in water, herbicides such as dicamba, 2,4-d and glyphosate move readily through agricultural environments into soil, aquatic and atmospheric ecosystems (Battaglin *et al.*, 2014; Hua *et al.*, 2006). Glyphosate and its major breakdown product, aminomethylphosphonic acid (AMPA), have both been identified in agricultural soils (Kremer & Means, 2009) as well as in surface and groundwater associated with urban catchments (Van Stempvoort *et al.*, 2014). In a recent study glyphosate was detected in 39.4% and AMPA in 55% of samples taken from water and sediment across the USA

(Battaglin *et al.*, 2014). The frequency of identification was particularly high for ditches, drains and in rain samples as well as in rivers and streams (Battaglin *et al.*, 2014). In addition, Chang *et al.* (2011) found glyphosate in 60-100% of samples taken weekly across the USA states of Mississippi and Iowa over two growing seasons. More than 50% of samples of surface waters in California were found to contain 5 or more different pesticides and two of the most commonly detected herbicides were 2,4-d and dicamba (Ensminger *et al.*, 2013). Both herbicides were also found in farm runoff and in aquatic environments closely associated with farmland (Kuo *et al.*, 2012). Dicamba and 2,4-d were identified in drinking water reservoirs in the Northern Great Plains, although the concentrations detected were below established guidelines (Donald *et al.*, 2007). They have also been found at levels higher than the Canadian Drinking Water Guidelines allow in samples of rainfall taken during agricultural seasons in Alberta (Filkowski *et al.*, 2003).

Human exposures can come from both the environment and household products. Both dicamba and 2,4-d were among the most frequently detected pesticides in carpet dust samples taken from four areas in the USA (Colt *et al.*, 2004). In addition, it seems likely that herbicide residues are entering the food supply. Relatively high residues of glyphosate and AMPA were detected on genetically modified soybeans from Iowa (Bøhn *et al.*, 2014) and 5.6% of tested bread samples in the UK were found to contain concentrations of glyphosate of up to 0.5 ppm (Benbrook, 2012b).

Herbicides and some of their breakdown products are toxic to humans and animals, as well as to plants. One exposure route is through food. As herbicides are so readily able to leach into unintended environments, and as manufacturers tend to recommend application rates that are high enough ensure adequate control of a wide range of weeds (Crespo, 2011), it is

necessary to regulate the concentrations of herbicides allowed to be present on human food or animal feed (Horváth *et al.*, 2014). Maximum residue limits (MRLs) are set by many countries for the various pesticides they wish to regulate (Horváth *et al.*, 2014). MRLs are also set for traded commodities by the Codex Alimentarius Commission (Codex Alimentarius Commission, 2012). They are determined based on the results of standardised trials and are aimed at minimising any risks to human health and the environment (Horváth *et al.*, 2014; MacLachlan & Hamilton, 2010).

Although herbicides are designed to target plants, they are biocides and there is potential that they will have off-target effects on other organisms. This includes, but is not limited to, DNA-damage, reduction in respiration efficiency, cell-cycle delays and mortality (González *et al.*, 2007; Malatesta *et al.*, 2008; Soloneski & Larramendy, 2011). In an ecological context, small shifts in one species can have severe consequences on the community as a whole (Relyea, 2005). Despite being considered environmentally safe if used as directed by regulatory agencies and panels (Dill, 2005; Munro *et al.*, 1992), a number of studies demonstrating the effects of herbicides on off-target organisms have been carried out since these chemicals became pivotal members of the agrichemical family.

Perhaps most well studied are the effects of herbicides on humans and other mammals. Both dicamba and 2,4-d have been shown to have genotoxic effects on human cell lines as well as other mammalian models. A formulation of 2,4-d increased chromatid and chromosomal breaks in human lymphocytes (Zeljezic & Garaj-Vrhovac, 2004), while dicamba caused increased rates of sister chromatid exchange as well as affecting the cell-cycle in a similar cell line (Gonzalez *et al.*, 2006) and in Chinese Hamster ovarian (CHO) cells (González *et al.*, 2007). Further work showed that this was likely due to the delivery of reactive oxygen

species (González *et al.*, 2009). 2,4-d has also been shown to be moderately genotoxic to mice, with increases in the rates of sister chromatid exchange (Madrigal-Bujaidar *et al.*, 2001). Furthermore, rates of both B-cell and diffuse B-cell lymphoma have been positively associated with the use of phenoxy herbicides such as 2,4-d and dicamba.

The International Agency for Research on Cancer (IARC) has said that there is sufficient evidence to conclude that glyphosate is probably carcinogenic. A formulation of glyphosate has been shown to increase the presence of tumours in rats (Séralini *et al.*, 2014) as well as to be toxic to human liver and placental cells in vitro (Gasnier *et al.*, 2009; Richard *et al.*, 2005).

Moreover, the formulation Roundup has been demonstrated to have inhibitory effects on mitochondria. Isolated rat liver mitochondria, when exposed to Roundup, experienced a range of inhibitory effects that ultimately resulted in inhibition of respiration (Peixoto, 2005). These effects included changes to membrane permeability and inhibition of both succinate oxidation as well as respiration complexes II and III (Peixoto, 2005). The effect is reduced efficiency of the electron transport chain (Peixoto, 2005). Roundup also caused dose-dependent reductions in transmembrane potential of the mitochondria, at concentrations of up to 1691ppm. In contrast, pure glyphosate had only minimal effects (Peixoto, 2005). Similar responses have been seen with hepatoma tissue culture cells. Roundup-treated cells experienced a decrease in mitochondrial potential and electron micrographs showed a reduction in the inner to outer membrane ratio (Malatesta *et al.*, 2008). The authors also noted an increase in the presence of lysozyme, which may be indicative of cytoplasmic damage, particularly as the quantity of vacuoles containing cellular debris also increased (Malatesta *et al.*, 2008).

Direct effects on organisms in the environment that are likely to come into contact with these chemicals, as well as broader, ecological scale effects, are also of concern. Recently, work has been carried out on honey bees (*Apis mellifera*), as concerns for this important pollinator grow. Samples of bees taken in Colorado revealed the presence of 19 different pesticides in total (Hladik *et al.*, 2016). Glyphosate has been shown to affect associative learning and sensitivity to nectar, although no effects on navigation were observed, suggesting that contaminated pollen could still be transported back to the hive and into contact with other individuals (Herbert *et al.*, 2014). Dicamba has been shown to reduce visitations to alfalfa by pollinators (Bohnenblust *et al.*, 2015).

Another research focus has been the effects of herbicide runoff in waterways. Relyea (2005) looked at a freshwater ecosystem after exposure to Roundup and 2,4-d. Although 2,4-d had no effect on species richness, Roundup caused a 22% decrease overall with a 70% reduction in the number of tadpole species specifically (Relyea, 2005). It has also been shown to affect testes structure and hormone levels in male ducks (Oliveira *et al.*, 2007), while 2,4-d was found to be highly toxic to fingerlings (Arivu *et al.*, 2015).

Microbial ecosystems in soil are also important environments where organisms may be exposed to herbicides frequently over long periods of time. Banks *et al.* (2014) showed that small changes in the community occurred after applications of glyphosate or atrazine and proposed that repeated exposures in the long-term may have accumulative effects. Indeed, Zabaloy *et al.* (2010) showed that exposure of soil ecosystems to 2,4-d provided a selective pressure that increased both community tolerance to the herbicide and the presence of bacteria capable of degrading it. Glyphosate and one of its formulations have been shown to affect fungal interactions with plant roots. Soybean and maize crops displayed reduced

nodulation but increased root-colonization by *Fusarium* after treatment with Roundup (Kremer & Means, 2009). It has also been observed that glyphosate can sometimes increase disease severity in crops. It was suggested that this is due to weakening of plant defences, thus benefitting fungal pathogens (Johal & Huber, 2009).

Herbicides can also be toxic to bacteria. Botelho *et al.* (2011) found that two commercial formulations of glyphosate had slight impacts on the growth rate of *E.coli* at concentrations below the recommended application rates (0.09 ppm). Other work has shown that higher levels are toxic to bacteria. The MIC of a Roundup formulation was found to be 7,400 ppm ae for a strain of *E. coli* and 6,190 ppm ae for *Salmonella Typhimurium* (Kurenbach *et al.*, 2015) while another study found that for a different formulation 84,550 ppm was necessary to completely inhibit soil bacteria on solid growth media (Busse *et al.*, 2000). In contrast, only 100 ppm Roundup was necessary to inhibit *Enterococcus faecalis*, while 10,000 ppm reduced the survival of *Clostridium botulinum* (Kruger *et al.*, 2013). Hillaker and Botsford (2004) measured the MICs of formulations of glyphosate, 2,4-d and dicamba to the nitrogen-fixing bacterium *Sinorhizobium meliloti* and found these to be 18.1, 347, and >1,200 ppm, respectively. A similar concentration, although in pure form, of 2,4-d was found to be toxic to *E. coli* (Balague *et al.*, 2001). For a different strain and a different formulation of 2,4-d the toxic concentration was approximately 10-fold higher (Kurenbach *et al.*, 2015). A formulation of dicamba appeared to be the least toxic of the three herbicides, with 14,000ppm necessary to inhibit growth of both *E. coli* and *S. Typhimurium* (Kurenbach *et al.*, 2015). The toxic effects of these herbicides vary greatly and depend on both the species and the formulation tested.

As well as being toxic to microorganisms, herbicides have also been shown to affect the response of bacteria to other toxic chemicals. In the first study of its kind, Kurenbach *et al.* (2015) demonstrated that sub-lethal concentrations of three commercial herbicide formulations induced changes in the response of both *E. coli* and *S. Typhimurium* to a range of different antibiotics. The herbicides tested were Kamba^{500}, which contains the active ingredient dicamba, 2,4-D amine 800 WSG (2,4-D), which contains 2,4-dichlorophenoxyacetic acid (2,4-d), and Roundup weed killer, which has the active ingredient glyphosate. These formulations caused both increases and decreases in the resistance of the two bacterial species to antibiotics. The effects occurred rapidly and no pre-exposure to the herbicide was required for the full response to be observed. The direction of this change was dependent on the combination of herbicide and antibiotic as well as the species tested. A few combinations did not produce a significant change in resistance.

In general, the two phenoxy-containing herbicides, Kamba and 2,4-D, induced similar changes in the MICs of the antibiotics for *S. Typhimurium*. Statistically significant increases in MIC were observed for the antibiotics ampicillin, chloramphenicol, ciprofloxacin and tetracycline. Only for kanamycin was there a difference in the effects of the two herbicides. Kamba induced a decrease in the MIC while 2,4-D had no statistically significant effect. For *E. coli*, non-significant results were seen more often; only for ciprofloxacin did both herbicides cause a significant change in the MIC. In several cases, Roundup induced a change in the MIC in the opposite direction to that induced by Kamba or 2,4-D. For *S. Typhimurium*, it caused a significant increase in the MIC of kanamycin but a decrease in that of tetracycline. In *E. coli*, Roundup induced significant decreases in the MICs of ampicillin,

chloramphenicol and tetracycline. Highly statistically significant increases in the MIC of ciprofloxacin were seen for every combination of herbicide and bacterial species. The concentrations of the herbicides necessary to induce the changes in response to the antibiotics were above the MRLs allowed under international trading laws (Codex Alimentarius Commission, 2012) but within the recommended application rates for commercial formulations tested.

As part of that study I used the broad-spectrum efflux pump inhibitor phenylalanine-arginine-β-naphthylamide (PAβN) to diagnose the contribution of efflux pumps to the response caused by the herbicides (Kurenbach *et al.*, 2015). In addition, *lacZ* fusion constructs were used to determine whether the global regulon *soxRS* was affected by the herbicides, thus contributing to the phenotype observed. Both of these assays indicated that efflux may be playing a part in the mechanism. Kamba was found to induce the expression of a *soxS* fusion gene and PAβN restored the susceptibility of *E. coli* to chloramphenicol and kanamycin when combined with Kamba or Roundup, respectively.

The formulations used in that study were a mixture of active ingredients and other additives that are not listed on the label (Vincent & Davidson, 2015). The active ingredients dicamba and 2,4-d are both auxinic herbicides. They mimic the action of the natural plant hormone indole-3-acetic acid, also known as auxin (Gleason *et al.*, 2011; Mithila *et al.*, 2011). Despite the use of auxinic herbicides in agriculture for more than 60 years, the exact mode of action is still not fully understood (Mithila *et al.*, 2011). However, the effects of these herbicides on plants can be broken down into three phases (Gleason *et al.*, 2011; Grossmann, 2010). The first phase is stimulation. Here, metabolic processes are activated, including the production of ethylene and abscisic acid, and growth is stimulated (Grossmann, 2010). This is followed

by the inhibition phase, where both root and shoot development is inhibited (Bukowska, 2006; Grossmann, 2010). In addition, the stomata close and the plant experiences reduced transpiration and carbon assimilation, as well as increased levels of reactive oxygen species (Grossmann, 2010). The final phase is tissue decay. Chloroplasts, membranes, and the vascular system break down resulting in wilting and eventually death (Grossmann, 2010).

Glyphosate acts through inhibiting the activity of 5-enolpyruvylshikimate-3-phosphate-synthase (EPSPS), which is an enzyme in the shikimate pathway (Sammons & Gaines, 2014). EPSPS catalyses an intermediate step in the production of the three amino acids phenylalanine, tryptophan and tyrosine (Nandula *et al.*, 2005; Service, 2007) and is an important factor in the feedback inhibition of the shikimate pathway (Duke & Powles, 2008). Although the exact mechanism by which glyphosate causes plant death is not known, there are two general theories (Duke & Powles, 2008). Firstly that the shortage of key amino acids has downstream effects on protein synthesis, and secondly that the loss of negative feedback that causes high production of shikimate-3-phosphate limits carbon availability for other essential metabolic pathways (Duke & Powles, 2008).

Adjuvants of herbicides are additives that are designed to improve the activity of the active ingredient (Vincent & Davidson, 2015). They act by increasing the emulsifying, sticking, spreading, absorbing and penetration abilities of the herbicide (Vincent & Davidson, 2015). These ingredients are often referred to as inert (Castro *et al.*, 2014) and indeed are often not reviewed by regulatory bodies (Navarro & Martinez, 2014; Vincent & Davidson, 2015). In the USA most states do not require registration of the adjuvants in a mixture, and of those that do, most do not require toxicity data, information that is usually required for active ingredients (Vincent & Davidson, 2015).

Adjuvants can be broken into categories based on their purpose. Surfactants, or surface-active agents, are responsible for increasing the spread of herbicide droplets (Vincent & Davidson, 2015). They alter the surface tension of the herbicide which increases the surface area in contact with vegetation (Vincent & Davidson, 2015). There are four different types of surfactants, classified by the nature of the hydrophilic group attached to the carbon chain (Castro *et al.*, 2014). Non-ionic surfactants are the most commonly used type in agrichemicals (Banks *et al.*, 2014). Herbicide formulations often contain 1-10% total surfactants and some commercial formulations of glyphosate have levels as high as 150 g/L (Castro *et al.*, 2014). When used in other industries, such as the food industry, surfactants can be identified under other names such as emulsifiers, bulking agents, detergents and stabilizers (Codex Alimentarius Commission, 2015). They are found in a wide range of foods, particularly processed foods such as ice cream (Goff, 1997), although they tend to be more tightly regulated in foodstuffs compared to agrichemical products (Codex Alimentarius Commission, 2015). However, their prevalent usage has raised concerns about indirect effects on human health. Swidsinski *et al.* (2009) found that carboxymethyl cellulose (CMC) caused overgrowth of intestinal bacteria in treated mice, as well as slight bowel inflammation. They suggest that consumption of emulsifiers and detergents may negatively affect the mucosal barrier in the gut and may contribute to the increased prevalence of irritable bowel disease (Swidsinski *et al.*, 2009). In addition, CMC and polysorbate 80 have been shown to induce inflammation and obesity in mice through interfering with the microbiota of the gut (Chassaing *et al.*, 2015). Herbicide adjuvants are also receiving more attention. In a number of the studies investigating the unintended effects of herbicides on off-target organisms it is noted that the active ingredient alone often did not cause as great an effect as did other components (Kroon *et al.*, 2015; Peixoto, 2005; Richard *et al.*, 2005).

The importance of not only testing the active ingredients but also the different adjuvants present in these mixtures has been demonstrated.

1.3 Objectives of this Study

The aim of this study is to further investigate the phenomenon, and to determine the mechanism, of the effects of herbicides on microorganisms.

To this end, I used the active ingredients in isolation and selected additives to test their influence on the response of *S. Typhimurium* to antibiotics. *S. Typhimurium* was selected as it had already been included in the previous study and is a leading cause of gastroenteritis in humans (McClelland *et al.*, 2001). In addition, it also infects a wide range of animal species (Stecher *et al.*, 2007) and is used in mice as a model for human typhoid fever (McClelland *et al.*, 2001). The active ingredients dicamba, 2,4-dichlorophenoxyacetic acid (2,4-d) and glyphosate as well as two surfactants, Tween80 and CMC were investigated. I started with three hypotheses:

1) The active ingredient is necessary to induce the change in antibiotic resistance.
2) The surfactants are necessary to induce the change in antibiotic resistance.
3) Either singularly, or in combination, the active ingredients and surfactants are sufficient to induce the change in antibiotic response.

Secondly, I investigated whether the effects on the antibiotic resistance of *S. Typhimurium* by Kamba (Kurenbach *et al.*, 2015) and salicylic acid (Rosner, 1985) can be additive. In different environments, bacteria may not be exposed to sufficient concentrations of these chemicals alone to induce the response. However, if chemicals such as these have an additive effect, then a mixture of low concentrations of multiple different compounds might

still induce resistance. Furthermore, I tested the effects of herbicides on the mitochondria of the eukaryotic microorganism, *Saccharomyces cerevisiae*.

Finally, I aimed to determine the mechanism by which herbicides were able to induce changes to the response of bacteria to antibiotics. I did this by identifying the effect of an efflux pump inhibitor and gene knockouts on the response observed when bacteria are exposed to both herbicides and antibiotics.

Chapter Two

Methods

2.1 Experimental methods

2.1.1 Bacterial strains, culture conditions and chemicals.

Strains of *Salmonella enterica* serovar Typhimurium and *Escherichia coli* used in this study are shown in table 2.1. Strains were stored long term at -80°C in glycerol solution (15%). Cultures were maintained for a week at a time on Luria-Bertani broth (LB) Agar and grown in (LB) at 37°C in a gyratory water bath shaker at 215 rpm prior to experiments. Luria-Bertani base (Lennox-L-Broth Base, Invitrogen (USA)) and agar (Bacteriological Agar No.1, Oxoid (UK)) were used.

S. cerevisiae strain SY1229 was also stored long term at -80°C in glycerol solution (15 %). Cultures were maintained on yeast extract peptone dextrose (YPD) agar (Sambrook *et al.*, 1989) and grown in YPD or yeast extract peptone glycerol (YPG) broth (Sambrook *et al.*, 1989) at 30°C in a gyratory water bath shaker at 215 rpm before experiments. Yeast extract and bacteriological peptone were both purchased from Oxoid (UK), D(+)-glucose anhydrous from Applichem (Germany) and glycerol from LabServ (NZ).

Table 2.1: Bacterial and yeast strains used in this study.

Strain	Genotype	Reference
E. coli		
JB578	HfrH Su+ *thi gal* r^{-} $m^{+}Rif^{r}$	Laboratory strain
BW25113	F^{-}, λ^{-}, Δ*(araD-araB)567*, *ΔlacZ4787(::rrnB-3), rph-1,*Δ*(rhaD-rhaB)568, hsdR514*	(Baba *et al.*, 2006)
CR5000	BW25113 Δ*acrB*	(Baba *et al.*, 2006)
CR7000	BW25113 Δ*acrA*	(Baba *et al.*, 2006)
JW0912	BW25113 Δ*ompF::kan*, Kan^{R}	(Baba *et al.*, 2006)
JW2454	BW25113 Δ*acrD::kan*, Kan^{R}	(Baba *et al.*, 2006)
JW5503	BW25113 Δ*tolC::kan*, Kan^{R}	(Baba *et al.*, 2006)
BW25113 (*nptII*)	BW25113 F42::miniTn-Kan	This study
S. Typhimurium		
SL3770	LT2, pyr^{+}, rfa^{+}	(Roantree *et al.*, 1977)
Saccharomyces cerevisiae		
SY1229	*MATa leu2-3 leu2-112 ura3 his3*	Laboratory strain

The antibiotics used in these experiments were ampicillin (stock concentration – 100 mg/ml), chloramphenicol (20 mg/ml), ciprofloxacin (10 mg/ml), kanamycin (40 mg/ml), streptomycin (50 mg/ml), and tetracycline (5 mg/ml). Stock solutions were stored at -20°C. Ampicillin sodium salt was purchased from Applichem (Germany), chloramphenicol, streptomycin sulfate salt and tetracycline hydrochloride from Sigma-Aldrich (USA), ciprofloxacin hydrochloride from Pentax (USA), and kanamycin sulfate from Life Technologies (USA). Salicylic acid powder was purchased from Sigma-Aldrich (USA), dissolved in ethanol to a concentration of 1M and the pH was neutralised to 7.

Three herbicide active ingredients; glyphosate, 2,4-dichlorophenoxyacetic acid (2,4-d) (sodium salt monohydrate) and dicamba, were purchased from Sigma-Aldrich (USA).

Glyphosate and 2,4-d were dissolved in double-distilled, autoclaved water to the concentrations 10,000 and 45,858 ppm ae respectively and stored at room temperature. Dicamba was dissolved in 98.9 % absolute ethanol (analytical reagent grade) to 415,000 ppm ae and was kept at 4°C. Polyoxyethylenesorbitanmonooleate (commonly known as Polysorbate-80 or Tween80) and carboxymethyl cellulose (CMC), both potential herbicide surfactants, were purchased from BDH (UK) and Sigma-Aldrich (USA), respectively. Both were stored at room temperature and dissolved directly into growth media to the desired concentration when required.

Two commercial herbicides were purchased from PGG Wrightson in a liquid form. Kamba 500 (Nufarm, NZ) contains the active ingredient dicamba as a dimethylamine salt (500 g/L). Roundup weedkiller concentrate (Monsanto, Australia) contains the isopropylamine salt form of glyphosate (360 g/L) as the active ingredient. Both were stored at room temperature and added into growth media to the necessary concentration when used.

The commonly used efflux pump inhibitor phenylalanine-arginine-β-naphthylamide (PAbN) was purchased from MP Biochemicals (NZ) and dissolved in 98.9 % absolute ethanol to a stock solution of 25 mg/ml. It was stored at -20°C.

2.1.2 Determining the effect of herbicide components on antibiotic resistance.

To determine whether the herbicide ingredients had an effect on antibiotic resistance a killing curve assay was carried out. This entails plating bacteria on a range of antibiotic concentrations in the presence and absence of the herbicide components. The minimum inhibitory concentration (MIC) can then be determined for each antibiotic with and without

the herbicide components. For this work, I define MIC as the minimum concentration of antibiotic necessary to cause a 1000-fold reduction in the efficiency of plating (EOP).

A concentration was selected for each herbicide ingredient that did not inhibit growth. This was determined before beginning this set of experiments and was different for each antibiotic and component combination tested. A control was included to demonstrate the selected ingredient concentration alone had no toxic effects on the bacteria.

LB Agar plates were supplemented with a range of antibiotic concentrations both with and without the herbicide component at the selected concentration. Plates were freshly poured and dried for at least an hour in a laminar flow cabinet before inoculation.

SL3770, grown in LB broth at 37°C until the absorbance of the culture at a wavelength of 600 nm (OD_{600}) reached approximately 1, was serially diluted 10-fold and 10 µl droplets were applied to agar plates to final culture dilutions of 10^{-2} – 10^{-8}. Spots were allowed to dry and plates were incubated for up to four days at 37°C and checked daily. Colonies were counted and converted into titres which were then used to calculate EOP values. This calculation normalises for day to day variation in growth of the initial broth culture used to inoculate the plates. It is calculated using the formula shown in figure 2.1.

$$EOP = \frac{Titre\ of\ Treatment\ Plate}{Titre\ of\ No\ Treatment\ Control\ Plate}$$

Figure 2.3: Formula to calculate the Efficiency of Plating (EOP). This is used for plate count data to normalise for day to day variations in growth of the broth culture used for inoculation.

Each experiment was carried out three times using three independent cultures of bacteria. Statistical analysis is described in section 2.2.1 on page 35.

2.1.3 Dose response assay.

An assay was carried out to determine the minimum concentration of the herbicide components necessary to induce the change in antibiotic resistance. Each component was diluted in LB agar plates to a range of sub-inhibitory concentrations in combination with an antibiotic. Antibiotic concentrations remained constant throughout each experiment and were selected based on results from section 2.1.3. Plates were freshly poured and dried for at least an hour in a laminar flow cabinet. Plates were inoculated with *S. Typhimurium* SL3770 in the same manner as in section 2.1.2. Plates were incubated at 37°C for up to four days and checked daily. Colonies were counted and converted into titres. This was used to calculate EOP values. Statistical analysis is described in section 2.2.2 on page 35.

2.1.4 Chemicals in combination: Kamba and salicylic acid.

Previous work has shown that both herbicides and salicylic acid produce a change in the antibiotic resistance of *S. Typhimurium* and *E. coli* when present at certain concentrations. This experiment was designed to test whether the effect of Kamba and salicylic acid on the response of *S. Typhimurium* to chloramphenicol was additive.

Concentrations of each chemical too low to induce a response were combined in LB Agar plates to a total concentration of 250 ppm ae along with 4.4µg/ml of chloramphenicol. 250 ppm ae was chosen as the total as it is above the minimum inducing concentration for both chemicals. Controls were also included to confirm this and to demonstrate that the levels of Kamba and salicylic acid used in the combination were not sufficient on their own to induce a response. Plates were freshly poured and allowed to dry in a laminar flow cabinet before inoculation with SL3770 in the same manner as in section 2.1.2. They were incubated at

37°C for up to four days and checked for new colonies daily. Titres were used to calculate EOP values. Statistical analysis is described in section 2.2.3 on page 36.

2.1.5 Determining the effects of herbicides on mitochondrial function in S. cerevisae.

Two herbicide formulations, one active ingredient and two surfactants were tested for effects on the mitochondrial function of *S. cerevisiae*.

Two 50ml conical flasks containing YPD and YPG broth were inoculated with SY1229 and grown at 30°C for 48 hours in a gyratory water bath at 215 rpm. YPD and YPG agar plates were prepared using the top agar overlay method (Schmalz, 1988). 150µl of SY1229 liquid culture was added to 6ml 0.7% molten agar which was then poured evenly over the agar plate to form a homogenous lawn. This was allowed to dry for 20 minutes before a 6 mm sterile filter (Whatman) was placed in the centre of each plate and 30 µl of the test solution added.

The following concentrations of herbicides were tested: Kamba, 3,600, 36,000 and 360,000 ppm ae; Roundup, 4,150, 41,500, 415,000 ppm ae. Glyphosate was used at 10,000 ppm ae and CMC at 10 g/L, which in both cases was the highest concentration that could be easily solubilised in water. Tween80 was used directly without dilution but was so viscous that sterile filters could not be used. It was applied directly to the top of the top agar. Sterile water was used as a control for cells being washed away from the filter by liquid drops. All solutions and water were tested in triplicate on both YPD and YPG agar plates. This was replicated three times with independent cultures of SY1229.

Plates were incubated for 48 hours at 30°C. Any toxicity could be seen as a halo of no growth in the lawn of *S. cerevisiae* around the filter. Three measurements were taken of

each halo using a standard 30 cm ruler. The diameter of the filter was subtracted from the diameter of the halo and the three measurements were then averaged. Statistical analysis for these experiments is described in section 2.2.4 on page 36.

2.1.6 Determining the relevance of efflux to herbicide-induced changes in antibiotic resistance of bacteria.

To determine if changes in the expression of efflux pumps could be responsible for the effects on antibiotic resistance induced by the herbicides, the general efflux pump inhibitor PAβN was used (Lomovskaya *et al.*, 2001). The method is outlined in figure 2.2. Two cultures of *E. coli* JB578 were growth in LB broth to an $OD_{600} \approx 1$. PAβN was added to one culture to a final concentration of 25 μg/ml. Both cultures were incubated at 37°C for 30 minutes. Agar plates were poured containing either Kamba (1,380 ppm ae) or Roundup (1,250 ppm ae) along with two antibiotics, chloramphenicol (10 μg/ml) or kanamycin (5 μg/ml), both with and without PAβN (25 μg/ml). No treatment and PAβN only plates were included as controls.

The two cultures were serially diluted 10-fold and 10 μl droplets were applied to the agar plates to give the dilution factors $10^{-2} – 10^{-8}$ on each plate. The culture incubated with PAβN was used to inoculate the PAβN-containing plates and all others were inoculated from the other culture.

Plates were incubated at 37°C and checked daily for up to four days. Colonies were counted as they arose. Titres were used to calculate the EOP as in equation 1. Statistical analysis is described in section 2.2.5 on page 37.

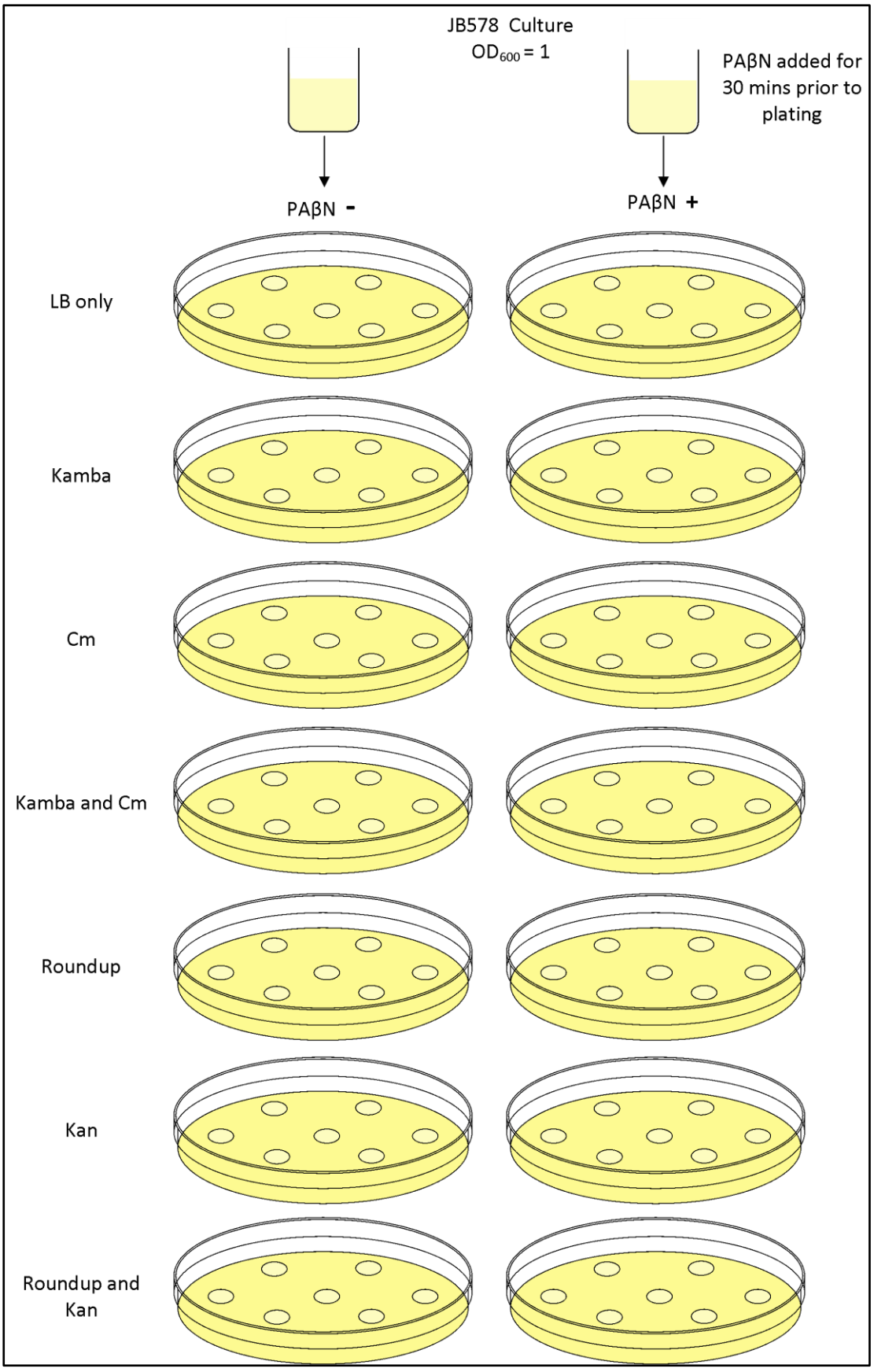

Figure 2.4: Schematic of the protocol used to test the effect of the efflux pump inhibitor, PAβN, on the changes in response of *S. Typhimurium* to chloramphenicol (Cm) and kanamycin (Kan) caused by the herbicides Kamba and Roundup.

2.1.7 Identification of specific genes involved in the intrinsic resistance of **E. coli** *to herbicides and antibiotics.*

To further elucidate the mechanism of the effect induced by the herbicides, a selection of *E. coli* strains from the Keio collection was obtained from the Stuart Levy's laboratory (Tufts University, Boston, MA, USA) and the Institute for Advanced Biosciences (Keio University, Japan). These strains each have a different efflux or influx related gene replaced by a kanamycin-resistance cassette (Baba *et al.*, 2006). The minimum inhibitory concentrations (MICs) of two herbicides, Kamba and Roundup, and three antibiotics, ciprofloxacin, streptomycin and tetracycline, were determined for each strain.

LB agar plates were supplemented with a range of concentrations of the herbicides and antibiotics separately. The strains were cultured at 37°C in LB broth to an $OD_{600} \approx 1$. They were then serially diluted 10-fold in LB broth and 10 µl droplets of each dilution were applied to each agar plate to final dilutions of 10^{-2} – 10^{-8}. No treatment controls were included. Plates were allowed to dry, inverted, and incubated for up to four days. Colonies were counted daily. Final titres were converted to EOP values. All experiments were replicated at least three times using independent initial broth cultures. The MIC of the herbicide or antibiotic was determined separately for each replicate and used to determine the mean MIC and the standard error of the mean (SEM).

2.1.8 Identifying specific genes involved in herbicide-induced antibiotic resistance.

As described in section 2.1.3, killing curve assays were carried out for each of these strains, including the wild type (BW25113), with the herbicides Kamba and Roundup in combination with the antibiotics ciprofloxacin, streptomycin and tetracycline. Freshly poured agar plates containing a range of concentrations of the antibiotics in both the absence and presence of

each of the herbicides were allowed to dry in a laminar flow cabinet prior to inoculation. The antibiotic and herbicide concentrations used varied by strain, as some of the mutants were particularly susceptible due to having a key component of their innate resistance knocked out. Herbicide concentrations used are shown in table 2.2.

Table 2.2: Concentrations of the herbicides Kamba and Roundup used in the killing curve assays with each of the knockout strains.

	BW25113 (WT)	CR7000 (Δ*acrA*)	CR5000 (Δ*acrB*)	JW2454 (Δ*acrD*)	JW5503 (Δ*tolC*)	JW0912 (Δ*ompF*)
Kamba (ppm ae)	1380	1380	1380	1380	1380	1380
Roundup (ppm ae)	1250	25	25	1250	25	1250

Plates were inoculated as in section 2.1.2 and then incubated at 37°C for up to four days. Colonies were counted daily and final titres were converted to EOP values. All experiments were replicated at least three times using different initial broth cultures and the statistical analysis is described in section 2.2.6 on page 37.

2.1.9 Determining whether the kanamycin resistance gene **nptII** *provides cross-resistance to streptomycin.*

A culture of the wild-type *E. coli* strain BW25113 was transformed with the plasmid F42::miniTn-Kan (Schmidt *et al.*, 1995) according to Sambrook *et al.* (1989). This plasmid was chosen because it carries *npt*II and has a low copy number in *E. coli*. Hence it will replicate the effects of a chromosomal insertion of *npt*II more closely than would a high copy number plasmid. Transformants were selected for on LB agar plates supplemented with 40 µg/ml of kanamycin. Colonies that grew after 16 hours of incubation at 37°C were confirmed for kanamycin resistance in the same way.

The MIC of streptomycin was determined for both BW25113 and BW25113 (F42::miniTn-Kan) simultaneously as described in section 2.1.7.

2.2 Statistical Analysis

R was used for all statistical analyses (R Core Team, 2013).

2.2.1 Determining the effect of herbicide components on antibiotic resistance.

This method was used for data from the killing curve assays carried out with the herbicide components on *S. Typhimurium* in section 2.1.2 on page 27.

Synergistic effects of herbicides and antibiotics were tested for effects on the log-transformed EOP scores. This was done using a multifactor analysis of variance (ANOVA) by evaluating the significance of the antibiotic by herbicide interaction term. Antibiotic concentrations were treated as separate categories in the ANOVA. Plots of residuals were used to test for violations of assumptions.

To identify the antibiotic concentrations with significant differences among herbicide treatments, contrasts across herbicide concentrations when antibiotic levels were fixed were evaluated. This was done using the testInteractions function in the phia package in R (De Rosario-Martinez, 2013). A Bonferroni correction was used within each experiment for this procedure.

2.2.2 Dose response assay.

When the dose-response curves were determined for the herbicide ingredients, as described in section 2.1.3 on page 29, many data points were near or below the detection limit, and as a result, the residuals from a standard ANOVA were not normally distributed. Hence, the equivalent nonparametric test, a Kruskal-Wallis one-way ANOVA, was used to test for differences in log-transformed EOP scores among herbicide ingredient concentrations. The P value given is for a comparison of a null model where EOP is the same

across all ingredient concentrations versus an alternative model where EOP differs among some ingredient concentrations.

2.2.3 Additive effects of Kamba and salicylic acid.

A one-way ANOVA was used initially to test whether the total concentration of Kamba and salicylic acid was sufficient to explain the variance in log-transformed EOP (see section 2.1.4 on page 29). Another variable was then included in a multi-factor ANOVA in order to quantify how much additional variation in log-transformed EOP is explained by knowing the proportion of the total concentration that was Kamba.

2.2.4 Determining the effects of herbicides on mitochondrial function in S. cerevisiae.

The effect of the herbicides on growth of *S. cerevisiae* on two different media was examined using a multifactor ANOVA to determine the amount of variation in log-transformed halo diameter that could be explained by the interaction between these two factors (see section 2.2.5 on page 30). Statistical significance was decided by the *P* value for the interaction term. Herbicide concentrations were treated as separate categories in the ANOVA. Plots of residuals were used to check for violations of assumptions.

Contrasts across the media type when herbicide concentrations were fixed were evaluated in order to identify individual herbicide concentrations where there was a significant difference in log-transformed halo diameter between the two media. This was achieved using the testInteractions function in the R package, phia (De Rosario-Martinez, 2013). A Bonferroni correction was used within each experiment to correct for the increased chance of getting false-positive results when testing multiple comparisons on the same data.

2.2.5 Efflux pump inhibition assay.

The effect of an efflux pump inhibitor PAβN on the survival of *E. coli* in the presence of herbicides and antibiotics was examined using a paired t test to determine if there was a difference in the means of EOPs in the presence and absence of the inhibitor. Although the data was found to be non-normally distributed the non-parametric equivalent of the paired t test, the paired Wilcoxon rank-sum test, was not appropriate due the small sample size of the data set. To account for this, and to increase confidence in the results of the test, the EOP data collected as described in section 2.1.6 on page 31 was log-transformed.

2.2.6 Identifying specific genes involved in herbicide-induced antibiotic resistance.

The effects of the herbicides on the antibiotic resistance phenotype of a series of *E. coli* knockout strains were determined in section 2.1.8 on page 33. The data from this set of experiments was analysed using the statistical method outlined in section 2.2.1.

Chapter Three

Investigating the different effects of herbicides and their components on microorganisms

3.1 Introduction

Previous work by myself and other members of the research group showed that commercial formulations of three herbicides at sub-lethal concentrations could induce changes in the response of *Salmonella enterica* and *Escherichia coli* to five antibiotics from different classes (Kurenbach *et al.*, 2015). The aim of this chapter was to 1. further investigate the different effects herbicides may have on microorganisms; and to 2. identify the components of the formulations that may be sufficient to cause those effects.

The three formulations tested were based on the active ingredients dicamba, 2,4-d, and glyphosate. However, herbicide formulations also contain other components such as surfactants. Two surfactants, polysorbate-80 (Tween80) and carboxymethyl cellulose (CMC), were identified from patent applications as potential herbicide ingredients (Grundman, 2014; Ushiguchi *et al.*, 2006) and are also known to be used in food (Chassaing *et al.*, 2015). They were used in this study as representatives of this class of compounds.

The effects of the active ingredients and surfactants on the response of *S. enterica* serovar Typhimurium to antibiotics were investigated in an attempt to identify which components of the herbicide formulations could explain our previous observations. In addition, the minimum concentrations of the components necessary to induce any effects were determined and the additive nature of the phenomenon was also investigated. Other studies have shown that the herbicide Roundup can inhibit the function of rat liver

mitochondria (Peixoto, 2005). *Saccharomyces cerevisiae* is a convenient model eukaryotic organism to study loss of capacity for aerobic respiration, a marker for function of the mitochondria. Both herbicide formulations and potential components were tested for effects on yeast respiration.

3.2 Results

3.2.1 Effect of purified active ingredients on the antibiotic response of S. Typhimurium.

The effects of herbicide active ingredient exposure on the response of *S. Typhimurium* to five antibiotics were tested. The bacteria were grown on plates a range of different concentrations of each antibiotic in the presence and absence of the active ingredient (at a constant concentration) (see chapter 2 section 2.1.2). The ingredient concentrations used were below those that would affect bacterial growth. Titres were used to calculate the efficiency of plating (EOP) before statistical analysis and graphing. The detection range was an EOP of $1 – 10^{-7}$.

In the absence of the active ingredients, EOP is high at low concentrations of the antibiotic. The closer the concentration of the antibiotic gets to the minimum inhibitory concentration (MIC), the lower the EOP.

Differences between survival following antibiotic exposure and survival following exposure to both the antibiotic and herbicide active ingredient were compared. Active ingredients both increased and decreased the MIC depending on the antibiotic. As well, in some cases, there was either no statistically significant difference in growth upon exposure to the active

ingredient, or there was a statistically significant difference at one concentration of the antibiotic but this had no effect on the overall MIC.

Dicamba induced statistically significant responses to all of the antibiotics tested (figure 3.1). The MICs of ampicillin (p = 4.0 x 10^{-3}), chloramphenicol (p = 1.2 x 10^{-8}), ciprofloxacin (p = 3.1 x 10^{-8}) and tetracycline (p = 2.0 x 10^{-3}) for *S. Typhimurium* were increased .The largest changes were observed for dicamba which changed the MICs for chloramphenicol, ciprofloxacin, tetracycline and ampicillin by, 7.0-fold, 3.5-fold, 2.7-fold and 1.3-fold, respectively (table 3.1). There was also a statistically significant difference for kanamycin (p = 3.0 x 10^{-3}) at one concentration of the antibiotic. However, this had no effect on the MIC.

Pure 2,4-d induced statistically significant increases in the MICs for the antibiotics chloramphenicol (p = 4.1 x 10^{-9}) , ciprofloxacin (p = 8.2 x 10^{-13}) and tetracycline (p = 9.0 x 10^{-4}) (figure 3.2). The magnitudes of these changes were 2.5-, 1.8- and 2.2-fold, respectively (table 3.1). The MIC of kanamycin decreased 4.0-fold (p = 2.0 x 10^{-4}). There was no significant change in response to ampicillin (p = 0.1) upon exposure to thus active ingredient.

The addition of glyphosate resulted in statistically significantly higher MICs for ampicillin (p = 1.1 x 10^{-5}), ciprofloxacin (p = 1.2 x 10^{-5}) and kanamycin (p = 4.1 x 10^{-10}) compared to those grown without glyphosate (figure 3.3). The magnitudes of these changes were 1.8-, 2.0- and 5.0-fold, respectively (table 3.1). In contrast, the MIC for chloramphenicol decreased 1.5-fold (p = 1.0 x 10^{-2}) and that of tetracycline decreased 1.4-fold (p = 1.0 x 10^{-3}) upon co-exposure to glyphosate.

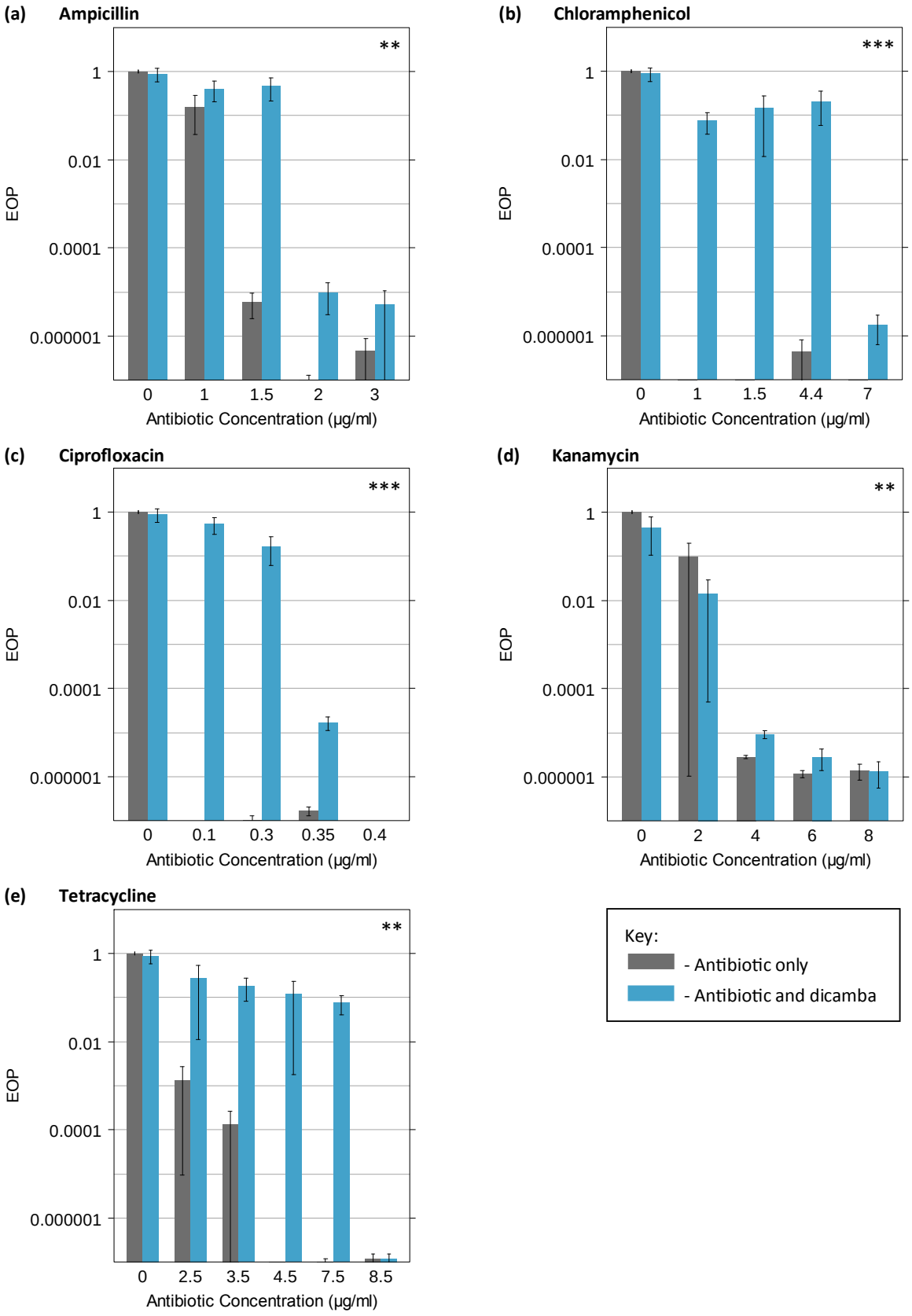

Figure 3.1: Survival of *S. Typhimurium* on a series of concentrations of (a) ampicillin, (b) chloramphenicol, (c) ciprofloxacin, (d) kanamycin and (e) tetracycline +/- dicamba. Survival is reported as EOP. Error bars are standard error of the mean (SEM). Asterisks in top right corner of graphs denote significance level. (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$.

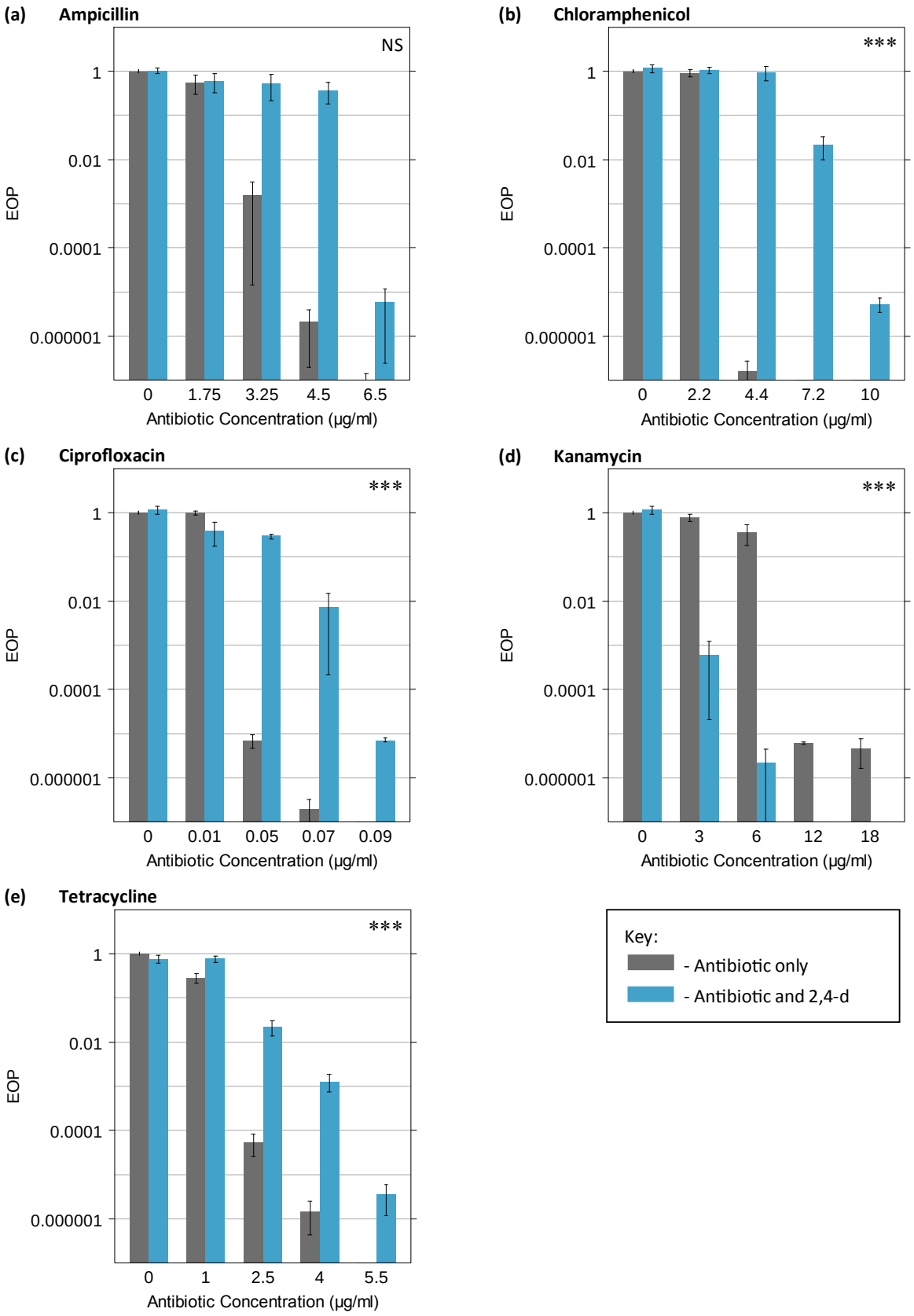

Figure3.2: Survival of *S. Typhimurium* on a series of concentrations of (a) ampicillin, (b) chloramphenicol, (c) ciprofloxacin, (d) kanamycin and (e) tetracycline +/- 2,4-d. Survival is reported as EOP. Error bars are SEM. Asterisks in top right corner of graphs denote significance level. (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$.

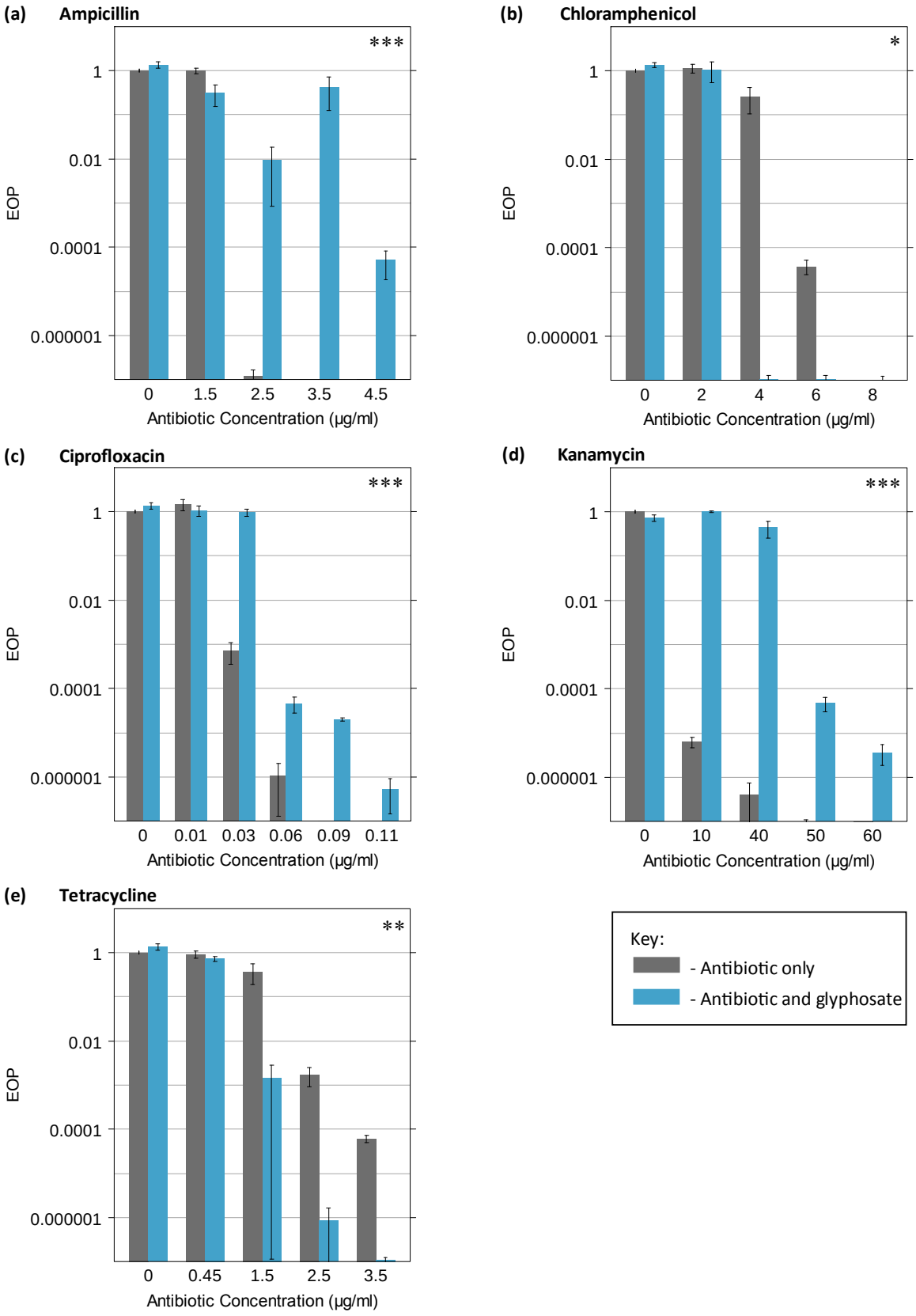

Figure 3.3: Survival of *S. Typhimurium* on a series of concentrations of (a) ampicillin, (b) chloramphenicol, (c) ciprofloxacin, (d) kanamycin and (e) tetracycline +/- glyphosate. Survival is reported as EOP. Error bars are SEM. Asterisks in top right corner of graphs denote significance level. (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$.

Table 3.1: Fold change in antibiotic concentration necessary to cause a 1000-fold reduction in EOP upon exposure to the active ingredients. Ns: not significant, 0: killing curves show statistically significant differences, but the drop below EOP 0.001 occurs at the same antibiotic concentration for treatment and no treatment plates. Concentrations of active ingredients (ppm ae) used are shown in parenthesis.

	Amp	Cm	Cip	Kan	Tet
dicamba	1.3 (1500)	7.0 (1500)	3.5 (1500)	0 (1500)	2.7 (1500)
2,4-d	NS	2.5 (600)	1.8 (5000)	4.0 (6000)	2.2 (500)
glyphosate	1.8 (3000)	1.5 (3000)	2.0 (200)	5.0 (200)	1.4 (3000)

3.2.2 Effect of surfactants on antibiotic resistance in S. Typhimurium.

The effect of the surfactants on the antibiotic resistance phenotype of *S. Typhimurium* was tested using the same experimental setup as above. The data is shown in figures 3.4 and 3.5.

Both Tween80 and CMC induced statistically significant increases in resistance to several of the antibiotics (figure 3.4 and 3.5). Resistance to chloramphenicol, ciprofloxacin, kanamycin and tetracycline was increased by Tween80 and resistance to ampicillin, kanamycin and tetracycline was increased by CMC. No decrease in resistance was observed for any combination of surfactant and antibiotic. For the combination of Tween80 and ampicillin, and CMC and chloramphenicol or ciprofloxacin, no significant difference in growth between the antibiotic only and antibiotic and surfactant treatment plates was detected.

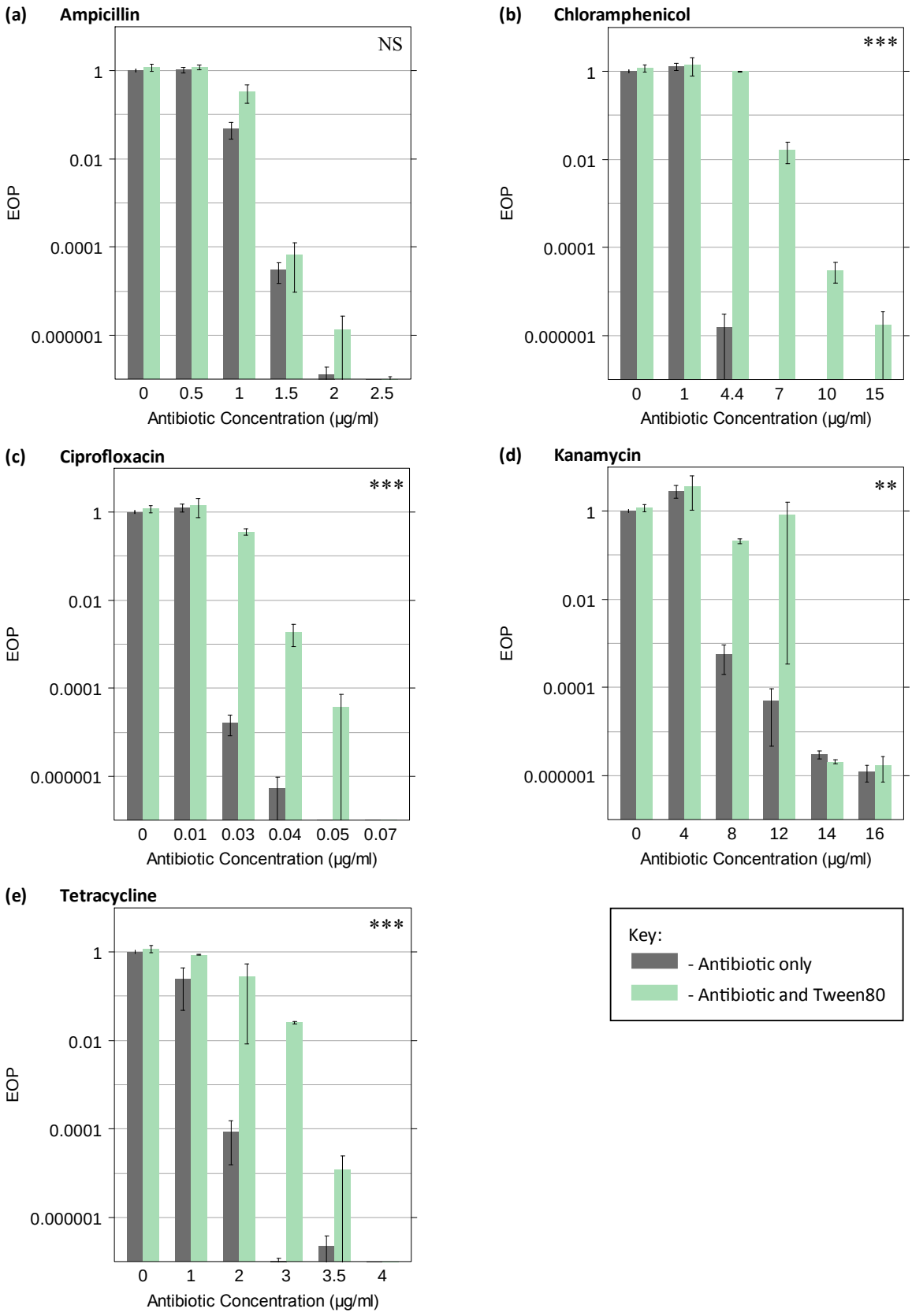

Figure3.4: Survival of *S. Typhimurium* on a series of concentrations of (a) ampicillin, (b) chloramphenicol, (c) ciprofloxacin, (d) kanamycin and (e) tetracycline +/- Tween80. Survival is reported as EOP. Error bars are SEM. Asterisks in top right corner of graphs denote significance level. (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$.

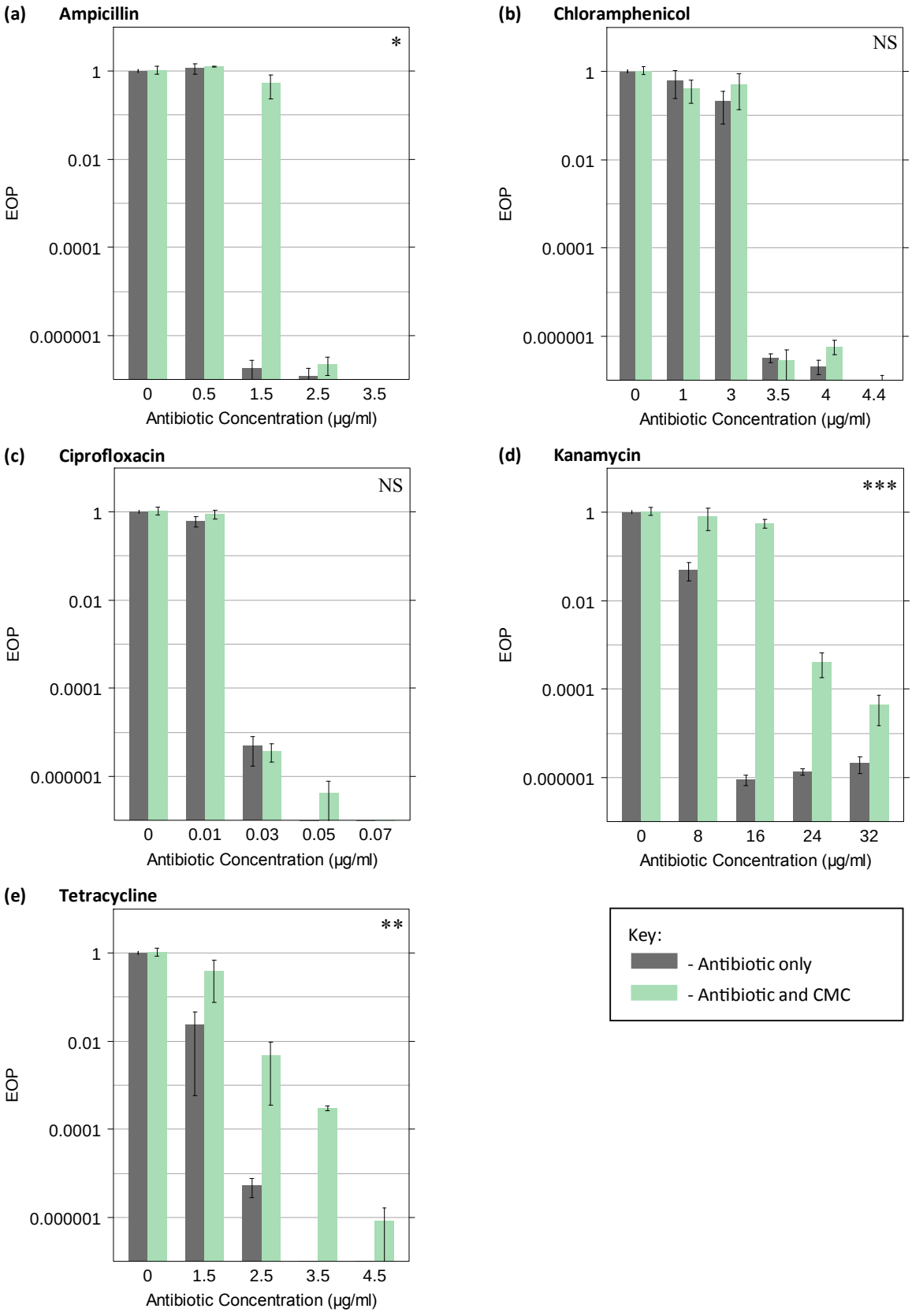

Figure 3.5: Survival of *S. Typhimurium* on a series of concentrations of (a) ampicillin, (b) chloramphenicol, (c) ciprofloxacin, (d) kanamycin and (e) tetracycline +/- CMC. Survival is reported as EOP. Error bars are SEM. Asterisks in top right corner of graphs denote significance level. (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$.

The differences in magnitude of the effects seen are shown in table 3.2 as the fold-change in MIC for each antibiotic with and without the surfactants. This fold-change, for most combinations, ranged from between 1.2- to 2.3-fold. The largest change seen was 2.3-fold for the combination of Tween80 and chloramphenicol ($p = 2.2 \times 10^{-11}$).

Table 3.2: Fold-change in the antibiotic concentration necessary to cause a 1000-fold reduction in EOP upon exposure to the surfactants. Concentrations of Tween80 and CMC used were 2% and 1%, respectively. NS: Not statistically significant.

	Amp	Cm	Cip	Kan	Tet
Tween80	NS	2.3	1.2	1.8	1.8
CMC	1.7	NS	NS	1.5	1.4

3.2.3 Dose response.

After determining whether there was an effect of the herbicide components on responses to antibiotics, the next step was to find the minimum concentration necessary to induce the response. This is defined as the minimum concentration of the herbicide component sufficient to cause an at least 100-fold change in the EOP value. Bacteria were grown in a series of plates on solid media representing a range of concentrations of the herbicide components and the same concentration of the antibiotic. Antibiotic concentrations that had shown the largest difference in EOP between treatments in the previous section were used for these experiments and thus were different for each combination of herbicide and antibiotic. They are shown in parentheses in table 3.3. EOP values were determined for every combination of antibiotic and herbicide component and can be found in the graphs contained in appendix A. Table 3.3 shows the minimum inducing concentrations for each combination where a significant change in antibiotic resistance was detected in the previous experiments.

Table 3.3: Minimum concentration of each herbicide component necessary to induce a 100-fold change in the EOP of the bacteria in the presence of the antibiotics ampicillin (Amp), chloramphenicol (Cm), ciprofloxacin (Cip), kanamycin (Kan) and tetracycline (Tet) for *S. Typhimurium.* This was not determined for combinations where a significant change in MIC was not detected in the previous section and is denoted by (-). Antibiotic concentrations (μg/ml) used shown in parentheses.

	Amp	Cm	Cip	Kan	Tet
Dicamba	100 (1.5)	1000 (4.4)	1000 (0.1)	-	50 (3.5)
2,4-d	-	100 (4.4)	7000 (0.05)	6000 (6)	300 (2.5)
Glyphosate	1500 (2.5)	500 (4)	200 (0.05)	125 (12)	50 (2.5)
Tween80	-	<0.05% (4.4)	1% (0.03)	2% (12)	2% (2)
CMC	1% (1.5)	-	-	0.25% (12)	1% (2.5)

Of the herbicide active ingredients, the minimum inducing concentrations were generally the lowest for glyphosate (table 3.3, row 3), followed by dicamba (row 1), and then 2,4-d (row 2). All of the concentrations determined were statistically significant.

2% Tween80 was necessary to induce resistance to kanamycin (p = 2.4 x 10^{-2}) and tetracycline (p = 8.9 x 10^{-4}). Resistance to ciprofloxacin was induced by 1% (p = 3.2 x 10^{-5}). Chloramphenicol resistance was induced at the lowest concentration of Tween80 (0.05%, p = 6.3 x 10^{-3}).

The lowest concentration of CMC to induce an effect was 0.25% for kanamycin (p = 2.0 x 10^{-5}). 1% CMC induced the required 100-fold change in the EOP for ampicillin (p = 1.1 x 10^{-1}) and tetracycline (p = 1.1 x 10^{-2}), however, the p-value for ampicillin was not statistically significant. This suggests that the effect induced by CMC on the ampicillin resistance phenotype at the concentrations tested was small. It may be that at higher concentrations of CMC a statistically significant minimum inducing concentration for ampicillin resistance could be determined.

3.2.4 Kamba and salicylic acid additively induce antibiotic resistance in S. Typhimurium.

Both the herbicide Kamba (Kurenbach *et al.*, 2015) and salicylic acid (Kurenbach *et al.*, 2015; Marjoshi, 2014; Rosner, 1985) cause changes in the response of *S. Typhimurium* to a number of antibiotics including chloramphenicol. Both of these chemicals could induce the full response at a minimum concentration of 250 ppm ae. In different environments, bacteria may not be exposed to sufficient concentrations of these chemicals alone to induce the response. However, if chemicals such as these have additive effects, then a mixture of low concentrations of multiple different compounds might still induce resistance.

To test this hypothesis, *S. Typhimurium* was exposed to Kamba and salicylic acid at the same time. The concentrations of each compound varied, but the two added up to a total of 250 ppm ae, the concentration for which full induction with either substance had been demonstrated. Calculated EOPs for the combinations and controls are shown in the graph in figure 3.6.

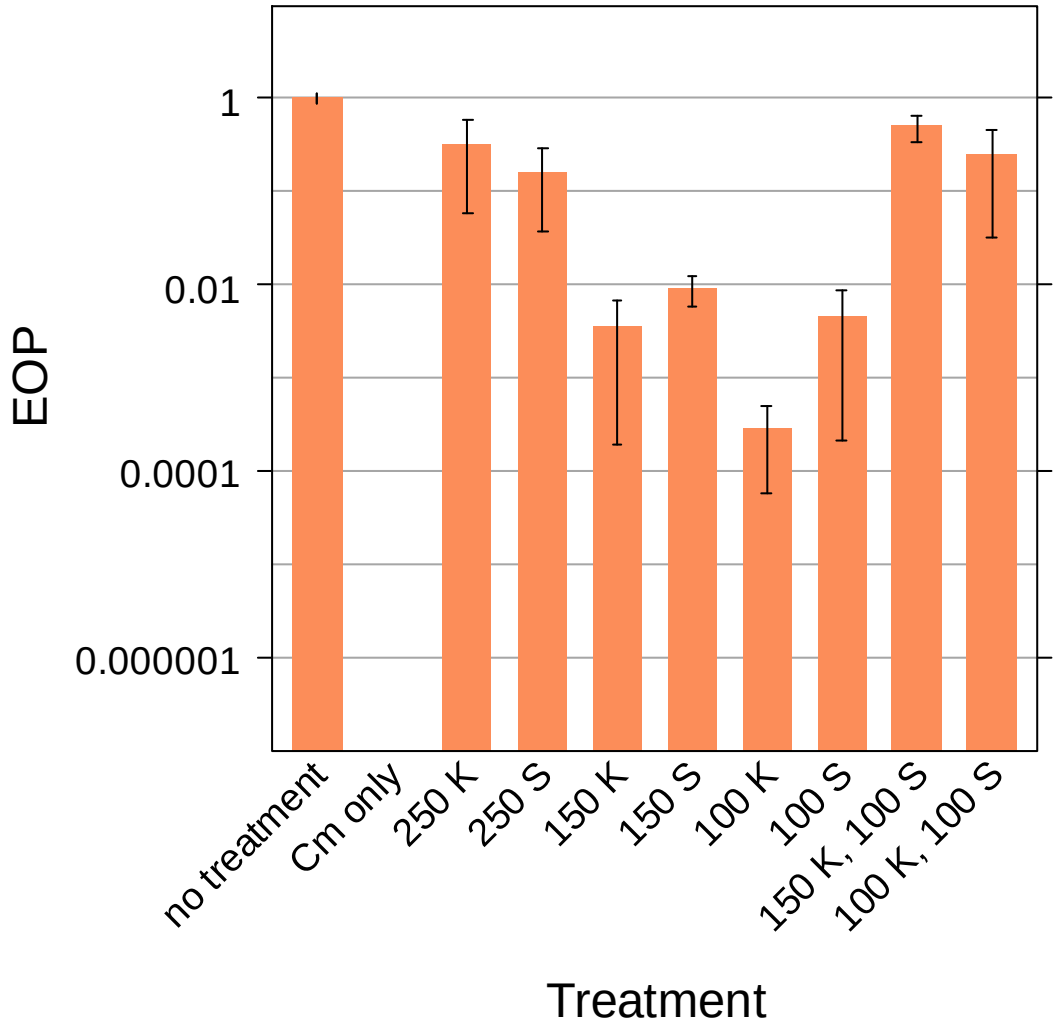

Figure 3.6: Survival of *S. Typhimurium* exposed to chloramphenicol (Cm) in combination with a range of concentrations of Kamba (K) and salicylic acid (S). Only when the total concentration of Kamba and salicylic acid reached 250 ppm ae was near complete survival of the bacteria in the presence of chloramphenicol achieved. If this concentration was reduced to 100 or 150 ppm ae survival was reduced.

The data confirms that treatment with 4.4 µg/ml chloramphenicol reduced the EOP to the detection limit (less than 1×10^{-7}), but that 250 ppm ae of either Kamba or salicylic acid was sufficient to increase survival almost to the same level as the no treatment control (EOP = 1). When bacteria were exposed to chloramphenicol and lower concentrations (100 or 150 ppm ae) of either chemical the EOP was between 1×10^{-2} and 1×10^{-4}. Thus, these concentrations were sufficient to partially increase survival in the presence of the antibiotic. Both combinations of Kamba and salicylic acid tested were sufficient to increase

survival to EOPs comparable to that of the no treatment control and to those produced when both chemicals were used singularly at 250 ppm ae. The total concentration of inducing chemicals (250 ppm ae) explained 52% of the variation in log-transformed EOP (p = 2.8 x 10^{-4}). Accounting for the proportion that was Kamba did not significantly improve the model (p = 0.46). This indicates that the change in the chloramphenicol resistance phenotype observed depended on the total amount of inducing compound, not the individual proportions of Kamba and salicylic acid.

3.2.5 Observing the effects of herbicides on aerobic respiration in S. cerevisiae.

Roundup has been reported to have effects on mitochondria of rat liver cells (Peixoto, 2005). Mitochondria are directly descended from free living bacteria. Thus, we sought to determine if they had any detectable separate response to herbicide exposures.

Mitochondrial activity can be easily assayed using differential growth media and the single-celled eukaryotic yeast *S. cerevisiae*. Carbon sources such as glycerol cannot be fermented and thus yeast provided with glycerol must grow aerobically via the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. Both of these pathways are restricted to the mitochondria. In contrast, glucose is a fermentable carbon source and providing yeast with glucose allows them to grow anaerobically.

The top agar overlay method was used to create homogenous lawns of *S. cerevisiae* on both YPD (glucose) and YPG (glycerol) solid media. 30 µl of the test chemical was then placed onto a filter in the centre of the lawn. Three different concentrations of each herbicide were tested on both YPD and YPG. Differences in the diameter of the halo produced were then compared between the two media (figure 3.7).

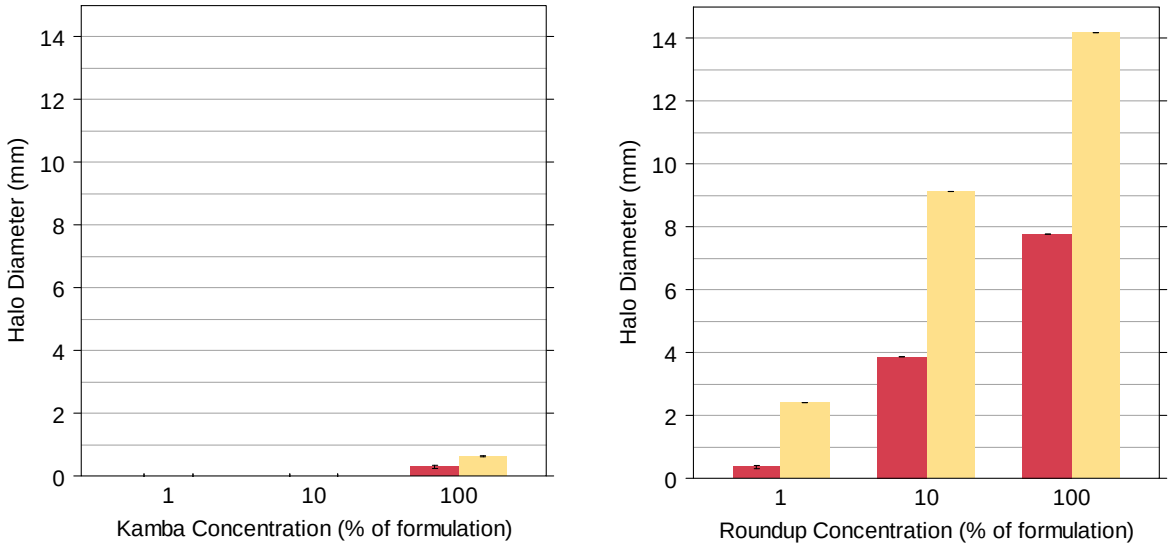

Figure 3.7: Inhibition of the growth of *S. cerevisiae* by Kamba (left) and Roundup (right) on both YPD (red) and YPG (yellow) media.

The herbicide Kamba was only toxic to *S. cerevisiae* at the highest concentration tested (100% of the formulation or 415,000 ppm ae dicamba) (p = 1.1 x 10^{-3}). There was no statistically significant difference in growth inhibition between the two media (p = 0.40). Treatment with either 1% or 10% of the formulation (4,150 ppm ae and 41,500 ppm ae of dicamba, respectively) did not produce visible halos around the filters.

Halo diameter significantly increased with Roundup concentration, although growth inhibition was observed at all concentrations tested. Differences in diameter between the YPD and YPG media were statistically significant overall (p = 5.3 x 10^{-4}) as well as at all three concentrations, 1% (2,664 ppm ae), 10% (26,640 ppm ae) and 100% of the formulation (266,400 ppm ae), individually (p = 1.6 x 10^{-3} for each concentration). When glycerol was the only carbon source, Roundup caused larger halos than when glucose was present. This indicates that Roundup had an inhibitory effect on respiration in *S. cerevisiae*.

In order to determine whether the effect seen was caused by the glyphosate, 10,000 ppm ae glyphosate was used instead of the commercial formulation. This was the highest

concentration that could be solubilised in water and corresponds to using about 3.75% strength of the formulation. No growth inhibition of *S. cerevisiae* was observed at 10,000 ppm ae on either YPD or YPG media suggesting that glyphosate is either unlikely to be the cause of the effect seen with Roundup, or requires other components of the formulation to reach its target.

Other herbicide components with potential to cause an effect include the surfactants tested for antibiotic resistance inducing effects in section 3.2.2. Both Tween80 and CMC were tested in the same manner as above. 30 µl of Tween80 was pipetted directly on top of the yeast lawn without dilution whereas a 1% solution of CMC was used with a filter. As with glyphosate, neither of these caused the formation of a halo on either media.

3.3 Discussion

Herbicide use has increased globally over the decades with some of the most intensive use associated with herbicide-tolerant crops (Editor, 2014). With this increased use it is important to identify and understand any unintended effects. The purpose of this chapter was to further investigate the different effects of herbicides on microorganisms including changes in the antibiotic resistance of bacteria and the loss of mitochondrial function in yeast. This study provides evidence that antibiotic resistance induced by herbicides can potentially be caused by several components and also that different compounds may have additive effects. In addition, the inhibitory effect of Roundup on mitochondria was demonstrated for the eukaryote, *S. cerevisiae.*

3.3.1 Herbicide components are sufficient to cause changes in the response of S. Typhimurium *to antibiotics.*

Commercially available formulations of commonly used herbicides have previously been shown to induce changes in the response of *E. coli* and *S. Typhimurium* to a range of antibiotics. A key aim of this chapter was to identify which components of the herbicide formulations might be responsible for these effects. Three active ingredients and two surfactants were tested for the ability to induce changes in the resistance of *S. Typhimurium* to five antibiotics.

Table 3.4 shows a summary of the changes in antibiotic MICs induced by the different herbicide components. The three active ingredients are paired with the corresponding herbicide formulation for comparison (Kurenbach *et al.*, 2015). Dicamba caused increases in resistance that ranged from 1.3-fold (ampicillin) to 7.0-fold (chloramphenicol) and no change. 2,4-d induced increases in some of the antibiotic MICs (ranged from 1.8-fold to 2.5-fold) and decreased the MIC of kanamycin. Glyphosate induced both increases (ranged from 1.8-fold to 5.0-fold) and decreases (ranged from 1.4-fold to 1.5-fold) in the MICs of the antibiotics.

In general, the effects of the active ingredients follow in the same trend as the formulations. Exceptions to this rule were combinations where the effect changed from an increase or decrease to not significant or vice versa. For example, the MIC of kanamycin did not change as a result of exposure to dicamba, however an increase was observed upon exposure to Kamba. Glyphosate induced an increase in the MIC of ampicillin where Roundup caused no change. In addition, the herbicide formulation 2,4-D increased resistance to ampicillin but there was no statistically significant change caused by the active ingredient alone. In no

instance was the direction of the change in MIC induced by the active ingredient opposite to that induced by the formulation.

The magnitude of the changes in MIC induced by the herbicide formulations and the active ingredients cannot be directly compared due to differences in the concentrations used in the experiments. When commercial formulations were used it was possible to keep the herbicide concentrations consistent (Marjoshi, 2014) but solubility and cost made this impossible for the active ingredients. However, since the change, if there was one, was always in the same direction for the formulations and active ingredient, the active ingredient seems to dominate the overall effects of the formulations. Taken together, the evidence suggests but does not prove that the active ingredient is necessary for the observations of Kurenbach *et al.* (2015).

Table 3.4: Change in MIC of *S. Typhimurium* for each antibiotic when in combination with the herbicide components. Previously published results for the herbicide formulations (F) are shown next to the corresponding active ingredient (A) for comparison. Results for the two tested surfactants (S) are also shown. (>): increase in resistance, (<): decrease in resistance, (NS): not statistically significant, (0): statistically significant difference was detected at one concentration but this did not change the calculated MIC.

	Ampicillin	Chloramphenicol	Ciprofloxacin	Kanamycin	Tetracycline
Kamba (F)	>	>	>	<	>
Dicamba (A)	>	>	>	0	>
2,4-D (F)	>	>	>	<	>
2,4-d (A)	NS	>	>	<	>
Roundup (F)	0	<	>	>	<
Glyphosate (A)	>	<	>	>	<
Tween80 (S)	NS	>	>	>	>
CMC (S)	>	NS	NS	>	>

Both surfactants caused increases in the response of *S. Typhimurium* to a number of the antibiotics tested (table 3.4). Three exceptions, Tween80 and ampicillin, CMC and chloramphenicol, and CMC and ciprofloxacin, showed no change in the MIC. Increases

ranged from 1.2-fold to 2.3-fold. Unlike the active ingredients, no combination of surfactant and antibiotic resulted in a decrease in resistance. This constitutes a pattern of responses not seen for either the herbicide formulations or the active ingredients. In addition, the fold changes observed for the surfactants were at the lower end of the spectrum compared to both the herbicide formulations and active ingredients. In some cases the direction of the response induced by the surfactants contrasted with that induced by some of the active ingredients (table 3.4), although it was generally weaker. For example, commercial 2,4-D decreased the MIC of kanamycin, as did the active ingredient 2,4-d. However, Tween80 and CMC both caused an increase in the MIC.

The aim of this set of experiments was to determine which components of the herbicide formulations might be responsible for the change in the response of *S. Typhimurium* to antibiotics. The results suggest that the active ingredients are not only able to induce this effect; they may be the main determinant of the direction of the change in antibiotic resistance. But, because there are differences in the magnitude of the MIC change, other substances in the formulations must be modulating the response, either upwards or downwards in terms of resistance. Since the surfactants showed only increases in resistance, contrasting effects might occur at the same time. This makes it difficult or impossible to predict the exact outcome. Obviously none of these effects are strong enough to flip the observed phenotypic response the other way. Furthermore, the effect might vary in magnitude or direction with other surfactants and I did not know what surfactants were actually present in the herbicide formulations used in the previous work (Kurenbach *et al.*, 2015). However, the results of this study do hint that interactions between different

chemicals in these formulations may alter the individual effects resulting in the final response observed.

3.3.2 Concentrations of herbicide components within potential exposure levels for bacteria are sufficient to cause a response.

Maximum residue limits (MRLs) for pesticides and maximum levels of additives in food are set by regulatory bodies such as governments, often based on guidance from international bodies such as the Codex Alimentarius Commission (Codex Alimentarius Commission, 2012). The Commission developed and now maintains the Codex Alimentarius which is a collection of guidelines, standards and recommendations that relate to the production and safety of foods. It was initially started by the Food and Agriculture Organisation of the United Nations (FAO) and was later joined by the World Health Organisation (WHO). The World Trade Organisation recognises the Codex Alimentarius as an international reference and so the regulations it contains pertain particularly to products which are traded internationally. The minimum concentrations of the active ingredients and surfactants necessary to induce changes in the response of *S. Typhimurium* to antibiotics were determined and compared to the internationally recognised guidelines and regulatory limits contained within the Codex Alimentarius.

A summary of the MRLs specified by the Codex Alimentarius for dicamba, 2,4-d and glyphosate are provided in table 3.5. Of the minimum inducing concentrations determined for the three active ingredients, none were within the MRLs for products intended for human consumption (Codex Alimentarius Commission, 2012). However, for some of the combinations of antibiotic and herbicide active ingredient tested, the inducing concentrations were within the MRLs for animal feed. Dicamba caused changes in the

response of *S. Typhimurium* to tetracycline at 50 ppm ae, which was equal to the highest MRL for this herbicide and the minimum inducing concentrations of 2,4-d for chloramphenicol (100 ppm ae) and tetracycline (300 ppm ae) were both below the MRL for grass hay and fodder of 400 ppm ae. Glyphosate induced changes in the responses to tetracycline (50 ppm ae), kanamycin (125 ppm ae), ciprofloxacin (200 ppm ae) and chloramphenicol (500 ppm ae) at concentrations that fall below the MRLs for alfalfa, pea or grass hay and fodder for this active ingredient. Moreover, these MRLs only apply to animal feed that is internationally traded. If it is used domestically, higher herbicide levels may be allowed.

Table 3.5: Summary of the MRLs of the herbicide active ingredients dicamba, 2,4-d and glyphosate for human food products and animal feed applied for international traded commodities by the Codex Alimentarius Commission (Codex Alimentarius Commission, 2012). Concentrations are given in ppm ae.

	Dicamba	2,4-d	Glyphosate
Human food products (ppm ae)	0.01 - 10	0.01 - 5	0.05 – 40
Animal feed (ppm ae)	0.6 - 50	0.01 - 400	50 - 500

MRLs are set for the final food product that is traded or sold (Horváth *et al.*, 2014). However, when pesticides are applied to a crop, the concentration recommended by the manufacturer must be sufficient to control pests and discourage the evolution of resistant weeds (Crespo, 2011). This means that the recommended application rates can be substantially higher than that found in the final product due to dilution or breakdown of the substance. Some example application rates for dicamba (Kamba^{500}, Nufarm), 2,4-d (2,4-D amine 800 WSG, Agpro) and glyphosate (Roundup Weed Killer, Monsanto) are given in table 3.6. All observed minimum inducing concentrations for the active ingredients fall within these levels.

Bacteria in a wide variety of environments might be exposed to herbicides and not just on the crops which are treated with these formulations. The active ingredients have been identified in soils (Kremer & Means, 2009) and in water (Battaglin *et al.*, 2014; Ensminger *et al.*, 2013; Filkowski *et al.*, 2003; Van Stempvoort *et al.*, 2014). For the observed effect on the antibiotic resistance phenotype to occur, bacteria were co-exposed to the herbicides and the antibiotics. Antibiotics have also been found in both soil and waterways due to the use of animal manure as fertiliser (Kemper, 2008; Mackie *et al.*, 2006). Other environments where bacteria might come into contact with both herbicides and antibiotics include humans, animals and insects. Humans and animals can come into contact with herbicides through eating or drinking contaminated food or water or through inhalation or skin contact from spray drift and direct herbicide use. In addition, both farm animals (Dolliver *et al.*, 2008; Shea, 2003) and bees (Tian *et al.*, 2012) are treated prophylactically with antibiotics. This provides several exposure pathways for bacterial communities to come into contact with both herbicides and antibiotics. Even if bacteria are not exposed to sufficient concentrations of herbicide active ingredients to induce changes in their response to antibiotics in human food or animal feed, if MRLs have been adhered to, those found on crop plants or in the nearby environment and exposed to application level concentrations may be.

Table 3.6: Examples of recommended application rates for three herbicide active ingredients. Value for dicamba was taken from a container of Kamba[500] (Nufarm, Otahuhu, New Zealand), for 2,4-d from 2,4-D amine 800 WSG (Agpro, Auckland, New Zealand) and glyphosate from Roundup Weed Killer (Monsanto, Australia). Concentrations are given in ppm ae.

	Dicamba	2,4-d	Glyphosate
Recommended application rate (ppm ae)	415 – 2,200	33,080	2,664 – 87,912

The concentration of adjuvants used in herbicide formulations are usually unregulated (Vincent & Davidson, 2015). Formulations often contain 1-10% of the adjuvants known as surfactants, although some glyphosate herbicides are known to have concentrations as high as 15% (Castro *et al.*, 2014; Lee *et al.*, 2009). Two surfactants, Tween80 and CMC, were shown to cause increases in the resistance of *S. Typhimurium* to some antibiotics. Both of these chemicals are also used in a wide range of different food products. Regulatory bodies, such as the Codex Alimentarius Commission, provide maximum limits and guidelines for their use in food (Codex Alimentarius Commission, 2015).

Tween80 induced resistance to kanamycin and tetracycline at 2% and to ciprofloxacin at 1%. The general standard of food additives by Codex provides limits for polysorbates across a wide range of foods. Most limits lie between 0.001% and 0.5%, however the maximum level for food supplements, such as vitamin or mineral tablets, is 2.5% (Codex Alimentarius Commission, 2015). The lowest minimum inducing concentration for Tween80 was for chloramphenicol. At the lowest concentration of the surfactant tested, 0.05%, the EOP of the bacteria was not reduced. This suggests that increased resistance to chloramphenicol could be caused by levels within the maximum limits set by Codex for products such as chewing gum, sauces and dairy-based desserts. For a full list of products the reader is directed to http://www.fao.org/gsfaonline/groups/details.html?id=99.

Codex also provides a substantial list of the different food categories in which CMC can be found (for a full list see: http://www.fao.org/gsfaonline/additives/details.html?id=51). There are no maximum limits for this additive as it is generally regarded as safe (GRAS), but it must be used under the standards of Good Manufacturing Practice (GMP) (Codex Alimentarius Commission, 2015). This states that the additive added to food or in the

manufacturing process be limited to the lowest levels necessary to produce the desired effects (Codex Alimentarius Commission, 2015). Cani and Everard (2015) state that both CMC and polysorbate-80 (Tween80) are used in food products at levels as high as 2%. CMC induced increased resistance of *S. Typhimurium* to ampicillin and tetracycline at 1% and to kanamycin at levels as low as 0.25%. This suggests that ingestion exposure may be sufficient to induce resistance in mouth or gut bacteria. In addition, surfactants such as polysorbates and CMC are widely used in cosmetics and personal hygiene products that we apply to our bodies on a daily basis. In these circumstances there are often no regulatory limits. Organisms on skin or hair and even in mouths could be exposed to these compounds at much higher levels than those shown here to change the response of *S. Typhimurium* to antibiotics.

3.3.3 The effects of inducing compounds can be additive.

Kurenbach et.al. (2015) found that the minimum amounts of the herbicides necessary to induce a 100-fold change in EOP at a given concentration of the antibiotics tested were, in most cases, above the MRLs for human food and animal feed allowed under international trading laws (Codex Alimentarius Commission, 2012). Similar results have been presented in this thesis for the pure active ingredients of these herbicides. This would suggest that, provided pesticide concentrations on food or feed are below the MRLs, there should be no significant phenotypic changes to the antibiotic resistance of gut microflora, if food and feed, and the herbicides, are the only exposures. However, this study provides evidence that the effects of different chemicals that induce similar changes in antibiotic resistance can be additive. The results show that a total concentration of 250ppm ae, irrelevant of whether it is made up of Kamba, salicylic acid or a combination of the two, was sufficient to increase the survival of *S. Typhimurium* in the presence of chloramphenicol.

This indicates that even if the concentrations of individual chemicals are within the MRLs, the use of multiple compounds may still lead to changes in antibiotic resistance of exposed bacteria. Adaptive responses such as these have been observed to be caused by bile salts (Prouty *et al.*, 2004), three commercially available herbicide formulations (Kurenbach *et al.*, 2015), their active ingredients, two surfactants and salicylic acid (this study, Rosner, 1985)). In addition, other biocides such as triclosan, chlorhexidine (Braoudaki & Hilton, 2004) and quaternary ammonium compounds (Buffet-Bataillon *et al.*, 2016) have also been shown to induce adaptive resistance to antibiotics. The range of different molecular structures tested so far suggests that other chemicals, that we as humans and the bacteria associated with us and our environment are exposed to regularly, may also have the potential to induce these changes in antibiotic response of bacteria and have not yet been identified. If bacteria are exposed to multiple compounds that are able to induce this response and the effects are additive, the exposure concentration of one compound may not be the only matter of importance for determining safe exposure limits.

3.3.4 Effects of herbicides on aerobic respiration in S. cerevisiae.

Roundup, but not glyphosate alone, has been shown to depress state 3 and uncoupled respiration in the mitochondria of rat liver cells (Peixoto, 2005). In this study I examined the effects of Kamba and Roundup formulations on *S. cerevisiae* growing in fermentable and non-fermentable conditions.

There appears to be no specific effect of Kamba on the mitochondrial function of *S. cerevisiae*. Only the undiluted Kamba formulation inhibited growth of the yeast. This occurred on both the fermentable (YPD) and non-fermentable (YPG) media, which hints at a mechanism of toxicity that is independent of mitochondrial function.

In contrast to Kamba, Roundup was toxic to *S. cerevisiae*. It inhibited growth on the fermentable medium (glucose) at the lowest concentration of Roundup tested, which was 1% of the formulation. Roundup also had an effect on the aerobic respiration. There were statistically significant differences in growth inhibition on the two media at all three concentrations of Roundup tested. This indicated that the toxic effects of this herbicide increased when fermentation was not an option for ATP production. Two of the concentrations (1% and 10%) at which Roundup had significant effects on mitochondrial activity were within the recommended application rates for this formulation which were 33% for wiper application and 1% for use as a foliage spray.

The active ingredient of Roundup, glyphosate, was also tested in the same way. As in previous work (Peixoto, 2005), it appeared to have no effect on respiration and had no effect on halo size when yeast were grown on either carbon source at the concentrations tested. However, due to its low solubility in water, it could not be used at levels comparable to the highest concentration in the Roundup formulation. Hence, glyphosate cannot be ruled out as a causal factor of the phenomenon in this model. It may require higher concentrations to show an observable effect.

As the active ingredient appeared to have no effect in isolation, the two surfactants were also tested. Neither undiluted Tween80 nor CMC (10 mg/ml) inhibited the growth of *S. cerevisiae* on either growth media. A further step would be to investigate whether polyethoxylated tallow amine (POEA), a known surfactant used in Roundup products (Mesnage *et al.*, 2013), causes similar effects to the formulation. POEA has been shown to be extremely toxic to fairy shrimp (*Thamnocephalus platyrus*) (Brausch & Smith, 2007) and to four species of frog found in North America (Howe *et al.*, 2004). It has also been shown to

have high toxicity to human cell lines (Mesnage *et al.*, 2013). In some cases it has demonstrated higher toxicity to the organisms than did some formulations of Roundup of which it was a component (Howe *et al.*, 2004; Mesnage *et al.*, 2013). Testing this surfactant with *S. cerevisae* may provide another clue to what component of the herbicide is responsible for the mitochondrial inhibition, particularly as at concentrations below those required for cell death, POEA has also been shown to have negative effects on the cellular respiration of human embryonic, hepatic and placental cell lines (Mesnage *et al.*, 2013). However, it is also possible that the effect of Roundup on respiration of S. cerevisiae is not due to one ingredient alone but to the combined effects of two or more. Unless regulators use studies specific to aerobic respiration, they may be underestimating the toxicity of herbicides.

Chapter 4

Investigating the mechanism by which herbicides induce changes in the antibiotic susceptibility of bacteria

4.1 Introduction

The changes in the responses of *E. coli* and *S. Typhimurium* to antibiotics are consistent with an adaptive response (Kurenbach *et al.*, 2015). The adaptive response usually involves a decrease in permeability and an increase in drug efflux. This can be achieved by a decrease in porins and new synthesis of efflux pumps. In this chapter, I tested the hypothesis that the adaptive response is caused by a change in antibiotic efflux or influx.

The aims of this chapter were to 1. assess whether efflux pumps are necessary for the herbicide-induced response to antibiotics and to 2. identify specific porins and efflux pumps that may be involved in the mechanism.

To achieve the first aim, I used the general efflux pump inhibitor phenylalanine-arginine-β-naphthylamide (PAβN) (Lomovskaya *et al.*, 2001). PAβN acts by competitively inhibiting access of the antibiotic to the efflux pump (Askoura *et al.*, 2011). Its use with a combination of herbicide and antibiotic was expected to confirm whether efflux pumps were an important part of the adaptive response mechanism.

To identify specific genes needed for the adaptive response, I tested various mutants. Those mutants were acquired from the Keio Collection (Baba *et al.*, 2006). The Keio collection was developed from *E. coli* line BW25113. The Keio strains are 'knockout' mutants, where

deletions are introduced into one gene in each strain and the kanamycin resistance gene *npt*II inserted as a marker. I tested strains with defined knockouts of particular efflux pump and porin genes. The strains were selected based on literature that indicated specific genes involved in adaptive changes in antibiotic resistance. The herbicides tested were Kamba, and Roundup. The antibiotics used were ciprofloxacin, streptomycin and tetracycline. These three antibiotics were selected from the original five used previously as representative examples of the different patterns of changes in antibiotic resistance that were observed (Kurenbach *et al.*, 2015). Streptomycin was used in place of kanamycin because the knockout strains were created using *npt*II, which grants resistance to kanamycin.

Before testing the knockout strains with combinations of herbicide and antibiotic, the MICs of each compound alone for each strain were determined. I hypothesised that knockout strains with a lower MIC of a herbicide or antibiotic than the wild-type strain were lacking a gene important for the intrinsic resistance of *E. coli* to that compound.

AcrAB-TolC and AcrAD-TolC are two key RND-efflux pumps in *E. coli* (Li & Nikaido, 2004). Strain CR5000 and JW2454 are Δ*acrB* and Δ*acrD*, respectively, and so neither can express the transporter protein for one of each of these pumps. Efflux is one of the known mechanisms of resistance to all of the antibiotics that were tested (Li & Nikaido, 2004; Nikaido, 1996; Rosenberg *et al.*, 2000). Thus, both strains were expected to have lower MICs of the antibiotics than the wild-type. AcrB is involved in the efflux of chloramphenicol, tetracycline, β-lactams and fluoroquinolones (Li & Nikaido, 2004; Nikaido, 1996) and so CR5000 was expected to be more susceptible to ciprofloxacin and tetracycline. JW2454 was expected to be more susceptible to streptomycin as AcrD has been implicated in the efflux of aminoglycosides (Rosenberg *et al.*, 2000). These pump complexes are often regulated

through stress response pathways (Amábile-Cuevas & Demple, 1991) and so it was expected that the MICs of the herbicides would also be lower for these strains than for the wild-type strain.

AcrA is the membrane fusion protein in both protein complexes and the gene for this protein was lacking from strain CR7000. This strain is therefore not able to produce functional complexes of either AcrAB-TolC or AcrAD-TolC and so was expected to have lower MICs than the wild-type for all herbicides and antibiotics.

TolC is the outer membrane channel of many efflux systems in *E. coli*, including the two mentioned above (Li & Nikaido, 2004). If the gene for this protein is knocked out, as it is in strain JW5503, the bacteria potentially lose the ability to remove substrates of many efflux pumps, including AcrAB-TolC and AcrAD-TolC, from the periplasm to the outside medium. This strain was expected to be the most susceptible to the widest range of antibiotics and herbicides.

In addition to the strains with reduced efflux capabilities, strain JW0912 with the genotype Δ*ompF* was also tested. It lacks the non-specific, outer-membrane porin OmpF. This porin has been implicated in the influx of tetracycline (Chopra & Roberts, 2001) and ciprofloxacin is also known to enter the cell through porins (Pagès *et al.*, 2008). JW0912 was expected to have higher resistance to these two antibiotics but not to streptomycin, which is thought to diffuse directly through the outer membrane lipid bilayer (Nikaido, 2003).

4.2 Results

4.2.1 Determining the relevance of efflux to herbicide-induced changes in antibiotic resistance phenotypes.

The efflux pump inhibitor PAβN is commonly used to diagnose resistance from efflux pumps (Lomovskaya *et al.*, 2001). The addition of the inhibitor fully or partially restores the efficacy of the antibiotic when resistance derives from the expression of efflux pump genes (Lomovskaya *et al.*, 2001). Combinations of herbicide and antibiotic were selected that resulted in increases in the antibiotic MIC (Kurenbach *et al.*, 2015). Solid media was supplemented with these compounds with or without PAβN, and then inoculated with *E. coli* strain JB578. Plate counts were converted into EOP values and significance was judged by the outcome of paired t tests on the log-transformed EOPs.

Table 4.1: EOP values for *E. coli* grown on the herbicides Kamba and Roundup alone and in combination with the antibiotics chloramphenicol (Cm) and kanamycin (Kan) both with and without PAβN. To determine whether there was a significant difference between the mean EOP values with and without PAβN, t tests were carried out and the corresponding *P* values are given.

Test condition	EOP (-PAβN)	EOP (+PAβN)	P value
LB	1.00	1.09	0.42
Kamba	1.42	0.292	0.058
Cm	2.28×10^{-3}	$< 1.00 \times 10^{-7}$	0.0010
Kamba + Cm	1.01	$< 1.00 \times 10^{-7}$	0.00012
Roundup	0.880	$< 1.00 \times 10^{-7}$	0.0058
Kan	8.69×10^{-5}	0.052	0.15
Roundup + Kan	1.44	$< 1.00 \times 10^{-7}$	0.00070

PAβN used alone had no effect on EOP (Table 4.1 row 1), suggesting that it had no effect on survival at the chosen concentration. In addition, when PAβN was used in combination with Kamba, the EOP was reduced by one order of magnitude, although this was not statistically significant (row 2). This suggests that at the concentration of Kamba tested (1380 ppm ae) active efflux was not an important factor in the intrinsic resistance of *E. coli* to this herbicide. Chloramphenicol alone decreased the EOP 1000-fold compared to the

combination of Kamba and chloramphenicol (row 3 vs. row 4, column 2), but the addition of PAβN reduced the EOP to the detection limit (row 4 column 3). This suggests that active efflux is important for Kamba to increase the resistance of *E. coli* to chloramphenicol.

PAβN increased Roundup toxicity. On media with Roundup and PAβN, the EOP was reduced by at least six orders of magnitude, falling to below the detection limit (row 5). This suggests that efflux is highly important to the intrinsic resistance of *E. coli* to this herbicide formulation. Kanamycin at the chosen concentration alone reduced the EOP from 1 to 8.69×10^{-5} (row 6 column 2). Surprisingly, PAβN increased the EOP approximately 1000-fold to 0.052 (column 3), although this difference was not statistically significant. This suggests either that PAβN does not inhibit all the relevant efflux pumps and the ones that are inhibited are not important for kanamycin resistance, or that active efflux itself is not important for resistance to kanamycin. The EOP of *E. coli* on both Roundup and kanamycin was 1.44. This was decreased to below the detection limit in the presence of PAβN (row 7). It is likely that this reduction is due to the high toxicity of Roundup alone in the absence of active efflux and so this experiment does not provide the information necessary to determine the importance of the efflux pumps to the increased resistance of *E. coli* to kanamycin when exposed to Roundup.

4.2.2 MICs of herbicides and antibiotics for E. coli *knockout strains.*

A collection of *E. coli* strains with knockouts of individual genes involved in the influx and efflux of antibiotics were used to investigate the mechanism by which herbicides affect antibiotic resistance in bacteria.

The first step for determining the importance of the influx and efflux components to the adaptive response mechanism was to measure individual MICs for all strains and all

compounds as a reference point for later experiments. In these experiments I define MIC as the minimum concentration of antibiotic necessary to reduce the EOP 1000-fold. Agar plates were supplemented with a series of concentrations of the herbicides and antibiotics and inoculated with *E. coli*. Plate counts were converted into EOP values and the MIC determined for each replicate. The mean MIC and the standard error of the mean (SEM) were calculated. Herbicide MICs are shown in table 4.2 and antibiotic MICs in table 4.3.

Table 4.2: The minimum concentration of each herbicide necessary to reduce the EOP by at least 1000-fold was determined separately for three different experiments. Values given are the mean ± SEM of these three experiments.

	MIC:					
Herbicide (ppm ae)	BW25113 (WT)	CR7000 (Δ*acrA*)	CR5000 (Δ*acrB*)	JW2454 (Δ*acrD*)	JW5503 (Δ*tolC*)	JW0912 (Δ*ompF*)
Kamba	14500 ± 0	7000 ± 0	7333 ± 333	12667 ± 333	3000 ± 0	14500 ± 0
Roundup	4000 ± 0	300 ± 0	300 ± 0	4000 ± 0	100 ± 0	4000 ± 0

The Δ*acrD*, Δ*acrA*, Δ*acrB* and Δ*tolC* but not Δ*ompF* genotypes had lower EOP values when exposed to Kamba. The MIC for the Δ*acrD* strain was higher than those for the Δ*acrA*, Δ*acrB* and Δ*tolC* strains. Kamba was most toxic to the Δ*tolC* strain. These results indicated that active efflux, and perhaps specifically AcrAB-TolC, was involved in the innate resistance of *E. coli* to Kamba. In addition, OmpF was not necessary for influx of Kamba into the cell. This herbicide may move through other outer membrane porins, diffuse directly through the membrane or be actively transported into the cell.

The MICs of Roundup were generally lower than those of Kamba and the strains were split into three groups. The MICs for the wild-type, Δ*ompF*, and Δ*acrD* strains were the highest followed by those for the Δ*acrA* and Δ*acrB* strains. The Δ*tolC* strain had the lowest MIC of Roundup. This indicated that again AcrAB-TolC may be largely responsible for the efflux of

Roundup from the cell and that this is a contributing factor to the intrinsic resistance of *E. coli* to this herbicide. As was seen for Kamba, OmpF did not appear to play an important role in the influx of Roundup.

Table 4.3: The minimum concentration of each antibiotic necessary to reduce the EOP by at least 1000-fold was determined separately for three different experiments. Values given are the mean ± SEM of these three experiments.

Antibiotic	MIC:					
	BW25113 (WT)	CR7000 (Δ*acrA*)	CR5000 (Δ*acrB*)	JW2454 (Δ*acrD*)	JW5503 (Δ*tolC*)	JW0912 (Δ*ompF*)
Ciprofloxacin (ng/ml)	10.0 ± 0.0	2.0 ± 0.0	3.3 ± 0.8	30.0 ± 0.0	1.0 ± 0.0	30.0 ± 0.0
Streptomycin (µg/ml)	5.8 ± 0.8	5.3 ± 0.7	8.0 ± 0.0	10.0 ± 0.0	2.0 ± 0.0	13.3 ± 3.5
Tetracycline (µg/ml)	1.0 ± 0.0	0.3 ± 0.0	0.8 ± 0.0	2.0 ± 0.0	0.5 ± 0.0	2.0 ± 0.0

The MICs of ciprofloxacin were lower for the Δ*acrA*, Δ*acrB*, and Δ*tolC* strains (row 1) than for the wild-type strain, indicating that these three pump components may be important for the efflux of ciprofloxacin. The MIC was three times higher for the Δ*ompF* strain than the wild-type, indicating that, as expected, OmpF contributes to the influx of ciprofloxacin. The MIC of ciprofloxacin was also three times higher for the Δ*acrD* strain, which lacks a component of the AcrAD-TolC efflux pump, than the wild-type. This suggests that this pump is not involved in removing ciprofloxacin from the cell.

There was less variation in the MICs of streptomycin across the strains (row 2). The MIC for the Δ*acrA* strain was similar to the wild-type and the MIC for the Δ*acrB* strain was slightly higher. The MIC of streptomycin for the Δ*tolC* strain was less than half that of the wild-type. Both Δ*acrD* and Δ*ompF* had MICs higher than the wild-type strain. As aminoglycosides have been found to be substrates of the AcrAD-TolC pump (Rosenberg *et al.*, 2000) but not of the AcrAB-TolC pump (Li & Nikaido, 2004), it was predicted that lower MICs would be observed

for the Δ*acrA,* Δ*acrD,* and Δ*tolC* strains compared to the wild-type. The results for the Δ*acrA* and Δ*acrD* strains conflicted with this hypothesis, although did suggest that TolC was involved in the efflux of streptomycin. In addition, the results also suggested that the loss of the OmpF porin could increase the resistance of *E. coli* to streptomycin.

The MIC of tetracycline was lower for the Δ*acrA*, Δ*acrB* and Δ*tolC* strains than the wild-type (row 3). The lowest MIC was for Δ*acrA*. Both the Δ*acrD* and Δ*ompF* strains had MICs for tetracycline that were twice as high as that of the wild-type. These results indicate that influx of tetracycline occurs through OmpF and that the AcrAB-TolC efflux complex is important for the removal of this antibiotic from the cell. Both of these observations are in agreement with previous findings (Baucheron *et al.*, 2004; Delcour, 2009).

4.2.3 Effects of herbicides on the response of the E. coli *knockout strains to antibiotics.*

The importance of five different porin or efflux complexes for the adaptive responses was determined. The response to three antibiotics, ciprofloxacin, streptomycin and tetracycline, and whether this changed in the presence of the herbicides Kamba and Roundup, was determined for each strain. Bacteria were grown on agar plates representing a range of concentrations of the antibiotic with and without the herbicide. The herbicides were used at a constant concentration chosen to not cause a decrease in the EOP value by itself. For the strains which were highly susceptible to Roundup (strains CR5000, CR7000 and JW2454), the concentration used was lower than for the wild-type. After incubation, titres were converted to an EOP value which was used for statistical analysis and graphing.

The MIC, defined here as the minimum concentration of antibiotic necessary to cause a 1000-fold decrease in the EOP, was determined for each strain and each antibiotic with and

without the herbicides. The magnitude and direction of the change in MIC demonstrated the effect of the herbicide on the antibiotic resistance phenotype, and can be used to identify the importance of each influx- and efflux-related gene to the overall phenomenon.

The hypothesis was that if a strain was mutant in a relevant gene, then the herbicide would not have an influence on the antibiotic MIC. This would be in contrast to the wild-type, where differences were detectable.

The wild-type strain, BW25113, was first tested to confirm that similar effects occurred in response to the herbicides as seen previously with *E. coli* JB578 and *S. Typhimurium* SL3770 (Kurenbach *et al.*, 2015) and also as a baseline for comparison to the knockout strains. Kamba induced statistically significant increases in the MICs of ciprofloxacin ($p < 2.0 \times 10^{-16}$) and tetracycline ($p < 2.0 \times 10^{-16}$) (figure 4.1). However, despite there being a highly significant difference in survival between the streptomycin only treatment and the streptomycin and Kamba treatment ($p = 1.5 \times 10^{-7}$) there was no change in the MIC because both dropped below the 1000-fold decrease in the EOP value at the same concentration. Overall, survival of BW25113 on streptomycin in the presence of Kamba decreased compared to media with no Kamba.

Statistically significant increases in MICs were also recorded for ciprofloxacin ($p < 2.0 \times 10^{-16}$) and streptomycin ($p < 2.0 \times 10^{-16}$) upon exposure to Roundup whereas a decrease was seen for tetracycline ($p < 2.0 \times 10^{-16}$). In all cases, the directionality of the changes in MICs was the same for *E. coli* strain BW25113 as observed previously for JB578 (Kurenbach *et al.*, 2015).

The magnitudes of the changes in MIC ranged from 1.3-fold (streptomycin and Kamba) to 16.7-fold (streptomycin and Roundup). Roundup and Kamba both induced a 2.0-fold shift in

the MIC of tetracycline and 5.0-fold and 3.0-fold changes in the MIC of ciprofloxacin, respectively.

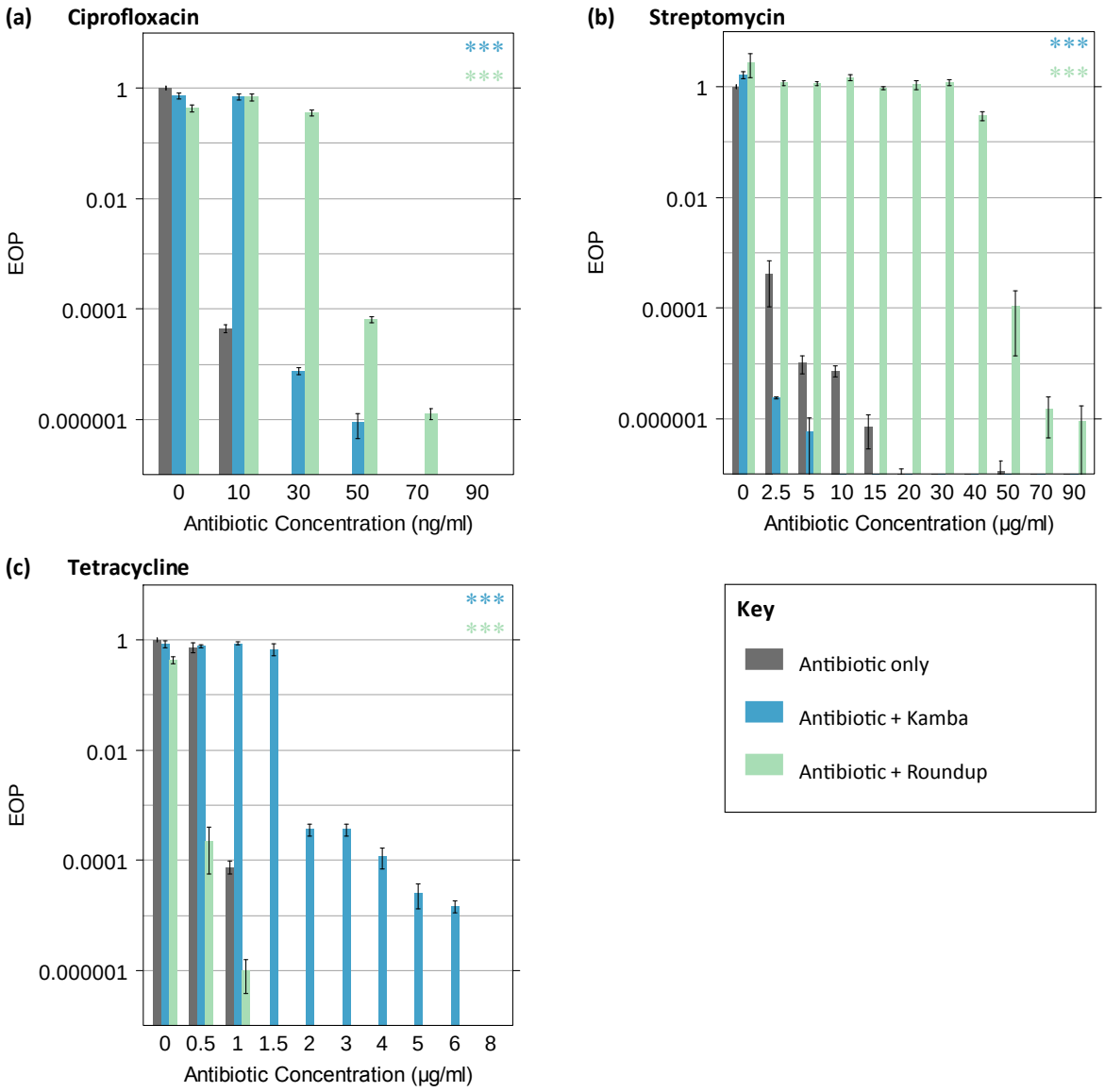

Figure 4.1: Survival of *E. coli* strain BW25113 (*WT*) on a series of concentrations of (a) ciprofloxacin, (b) streptomycin, and (c) tetracycline, +/- Kamba (blue) or Roundup (green). Survival is reported as EOP. Error bars are standard error of the mean (SEM). Asterisks in top right corner of graphs denote significance level. (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$.

The strain Δ*acrA* (CR7000) lacks the periplasmic membrane fusion protein, AcrA, which can link TolC to a number of transporter proteins including AcrB and AcrD.

Kamba induced statistically significant increases in the MICs of ciprofloxacin ($p < 2.0 \times 10^{-16}$) and tetracycline ($p = 7.4 \times 10^{-6}$) and decreased that of streptomycin ($p = 5.2 \times 10^{-7}$)

(figure 4.2). For ciprofloxacin and tetracycline the fold-changes in MIC for the Δ*acrA* strain (1.3-fold and 1.0-fold, respectively) were lower compared to the wild-type (3.0-fold and 2.0-fold, respectively). The involvement of this gene in the effects caused by Kamba cannot be ruled out. The MIC of streptomycin for the Δ*acrA* strain decreased 6.0-fold in response to Kamba which was a substantially larger effect than occurred for the wild-type strain where no change in MIC was observed. This suggested that the deletion of *acrA* allowed Kamba to have an increased effect on the cell, perhaps due to increased intracellular accumulation of the herbicide.

Roundup caused statistically significant increases in the MIC of ciprofloxacin (1.2-fold, $p = 4.1 \times 10^{-2}$) and streptomycin (1.3-fold, $p = 3.2 \times 10^{-2}$). The *P* values for these changes were larger than those for the effects induced by Kamba and there was no significant effect on the MIC of tetracycline. The fold-changes in MIC for ciprofloxacin and streptomycin were both much lower than those determined for the wild-type strain (5.0-fold and 16.7-fold, respectively). These differences in magnitude combined with the lack of significant effect on the MIC of tetracycline suggest that AcrA plays an important role in the mechanism by which Roundup alters the susceptibility of *E. coli* to all three antibiotics.

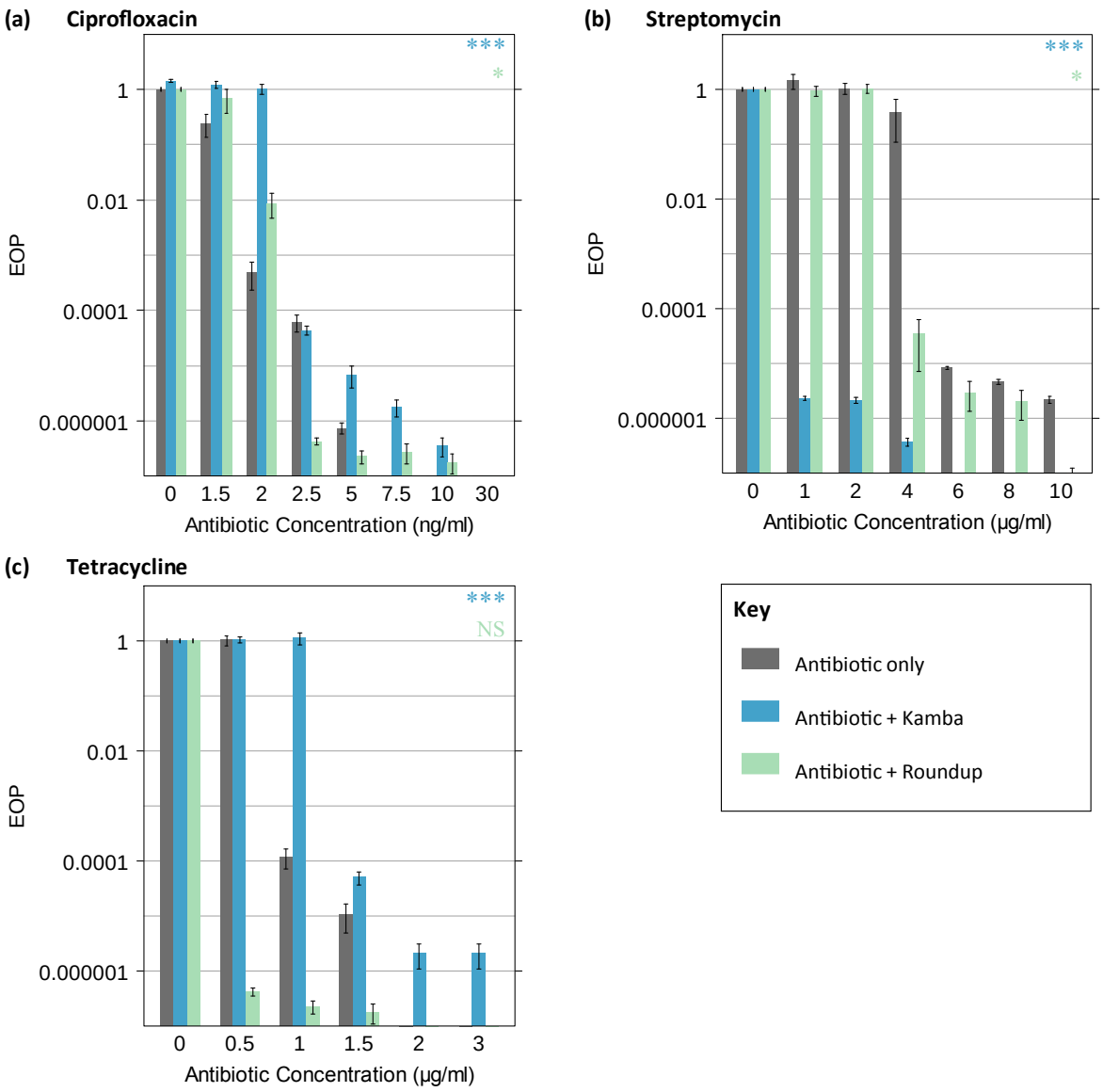

Figure 4.2: Survival of *E. coli* strain CR7000 (Δ*acrA*) on a series of concentrations of (a) ciprofloxacin, (b) streptomycin, and (c) tetracycline, +/- Kamba (blue) or Roundup (green). Survival is reported as EOP. Error bars are standard error of the mean (SEM). Asterisks in top right corner of graphs denote significance level. (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$.

Strain CR5000 lacks *acrB* which is the gene for the transporter component of the AcrAB-TolC efflux pump (Li & Nikaido, 2004).

In this strain, Kamba induced a statistically significant and large (8.0-fold) decrease in the MIC of streptomycin ($p < 2.0 \times 10^{-16}$), but had no effect on resistance to ciprofloxacin ($p = 8.8 \times 10^{-2}$) and only a minimal, although marginally significant, effect on the MIC of tetracycline (no change in MIC, $p = 4.9 \times 10^{-2}$) (figure 4.3). Kamba had a much larger effect

on the MIC of streptomycin for the Δ*acrB* strain than on the wild-type (no change in the MIC). This suggested that removal of *acrB* allowed Kamba to exert a stronger effect on the susceptibility of *E. coli* to streptomycin, as was also observed for *acrA*. In addition, the results indicated that AcrB may be important for Kamba to induce changes in resistance to ciprofloxacin and tetracycline.

The effects caused by Roundup were highly statistically significant for all three antibiotics, although the magnitudes of the changes in MICs were small (figure 4.2). The MIC of ciprofloxacin was increased 1.3-fold (p = 2.5 x 10^{-6}), of tetracycline 1.3-fold (p = 2.5 x 10^{-8}) and of streptomycin 1.2-fold (p = 9.0 x 10^{-10}). These changes in the MIC are substantially lower than those that were observed for the wild-type strain. Despite the statistical significance of the effects, the difference in magnitude of the effect of Roundup on the wild-type and Δ*acrB* strains hint at involvement of AcrB in the mechanism by which Roundup alters the response of *E. coli* to antibiotics.

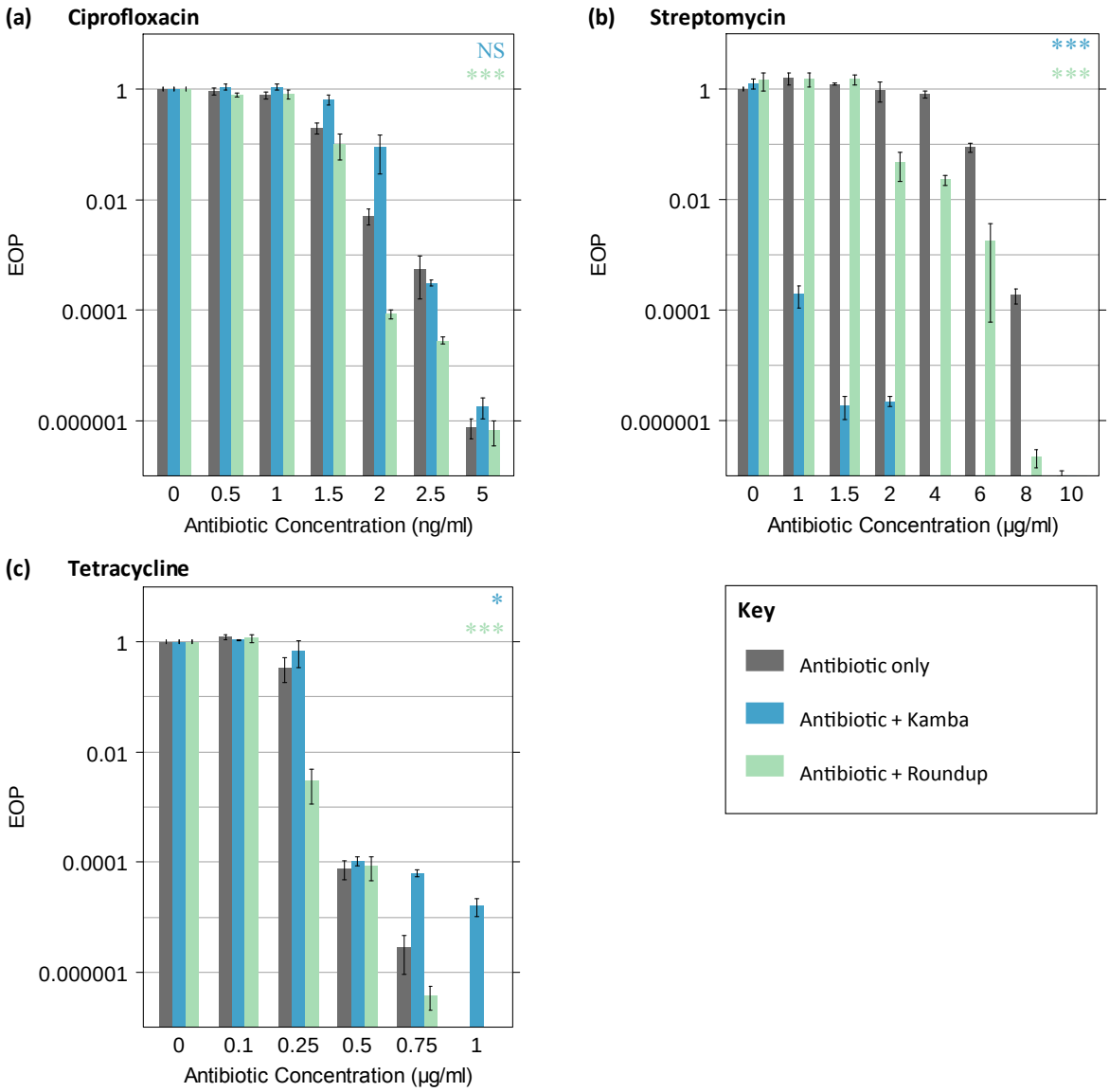

Figure 4.3: Survival of *E. coli* strain CR5000 (Δ*acrB*) on a series of concentrations of (a) ciprofloxacin, (b) streptomycin, and (c) tetracycline, +/- Kamba (blue) or Roundup (green). Survival is reported as EOP. Error bars are standard error of the mean (SEM). Asterisks in top right corner of graphs denote significance level. (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$.

JW2454 was used to investigate whether the AcrAD-TolC efflux pump is important to the mechanism by which the herbicides affect the antibiotic resistance of *E. coli*. This strain has the genotype Δ*acrD*, and so does not have functional forms of the AcrAD-TolC pump.

Both herbicides induced highly significant shifts in the MICs of all three antibiotics (figure 4.4). Kamba caused 2.1-fold and 2.7-fold increases in the MICs of ciprofloxacin ($p = 1.5 \times 10^{-6}$) and tetracycline ($p < 2.0 \times 10^{-16}$), respectively, as well as a 2-fold decrease in

the MIC of streptomycin ($p < 2.0 \times 10^{-16}$). The magnitude of the change in the MIC of ciprofloxacin was smaller for this strain compared to the wild-type, however, for both tetracycline and streptomycin, the magnitude of the change in MIC was larger for the Δ*acrD* strain. As for the Δ*acrA* and Δ*acrB* strains, this could suggest that the absence of this pump component allowed Kamba to exert a stronger effect on the antibiotic susceptibility of the bacteria.

When combined with Roundup, 2.3-fold and 4.0-fold increases in the MICs of ciprofloxacin ($p = 4.0 \times 10^{-10}$) and streptomycin ($p < 2.0 \times 10^{-16}$) were observed. In addition, the MIC of tetracycline decreased 2.0-fold ($p < 2.0 \times 10^{-16}$). For ciprofloxacin and streptomycin the fold-change in MIC was lower for the Δ*acrD* strain than for the wild-type, although this difference was much more substantial for streptomycin. AcrD could contribute to the effects of Roundup on the susceptibility of these two antibiotics. The magnitude of the change in MIC of tetracycline caused by exposure to Roundup for the Δ*acrD* strain was the same as the wild-type, indicating that it is unlikely this transporter was involved in the effects of this herbicide on tetracycline resistance.

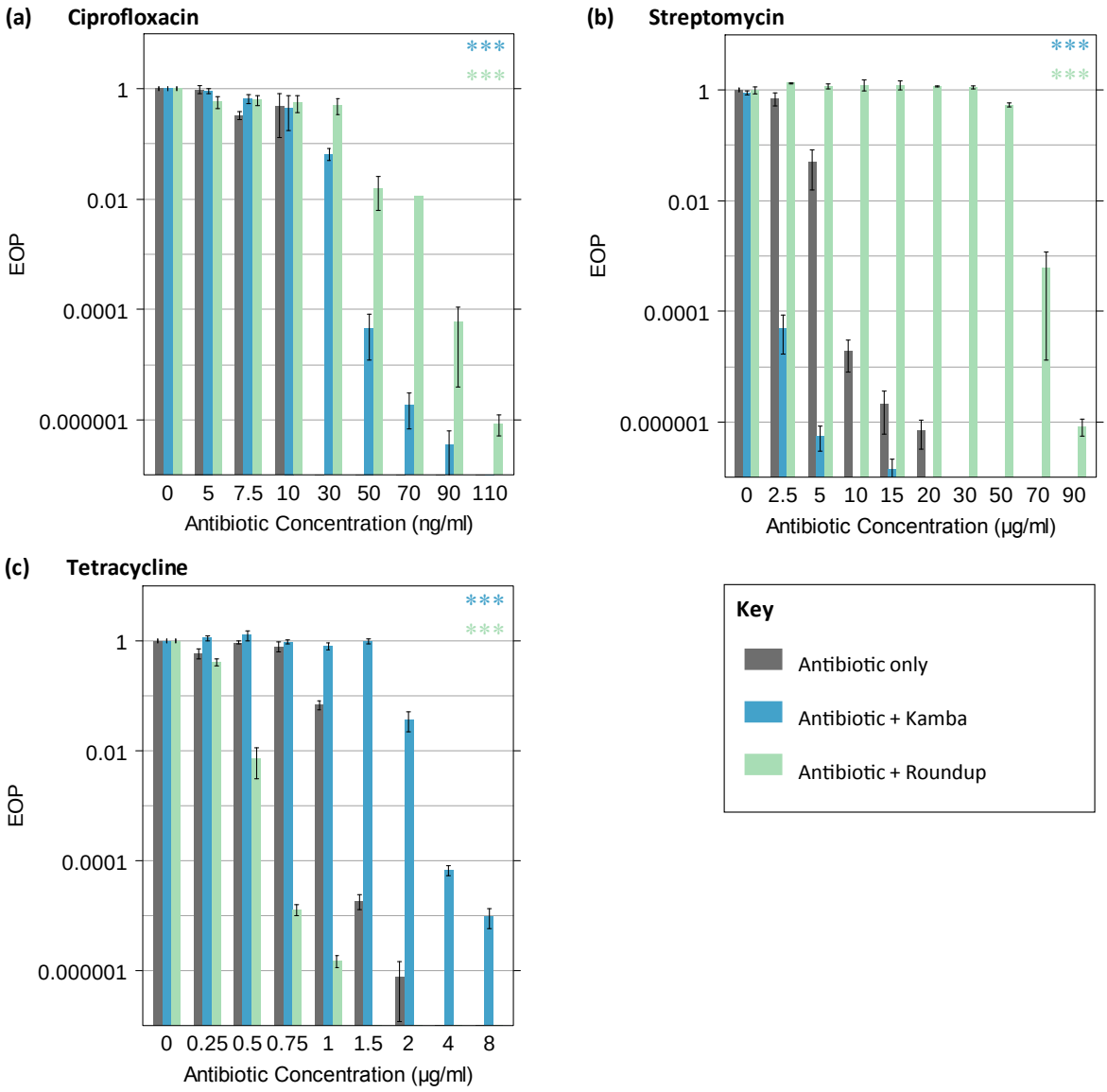

Figure 4.4: Survival of *E. coli* strain JW2454 (Δ*acrD*) on a series of concentrations of (a) ciprofloxacin, (b) streptomycin, and (c) tetracycline, +/- Kamba (blue) or Roundup (green). Survival is reported as EOP. Error bars are standard error of the mean (SEM). Asterisks in top right corner of graphs denote significance level. (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$.

TolC is the outer membrane channel for a number of efflux pumps in *E. coli*, including AcrAB-TolC and AcrAD-TolC.

The effects of the herbicides on the response of JW5503 (Δ*tolC*) to the antibiotics were varied (figure 4.5). Kamba induced a 2.0-fold increase in the MIC of ciprofloxacin ($p = 3.9 \times 10^{-12}$) an 8.0-fold decrease in the MIC of streptomycin ($p = 9.9 \times 10^{-15}$). For ciprofloxacin, this effect was weaker for the Δ*tolC* strain than for the wild-type strain,

indicating that TolC could be important for the Kamba-induced changes in the response of *E. coli* to this antibiotic. For streptomycin the effect on the MIC was larger for the Δ*tolC* strain than for the wild-type. In the case of tetracycline, the effect of Kamba was statistically significant ($p = 1.6 \times 10^{-4}$) for one concentration of the antibiotic, however, there was no shift in the overall MIC, indicating that TolC plays a role in the mechanism by which Kamba causes changes in the response of *E. coli* to this antibiotic.

Roundup had no significant effect on the MIC of ciprofloxacin ($p = 0.31$). There were significant differences in the EOP value with and without Roundup at two concentrations of streptomycin resulting in a 2.7-fold change in the MIC ($p = 8.9 \times 10^{-6}$), and one concentration of tetracycline ($p < 2.0 \times 10^{-16}$), however there was no shift in the MIC of this antibiotic. These effects were smaller than those observed for the wild-type strain, suggesting that TolC was important for the effects on antibiotic susceptibility caused by Roundup.

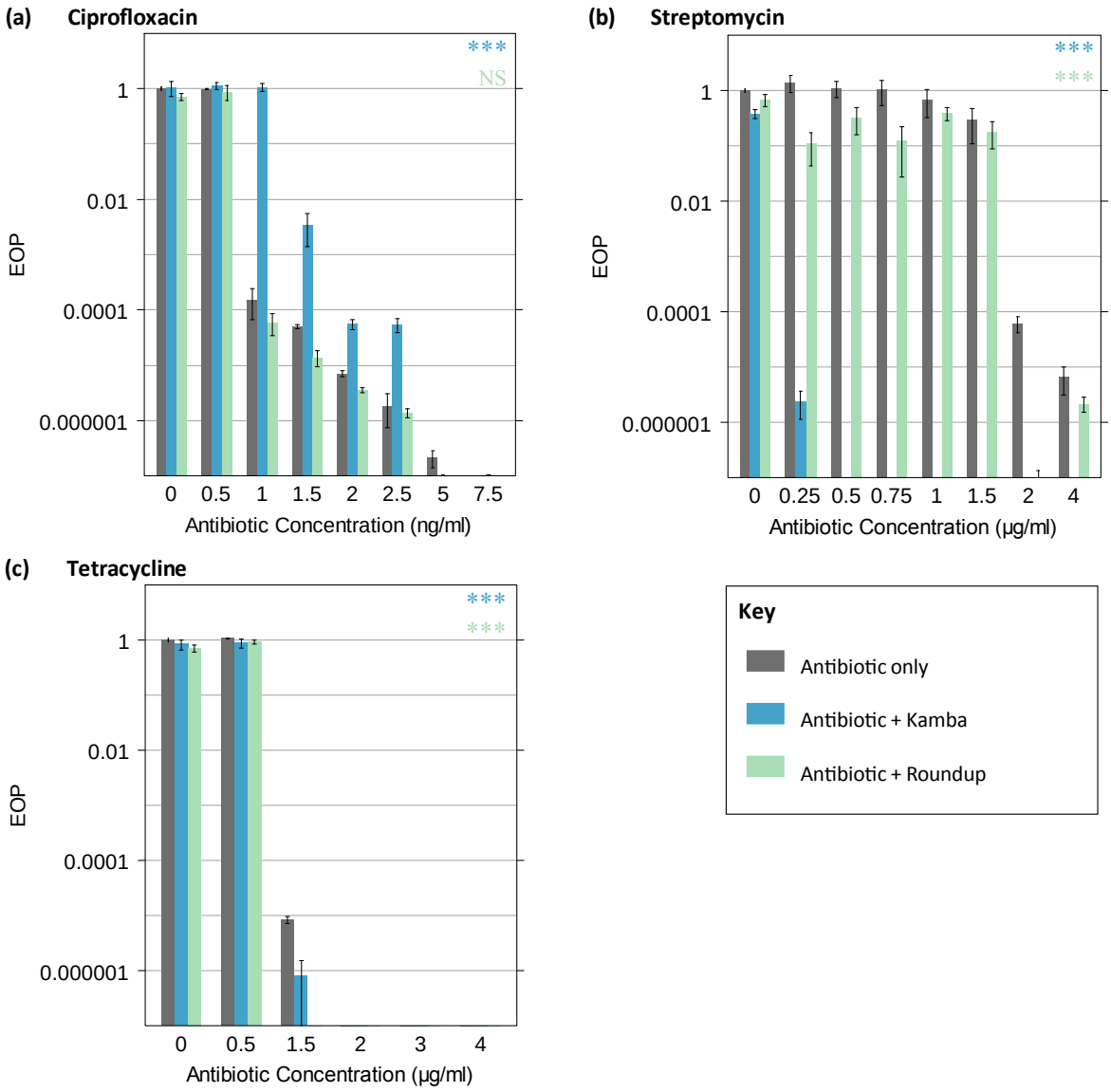

Figure 4.5: Survival of *E. coli* strain JW5503 (Δ*tolC*) on a series of concentrations of (a) ciprofloxacin, (b) streptomycin, and (c) tetracycline, +/- Kamba (blue) or Roundup (green). Survival is reported as EOP. Error bars are standard error of the mean (SEM). Asterisks in top right corner of graphs denote significance level. (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$.

OmpF is a key porin found in *E. coli* that provides an influx route for a number of antibiotics including the quinolones and tetracycline (Delcour, 2009). The Δ*ompF* strain was used to determine the importance of this influx route to the effects on antibiotic resistance by herbicides (figure 4.6).

The MICs of both ciprofloxacin and tetracycline for this strain increased in response to co-exposure to the antibiotic and Kamba (2.1-fold, $p < 2.0 \times 10^{-16}$ and 2.9-fold, $p = 6.2 \times 10^{-13}$,

respectively). The magnitudes of the changes in MICs of these two antibiotics differ slightly from those determined for the wild-type strain. The involvement of OmpF in the effects of Kamba on susceptibility to ciprofloxacin and tetracycline cannot be ruled out. In contrast, Kamba caused a 6.7-fold decrease in the MIC of streptomycin (p = 2.0 x 10^{-5}). This is a substantial effect compared to the 1.3-fold change in MIC induced in the wild-type strain. Kamba caused larger changes in the MIC of streptomycin for all five knockout strains than for the wild-type, where it had no effect. This was unexpected. One hypothesis is that the loss of the individual influx and efflux components allowed Kamba to have a stronger effect on the response of the bacteria to streptomycin. It could also be that the gene used to knockout the components, *npt*II, was having unforeseen effects on the experiment.

Roundup increased the MIC of ciprofloxacin by 4.5-fold (p = 2.0 x 10^{-16}) and streptomycin by 2.3-fold (p = 1.7 x 10^{-12}). It also induced a decrease in the MIC of tetracycline by 1.5-fold (p < 2.0 x 10^{-16}). For ciprofloxacin and tetracycline these changes are very similar to those observed for the wild-type strain. This suggests that OmpF does not play an important role in the effect of Roundup on susceptibility to these two antibiotics. In contrast, the fold-change in the MIC of streptomycin for the Δ*ompF* strain was much lower than the wild-type, indicating that this porin may be involved in the mechanism by which Roundup affects susceptibility to this antibiotic. This is surprising as streptomycin is not known to be a substrate of OmpF (Nikaido, 2003).

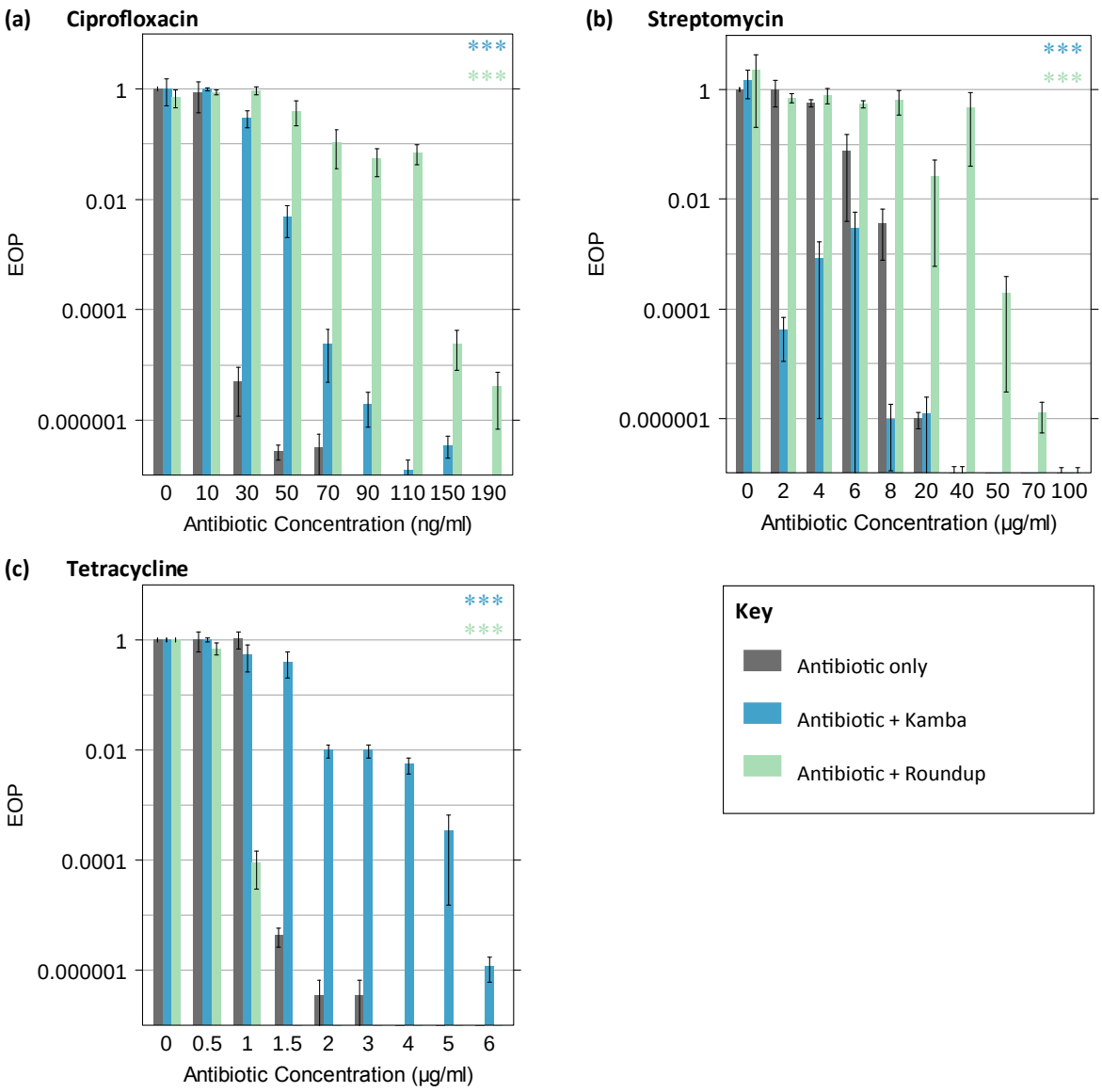

Figure 4.6: Survival of *E. coli* strain JW0912 (Δ*ompF*) on a series of concentrations of (a) ciprofloxacin, (b) streptomycin, and (c) tetracycline, +/- Kamba (blue) or Roundup (green). Survival is reported as EOP. Error bars are standard error of the mean (SEM). Asterisks in top right corner of graphs denote significance level. (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$.

4.2.4 Determining the effect of the nptII *gene on streptomycin resistance.*

The knockout strains used in the previous two sections were created by inserting the *npt*II gene, the product of which is aminoglycoside-3'-phosphotransferase II (aph(3')-II) (Fuchs *et al.*, 1993). This enzyme provides resistance to the aminoglycoside antibiotics, kanamycin and neomycin. Because of this resistance, streptomycin was used in the above experiments as a representative of the aminoglycoside family that would, theoretically, not be affected

by the product of *npt*II. However, the results collected from a number of the assays inconsistent with other studies. In particular, streptomycin has been shown to be a substrate of the AcrD transporter protein (Rosenberg *et al.*, 2000) and so the MIC for this antibiotic was expected to be lower for the Δ*acrD* strain than for the wild-type. The streptomycin MIC for the knockout strain was almost double the MIC for the wild-type. Other discrepancies were also noticed throughout section 4.2.3.

This raised concerns that the aph(3')-II activity in the knockout strains was conferring cross-resistance to streptomycin. This additional variable in the experimental system would make it challenging to interpret results.

To test this hypothesis, the wild-type strain BW25113 was transformed with a low copy number plasmid (F42::miniTn-Kan) carrying the *npt*II gene (Ferguson *et al.*, 2002). The MIC of streptomycin was then determined for both BW25113(*npt*II) and the wild-type, concurrently, using the same protocol as in section 4.2.2.

The MIC of streptomycin for BW25113 was found to be 6.0 μg/ml, which is very similar to that determined previously of 5.8 μg/ml. The MIC for BW25113(*npt*II) was found to be > 130 μg/ml. This result shows that it is possible for *npt*II to provide cross-resistance to streptomycin, at least in a system where it is present on a low copy number plasmid. In the knockout strains *npt*II is inserted into the chromosome. Although unlikely, it is possible that this could have an effect on the level of cross-resistance to streptomycin caused by aph(3')-II.

4.3 Discussion

Multidrug resistant bacteria are undermining the success of the treatment of infections world-wide (Alekshun & Levy, 2007). A major mechanism by which bacteria can become multidrug resistant is by upregulating or acquiring broad-substrate efflux pumps. Herbicides have been shown to induce changes in the responses of *E. coli* to a number of antibiotics from different classes (Kurenbach *et al.*, 2015). The aim of this chapter was to determine the mechanism by which these effects occur, with a particular focus on the efflux pumps AcrAB-TolC and AcrAD-TolC as well as the outer membrane porin OmpF.

4.3.1 Efflux Pumps contribute to the herbicide-induced changes in antibiotic response.

The efflux pump inhibitor PAβN was used to confirm that efflux pumps played a role in the adaptive resistance response to herbicide exposure (Kurenbach *et al.*, 2015). It was hypothesised that if pumps are indeed responsible for this phenomenon, then bacteria simultaneously exposed to PAβN, a herbicide and an antibiotic, would not display the adaptive response previously observed, because inhibition of the efflux pumps would counteract the inducing effect of the herbicide.

Co-exposure to PAβN and Kamba only marginally reduced survival in comparison to the Kamba only control. However, the effect range of PAβN is poorly characterised. It is possible that there are efflux pumps which are not inhibited by this molecule. It also changes the membrane potential of the cell (Lamers *et al.*, 2013). This could have pleiotropic effects on energy-dependent transport, not just the pumps that PAβN competitively inhibits. The innate resistance of *E. coli* to Kamba may be due to pumps that are outside of the influence of PAβN. In addition, a relatively low concentration of Kamba was used, well below the MIC

for this strain (13,883 ppm ae (Kurenbach *et al.*, 2015)). It could be that even without efflux there was simply not enough Kamba to be toxic.

Kamba induced an increase in the resistance of *E. coli* to chloramphenicol. Upon co-exposure with PAβN this effect was negated and bacterial survival was reduced in comparison to the control with no efflux pump inhibitor. This indicates that efflux pumps are a key part of the mechanism by which Kamba causes increased resistance to chloramphenicol.

In contrast to Kamba, co-exposure of *E. coli* to only Roundup and PAβN resulted in a reduction in the EOP to below the detection limit. This could indicate that the efflux pumps are very important to the intrinsic resistance of *E. coli* to Roundup. However, PAβN has also been shown to permeabilise the outer membrane, allowing bulky molecules such as vancomycin to cross the lipid bilayer (Lamers *et al.*, 2013). The dramatically increased toxicity precludes drawing conclusions from the resulting EOP of the PAβN, Roundup and kanamycin treatment, as the reduction in the EOP to below the detection limit was most likely due to the increased toxicity of Roundup in the presence of the efflux pump inhibitor rather than a loss of the adaptive response mechanism.

The difference in the effect of PAβN on each herbicide suggests different mechanisms of coping with the toxicity of these pesticides. It is interesting to note that intrinsic resistance to Roundup, which was more toxic than Kamba to both *E. coli* and *S. Typhimurium* (Kurenbach *et al.*, 2015), was most closely linked to the efflux pumps inhibited by PAβN. One transporter protein that PAβN is known to be a substrate for is AcrB (Edward *et al.*, 2005), making the AcrAB-TolC complex a viable candidate for active efflux of Roundup.

4.3.2 Loss of influx and efflux components can alter the susceptibility of **E. coli** *to both herbicides and antibiotics.*

The relative importance of a number of antibiotic influx and efflux related genes to the innate tolerance of *E. coli* strain BW25113 to two herbicides and three antibiotics was shown using a selection of strains from the Keio Collection (Baba *et al.*, 2006). The MICs of the wild-type strain (BW25113) for each herbicide and antibiotic was determined. This was then compared to the MICs of the antibiotics and herbicides for each knockout strain. The selected strains were each lacking one of the genes *acrA*, *acrB*, *acrD*, *tolC* and *ompF*, which are all involved in either influx or efflux. Most of the differences between MICs of the wild-type and those of the knockout strains were consistent with the predictions of the hypotheses outlined in section 4.1.

The results suggested that the AcrAB-TolC pump complex is important for the efflux of ciprofloxacin and tetracycline. The MICs of the antibiotics for the Δ*acrA*, Δ*acrB* and Δ*tolC* strains were lower than the for the wild-type strain. OmpF was also demonstrated to be involved in the influx of these two antibiotics. Removal of this porin caused increases in the resistance of *E. coli* to ciprofloxacin and tetracycline.

Interestingly, deletion of the gene for the transporter protein AcrD also resulted in increases in the resistance of *E. coli* to these antibiotics. The MIC of ciprofloxacin for JW2454 was three times as high as the MIC of the wild-type. For tetracycline, the MIC for JW2454 was twice as high as the MIC of the wild-type. A closer look at the relationships between *acrA*, *acrB* and *acrD* as well as their products provides a possible explanation. Like AcrB, AcrD forms a complex with AcrA and TolC to pump molecules from the cell. The genes *acrA* and *acrB* are components of the same operon and are transcribed as a polycistronic message

from the same promoter (Li & Nikaido, 2004). Upon sequencing, no transcriptional termination signal was identified downstream of *acrA* and upstream of *acrB* (Ma *et al.*, 1993) (note that in this publication *acrB* is referred to by its former name, *acrE* (Ma *et al.*, 1996)) within the 23 base pairs separating the two open reading frames (Benson *et al.*, 2012). Indeed, when BW25113 was grown without any selective pressure (no antibiotics or herbicides) mRNA levels of *acrA* and *acrB* mRNA were roughly equal (B. Kurenbach and Heinemann research group, personal communication, January 2016). *acrD*, on the other hand is located more than 2 kbp away (Benson *et al.*, 2012) and is regulated by BaeR and BaeS (Nishino *et al.*, 2005). When these two efflux pump complexes are formed, it is likely that AcrD competes with AcrB to bind with AcrA. If AcrD is removed from this competition, then presumably more AcrA is available to bind with AcrB, possibly producing slightly higher quantities of the AcrAB-TolC pump than the wild-type strain. This may explain the increases in resistance to ciprofloxacin and tetracycline, where increased resistance has been linked to the AcrAB-TolC pump (Li & Nikaido, 2004; Nikaido, 1996). However, the level of *acrD* mRNA in BW25113 grown without any selective pressure was much lower than the levels of *acrA* and *acrB* mRNA (B. Kurenbach and Heinemann research group, personal communication, January 2016). Whether the deletion of *acrD* indeed produces an increase in the quantity of AcrAB-TolC sufficient to cause the observed increases of the MICs of ciprofloxacin and tetracycline is possible to examine in future work.

TolC and possibly AcrA were found to be important for intrinsic resistance to streptomycin. However, neither of the two transporter proteins investigated in this study which form complexes with TolC and AcrA seemed to be involved. This was surprising because aminoglycosides have been shown to be a substrate for the AcrAD-TolC and deletion of *acrD*

resulted in *E. coli* that accumulated higher levels of streptomycin (Rosenberg *et al.*, 2000). However, as *acrD* transcription in the wild-type of this strain is low, deletion of this gene may not have had a substantial effect on the levels of the AcrD protein and thus the AcrAD-TolC complex. This highlights a limitation of using knockout strains as a system in which to determine the relevance of a particular gene. If that gene was not highly expressed in the wild-type then deleting it will have little effect on the phenotype. In the case of a gene that is not highly expressed, it may be more prudent to instead place it under the control of a known promoter that can then be overexpressed. This strain could then be compared to the wild-type to see if the effects are increased instead of decreased. In any case, it seemed likely that AcrAB-TolC was not responsible for efflux of streptomycin from the cell.

Deletion of OmpF resulted in a strain more resistant to streptomycin than the wild-type strain. This would indicate that streptomycin enters the cell through OmpF and so the loss of this protein reduced influx of the antibiotic. This was surprising as streptomycin is thought to diffuse directly through the lipid bilayer, rather than through porins such as OmpF (Hancock *et al.*, 1991; Nikaido, 2003).

Inconsistencies of the results for streptomycin with previous studies led to the hypothesis that the *npt*II gene, which was used to create the knockout strains, was providing some cross-resistance to streptomycin. This was tested by transforming the wild-type strain with a low copy number plasmid carrying *npt*II and then determining the MIC of streptomycin for both strains. The results of this experiment confirmed that aph(3')-II, the product of *npt*II, was likely causing cross-resistance to streptomycin. This resistance was substantial. The MIC of streptomycin was increased from 6 µg/ml for the wild-type to >130 µg/ml after transformation with the *npt*II carrying plasmid. This cross-resistance confounds

interpretation when testing because it is impossible to know how much of the MIC can be attributed to aph(3')-II and how much to the deletion of the efflux or influx component.

AcrAB-TolC was found to be important for the intrinsic resistance of *E. coli* to both Kamba and Roundup. This multidrug exporter is regulated by global activators such as MarA and SoxS, which are both members of stress-response regulons (Alekshun & Levy, 1999; Amábile-Cuevas & Demple, 1991). Stress induced by herbicides may result in upregulation of the *acrA*, *acrB* and *tolC* genes. Indeed, a formulation containing dicamba as the active ingredient has been shown previously to upregulate the expression of a *soxS::lacZ* fusion gene in *E. coli* (Kurenbach *et al.*, 2015).

OmpF was not involved in the influx of either Kamba or Roundup. Deletion of *ompF* did not have an effect on the MIC of either herbicide. Alternative mechanisms of influx include diffusion through the lipid bilayer (Delcour, 2009), passive entry through alternate porins such as OmpC (Chubiz & Rao, 2011) or active transportation. Large, hydrophobic molecules are known to be able to diffuse directly through the outer membrane lipid bilayer (Delcour, 2009) and this could be a viable method of entry for components of the herbicide formulation.

To summarise, AcrAB-TolC was found to be the most important factor in the intrinsic resistance of *E. coli* to these antibiotics and herbicides.

4.3.3 Loss of influx and efflux components can affect herbicide-induced changes in the antibiotic response of E. coli*.*

The knockout strains were then used to determine the contribution of AcrA, AcrB, AcrD, TolC and OmpF to the mechanism by which the herbicides, Kamba and Roundup, cause changes in the antibiotic resistance phenotype of *E. coli*. Each strain was grown on a range

of concentrations of each antibiotic with and without each herbicide. The magnitude of the response was measured as the fold-change in the MIC of the antibiotic and this was compared to the wild-type. A summary of the direction of the change in MIC and the associated statistical significance for each combination of herbicide, antibiotic and knockout strain can be found in appendix B.

Overall the data was highly variable. In three cases the observed statistical significance of the effects disappeared, indicating that here the knocked out function explains most if not all the adaptive response. For all other combinations, there was no obvious cut-off where it was clear that a gene was or was not involved in the phenomenon. This data is thus not sufficient to make definitive statements of the contribution of some components. However, in a number of cases it was quite clear that a component was involved and this made it possible to draw some general conclusions from the results.

The loss of *acrA*, *acrB* or *tolC* had the most obvious effects on the ability of Kamba and Roundup to alter the response of *E. coli* to ciprofloxacin and tetracycline. The AcrAB-TolC complex is the most well-studied of the efflux pumps present in *E. coli* (Nikaido, 2003). Both ciprofloxacin and tetracycline have been shown to be substrates of this pump (Li & Nikaido, 2004). It also contributes to the intrinsic resistance of *E. coli* to both Kamba and Roundup. Expression of AcrAB-TolC has been shown to be upregulated by salicylic acid through induction of the *marRAB* operon (Cohen *et al.*, 1993). Salicylic acid also induces phenotypic changes in the antibiotic resistance of *E. coli* (Rosner, 1985) and *S. Typhimurium* (Marjoshi, 2014). That components of the AcrAB-TolC complex might be up- or down-regulated due to the presence of the herbicides resulting in phenotypic changes in ciprofloxacin and

tetracycline resistance fits with the findings of previous studies (Cohen *et al.*, 1993; Rosner, 1985; Shen *et al.*, 2011).

Whether the transporter protein AcrD was involved in the phenomenon could not be deduced from the data, although it seemed unlikely. The fold-changes in the MIC of ciprofloxacin caused by Kamba and Roundup for the Δ*acrD* strain were lower than the wild-type, indicating that AcrD could be involved in these herbicide and antibiotic combinations. It is unlikely to be involved in the effects of either herbicide on tetracycline resistance. The fold-change in MIC of this antibiotic caused by Kamba for the Δ*acrD* strain was larger than for the wild-type. That the loss of an efflux component would allow or cause larger effects of the herbicides on the antibiotic resistance phenotype was unexpected and cannot be explained at present.

Loss of the porin OmpF had little to no effect on the ability of Kamba or Roundup to induce changes in the response of *E. coli* to ciprofloxacin or tetracycline. It was therefore unlikely that changes in the levels of influx via OmpF were responsible for the effects of the herbicides. One exception was the combination of ciprofloxacin and Kamba. The fold change in the MIC of ciprofloxacin for the Δ*ompF* strain was slightly lower than for the wild-type strain, indicating that OmpF may have a small role in the effects of Kamba on the phenotype of ciprofloxacin resistance.

A different pattern of results was observed for streptomycin. Kamba had a very small effect on the MIC of the wild-type strain. In comparison, larger effects were observed for all knockout strains. This suggested that deletion of each influx and efflux component tested allowed Kamba to exert stronger effects on the bacteria. For AcrA, AcrB and TolC, this could possibly be explained by the reduced efflux of Kamba, meaning higher concentrations of the

herbicide accumulated in the cell to potentially cause increased effects on the antibiotic resistance phenotype. However, for AcrD and TolC, which were shown to have little to no effect on the influx or efflux of Kamba, this explanation could not apply.

Roundup had a much larger effect on the MIC of streptomycin for the wild-type strain (16.7-fold) than either of the other antibiotics. This effect was much reduced for all knockout strains, suggesting that AcrAB-TolC, AcrAD-TolC and OmpF, were all involved in the mechanism by which Roundup induced changes in the response of *E. coli* to streptomycin.

However, as mentioned in section 4.3.2, *npt*II, which was inserted into the knockout strains when they were created, provides substantial cross-resistance to streptomycin. This was surprising as aph(3')-II is generally considered to cause resistance to kanamycin and neomycin (Fuchs *et al.*, 1993). No previous observations of *npt*II causing streptomycin resistance could be found in the literature. This cross-resistance makes it difficult to interpret the results for this antibiotic. It could explain why the pattern of fold-changes in the MIC of streptomycin across the different strains was so different compared to the other antibiotics. As this mechanism of increased resistance would have been working in the opposite direction to the increased susceptibility caused by the deletion of efflux pump components, it is likely that the results underestimate rather than overestimate the involvement of efflux in the phenomenon.

In conclusion, this study has demonstrated that efflux is a substantial if not sole mechanism by which the herbicides Roundup and Kamba alter the phenotypic susceptibility of *E. coli* to antibiotics. In particular, AcrAB-TolC seemed to play a role in the changes in resistance for most of the combinations of antibiotic and herbicide tested. Finally, the product of *npt*II was shown to provide high levels of cross-resistance to streptomycin.

Chapter 5

Summary and Future Direction

This study aimed to further explore the phenomenon and to determine the mechanism of the effects of herbicides on microorganisms. In particular, different components of herbicide formulations were tested in order to determine their effects on the antibiotic resistance phenotype of *S. Typhimurium*. In addition, the significance of efflux and influx to the mechanism by which herbicide formulations can affect the response of bacteria to antibiotics was investigated for *E. coli*.

The World Health Organisation (WHO) released a global report on the surveillance of antimicrobial resistance in 2014 which highlighted that antibiotic resistance is no longer a concern of the future (World Health Organization, 2014). Antibiotic resistance is a critical public health issue across the globe and without intervention the world is headed towards a post-antibiotic era (World Health Organization, 2014).

It is broadly known that the widespread use of antibiotics causes a strong selection pressure for the spread of resistance (Kunin, 1993), however, it has become apparent that bacteria are exposed to other human-made industrial products on large scales and these too may play a part in accelerating the evolution of resistance. Early last year Kurenbach *et al.* (2015) demonstrated that three commercial herbicide formulations, Kamba, 2,4-D, and Roundup, were able to induce phenotypic changes in the response of *S. Typhimurium* and *E. coli* to antibiotics. The results of this study raised some new questions. What components of the herbicide formulations were responsible for the phenotypic changes in antibiotic resistance

observed? And could the mechanism of this effect be linked to changes in the expression of influx porins or efflux pumps?

The results presented in this thesis showed that three herbicide active ingredients, dicamba, 2,4-dichlorophenoxyacetic acid (2,4-d), and glyphosate, as well as two potential herbicide adjuvants, carboxymethyl cellulose and Tween80, both of which are surfactants, were able to induce changes in the response of *S. Typhimurium* to antibiotics. Dicamba increased resistance to ampicillin, chloramphenicol, ciprofloxacin and tetracycline, but did not change the MIC of kanamycin. 2,4-d had no significant effect on the response to ampicillin but increased resistance to chloramphenicol, ciprofloxacin and tetracycline. It also caused an increase in susceptibility of *S. Typhimurium* to kanamycin. Glyphosate increased resistance to ampicillin, ciprofloxacin and kanamycin and increased susceptibility to chloramphenicol and tetracycline. The two surfactants both either increased resistance to a given antibiotic or had no statistically significant effect. These results suggested that the direction of the change in resistance to different antibiotics is determined by the active ingredient but is also modulated by other components of the formulation. In addition, contrasting effects may occur at the same time. This means that the effects on antibiotic resistance may vary depending on the compounds that are combined with the herbicide active ingredients. As the actual adjuvants present in Kamba, Roundup or 2,4-D were not known to us, it is difficult to predict what form these differences may take. One way of testing this would be to combine the active ingredients with Tween80 or CMC at levels at which they may be present in herbicide formulations and measure how the effect differs from the active ingredient alone or the herbicide formulations previously tested. Also of note is that the pattern of the surfactant effects on the resistance phenotype differed to that of the active

ingredients. This is indicative of a different underlying cause. The next step is to test Tween80 and CMC in combination with antibiotics and PAβN, to determine if efflux is also a likely mechanism. Transcriptome responses to surfactant exposures could also be used to identify affected genes.

Exposure to the herbicide at the same time as the antibiotic was sufficient for the bacteria to benefit from the protective effects of the herbicide. There are numerous examples of environments where both antibiotics and herbicides have been found, including soil, aquatic environments (Battaglin *et al.*, 2014; Hua *et al.*, 2006; Kemper, 2008) and effluent (Kemper, 2008; Mackie *et al.*, 2006; Manzetti & Ghisi, 2014), to name a few. Perhaps the most concerning environments where bacteria might come into contact with both of these compounds are humans, domestic animals and insects. Antibiotics are heavily used in some agricultural practices (Dolliver *et al.*, 2008; Shea, 2003), such as pig farming (Key & McBride, 2014; Mackie *et al.*, 2006). In addition, honeybee colonies in the US are treated prophylactically with oxytetracycline (Tian *et al.*, 2012). All of these organisms are likely to come into contact with herbicides and are home to complex communities of microorganisms. This raises concerns about the risks to people who come into contact with bacteria that have an altered antibiotic resistance phenotype which may result in decreases in treatment efficacy and ultimately, success.

E. coli and *S. Typhimurium* have been used in this study and the previous work as model organisms found in the gut. Ingestion is one possible exposure route to herbicides, antibiotics or bacteria with a prior exposure resulting in the altered phenotype. Other exposure routes include skin contact and inhalation, from herbicide spray drift or when applying herbicides. Bacteria, particularly Gram positive species, normally found in these

environments may respond differently. *Staphylococcus aureus* is a Gram positive, natural coloniser and important pathogen of the skin and nasopharynx environments (Chambers, 2001). Testing the effects of the herbicides on antibiotic resistance in this species is a natural next step for this project, particularly as concerns about methicillin resistance of this organism are rising.

The minimum concentrations of the active ingredients and surfactants necessary to change the antibiotic resistance phenotype were also determined. For the active ingredients these concentrations were all within potential application rates and some were within the MRLs recommended by the Codex Alimentarius Commission for animal feed products (Codex Alimentarius Commission, 2012). Surfactants are unregulated when used in herbicides but are reported to be at levels as high as 15% in some formulations (Lee *et al.*, 2009). All of the observed effects were induced at concentrations lower than this. In addition, resistance to a number of the antibiotics tested were induced at concentrations of CMC and Tween80 below what might be used in food (Codex Alimentarius Commission, 2015). These results suggest that bacteria associated with humans or animals may come into contact with sufficent quantities of inducing compounds, herbicides and/or processed foods, to cause changes to their susceptibility to antibiotics. This study also provided evidence that the effects of different chemicals that induce similar changes in antibiotic resistance can be additive. This suggests that bacteria exposed to low concentrations of multiple compounds that are able to induce this response may still experience the observed changes in antibiotic susceptibility. In addition, the range of different molecules that have so far been shown to induce this adaptive response in bacteria raises concerns about the effects of other compounds that we and our associated microorganisms are exposed to on a regular basis.

Further work is needed to identify what effects sub-inhibitory concentrations of other ingredients in biocides, cosmetic products and processed foods, to name a few, might be having on bacteria.

In this study Roundup, but not Kamba, was shown to affect aerobic respiration in *S. cerevisae* when applied at concentrations within the recommended application rate of the herbicide. This suggests that the toxicity of herbicides may be underestimated by regulatory authorities, unless studies take aerobic respiration specifically into account. In addition, glyphosate and the two surfactants, Tween80 and CMC, were also tested but were found to have no effect. This is in agreement with a previous study that demonstrated that Roundup but not glyphosate inhibited respiration in rat liver mitochondria (Peixoto, 2005). Another component of the formulation must be responsible for the response, or for making the glyphosate available to the mitochondria. A likely candidate is POEA, a known surfactant in Roundup products (Mesnage *et al.*, 2013). It has been shown to be highly toxic to a number of off-target species including fairy shrimp (Brausch & Smith, 2007), frogs (Howe *et al.*, 2004) and various human cell lines (Mesnage *et al.*, 2013).

The mechanism by which herbicides caused changes in the antibiotic resistance phenotype of bacteria was also investigated. The efflux pump inhibitor PAβN was used to demonstrate that efflux pumps were important to both the intrinsic resistance of *E. coli* to Roundup, and to the effects of Kamba on the response of *E. coli* to the antibiotic chloramphenicol.

The identification of active efflux as a likely mechanism of the altered antibiotic resistance phenotype led to a more targeted approach. PAβN is a broad-spectrum inhibitor known to competitively inhibit a number of efflux pumps (Lomovskaya *et al.*, 2001), although its full range of targets are unknown. It also alters membrane potential and permeabilises the

outer membrane (Lamers *et al.*, 2013). The transporter protein AcrB is a known target of PAβN (Edward *et al.*, 2005) and ciprofloxacin and tetracycline have both been shown to be substrates of the AcrAB-TolC efflux complex (Li & Nikaido, 2004; Nikaido, 1996). This made these proteins sensible targets for the next step. In addition, AcrD, the transporter protein in the AcrAD-TolC efflux complex (Li & Nikaido, 2004), which has been shown to transport aminoglycosides, including streptomycin, from *E. coli* (Rosenberg *et al.*, 2000), was tested. The outer membrane porin OmpF was also included as a representative of one mechanism of antibiotic influx, because both ciprofloxacin and tetracycline have been shown to move through this pore into the cell (Delcour, 2009).

To test these influx and efflux components, a selection of *E. coli* knockout strains from the Keio collection was obtained from Professor Stuart Levy's laboratory (Tufts University, Boston, MA, USA) and the Institute for Advanced Biosciences (Keio University, Japan (Baba *et al.*, 2006)). These strains were created by insertion of *npt*II, a neomycin and kanamycin resistance gene, into the desired site (Baba *et al.*, 2006). This resulted in a series of strains, each with a different gene deleted.

Initially the MICs of three antibiotics, ciprofloxacin, streptomycin and tetracycline, and two herbicides, Kamba and Roundup, were determined for each strain. These results showed that AcrAB-TolC was the most important factor for the intrinsic resistance of *E. coli* to ciprofloxacin, tetracycline, Kamba and Roundup. However, the results for streptomycin were inconsistent with previous studies (Li & Nikaido, 2004; Rosenberg *et al.*, 2000). This led to the hypothesis that the kanamycin resistance gene, *npt*II, could be causing cross-resistance to streptomycin in the knockout strains. Transforming the wild-type strain with a low copy number plasmid carrying *npt*II and then determining the streptomycin MIC

confirmed this hypothesis. This was unexpected as aph(3')-II, the product of *npt*II, is generally considered to phosphorylate neomycin and kanamycin (Fuchs *et al.*, 1993) and no references to streptomycin cross-resistance could be found in the literature. It confounds the interpretation of the results for streptomycin as it is impossible to know how much of the resistance can be attributed to the deletion of the influx and efflux component.

This finding also had ramifications for a second set of experiments using the knockout strains. The strains were grown on combinations of herbicide and antibiotic, with the idea that for each strain the fold-change in MIC of the antibiotic could be determined and compared to the wild-type. If a component was involved in the adaptive resistance response, we would expect to see a smaller effect for the strain in which it was knocked out than for the wild-type. Cross-resistance from *npt*II caused the experimental system to be more complex than originally thought and made it difficult to interpret the results for streptomycin. In future, it would be prudent to replace the *npt*II cassette with a marker unrelated to antibiotic resistance, such as *gfp* or *lacZ*, and repeat the experiments with streptomycin.

In addition, the data from the total second set of experiments was highly variable. It was difficult to make a definitive statement as to whether a component was or was not involved in the phenomenon and this data was not of adequate quality to be used in this way. Instead, it can be used as an indication of likely candidates for this mechanism which can then be targeted using different techniques.

Overall, at least with respect to ciprofloxacin and tetracycline, AcrA, AcrB and TolC seemed to be the most likely components, of those tested, to be involved in the adaptive resistance response induced by Kamba and Roundup. Involvement of AcrD could not be ruled out but

seems unlikely as it is not thought to be involved in the efflux of either ciprofloxacin or tetracycline (Li & Nikaido, 2004; Nikaido, 1996), or either herbicide (this study). It is unlikely that OmpF plays a role in the observed effects. There was little difference in the fold-change in MIC between the Δ*ompF* and wild-type strains.

Further work is needed to elucidate the mechanism by which herbicides induce changes in the response of bacteria to antibiotics. The results presented in this thesis demonstrate that AcrAB-TolC is likely to be involved, although it is not possible to know how much of the effect it accounts for. It is also possible that multiple influx or efflux mechanisms were affected in concert to produce the resulting change in antibiotic resistance phenotype, a possibility not excluded by the methods used. In addition, only specific efflux pump components were tested. How the herbicides may be altering the function of these components was not investigated. Whole transcriptome sequencing could provide an overview of mRNA levels of most genes that might be affected by the herbicides. This would both allow an expanded view across other efflux and influx mechanisms that were not focussed on in this project, as well as a closer look at the regulatory genes associated with them, such as *soxRS* (Amábile-Cuevas & Demple, 1991) and *marRAB* (Li & Nikaido, 2004) . This type of work has been used previously for investigations in adaptive antibiotic resistance in *E. coli* (Pomposiello *et al.*, 2001). The most important problem is that changes in transcript level are poor predictors of changes at the protein level (Chen *et al.*, 2002; Hegde *et al.*, 2003). Phenomenon such as post-transcriptional regulation and protein-herbicide interactions cannot be identified through this method. But it is a good place to start.

A post-antibiotic present is fast becoming a reality. An understanding of resistance and the different factors affecting its evolution is imperative. The results presented in this thesis highlight that a range of other anthropogenic environmental inputs, which we and our microflora are exposed to regularly, can impact on antibiotic resistance and potentially treatment success. Furthermore, the mechanism by which these effects occur is complex and difficult to untangle. The effects of non-antibiotic compounds on adaptive antibiotic resistance cannot be ignored and more work is needed before this phenomenon can be fully understood.

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Appendices

Appendix A: Herbicide ingredient dose response curves

A1 2,4-d dose response curves.

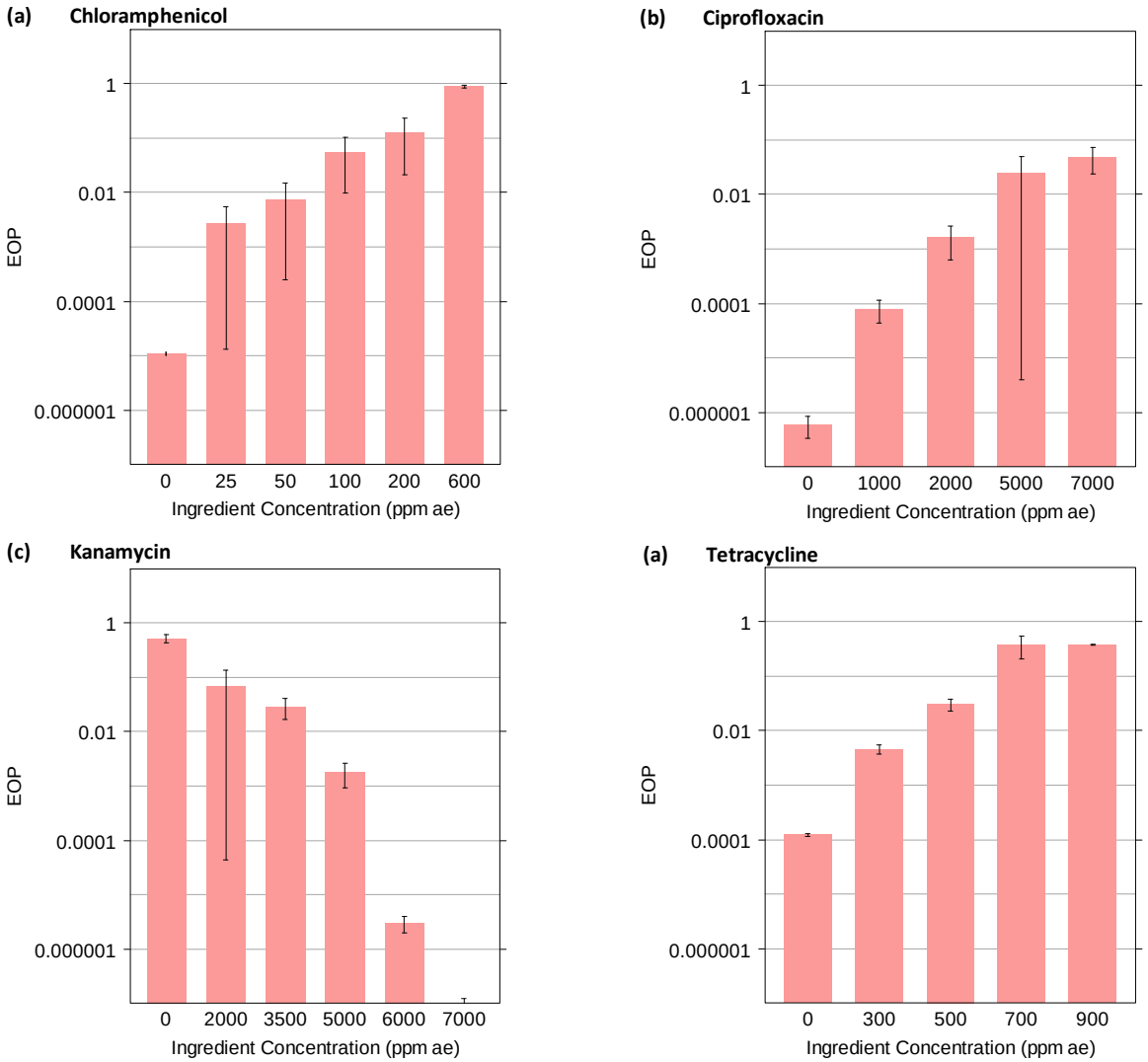

Figure A1: Survival of *S. Typhimurium* on (a) chloramphenicol, (b) ciprofloxacin, (c) kanamycin and (d) tetracycline in the presence of a range of concentrations of 2,4-d. Survival is reported as EOP. Error bars are Standard Error of the Mean (SEM). These curves were used to determine the minimum concentration of each herbicide component necessary to induce a 100-fold change in the EOP that was reported in Chapter 3, section 3.2.3.

A2 Dicamba dose response curves.

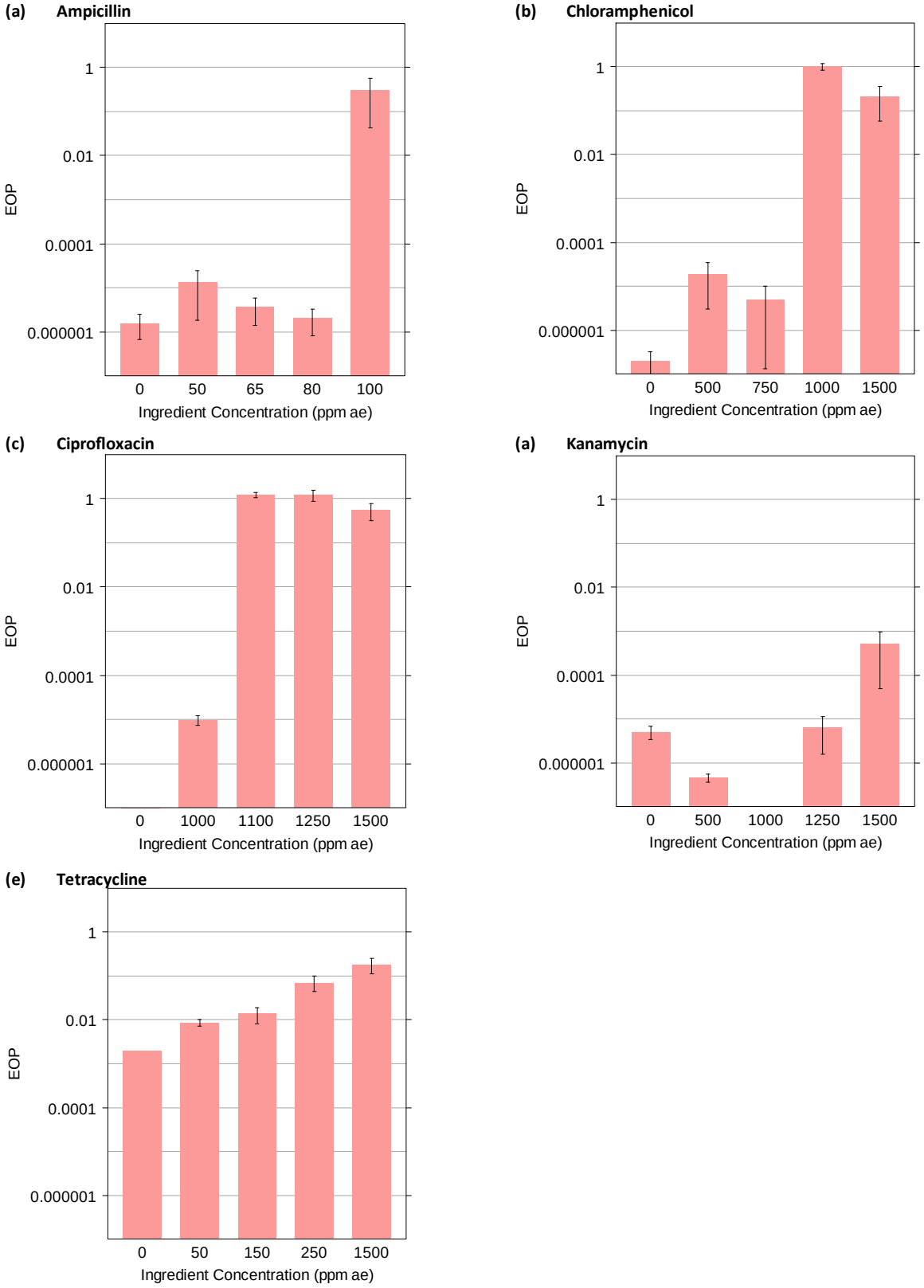

Figure A2: Survival of *S. Typhimurium* on (a) ampicillin, (b) chloramphenicol, (c) ciprofloxacin, (d) kanamycin and (e) tetracycline in the presence of a range of concentrations of dicamba. Survival is reported as EOP. Error bars are SEM. These curves were used to determine the minimum concentration of each herbicide component necessary to induce a 100-fold change in the EOP that was reported in Chapter 3, section 3.2.3.

A3 Glyphosate dose response curves.

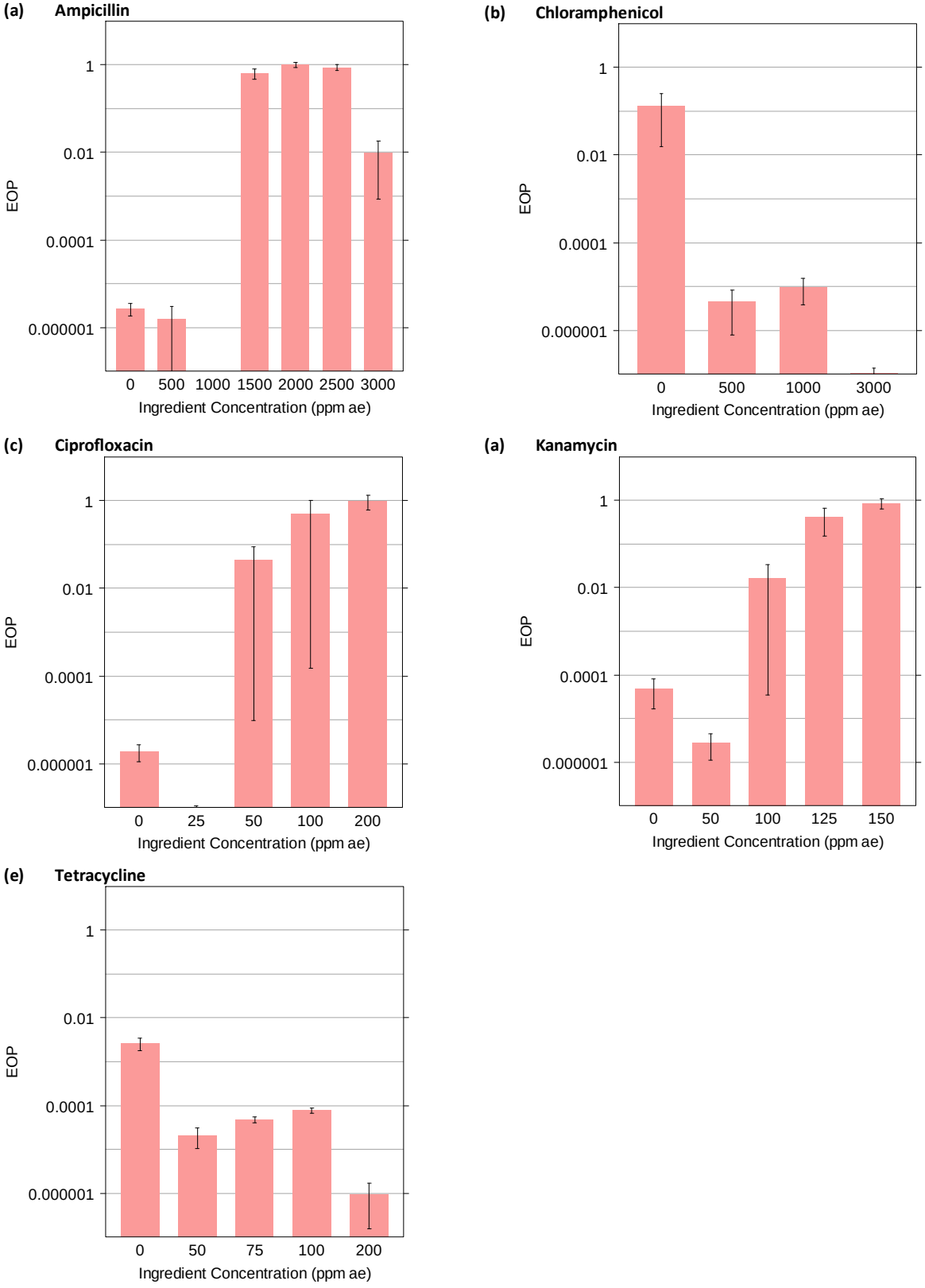

Figure A3: Survival of *S. Typhimurium* on (a) ampicillin, (b) chloramphenicol, (c) ciprofloxacin, (d) kanamycin and (e) tetracycline in the presence of a range of concentrations of glyphosate. Survival is reported as EOP. Error bars are SEM. These curves were used to determine the minimum concentration of each herbicide component necessary to induce a 100-fold change in the EOP that was reported in Chapter 3, section 3.2.3.

A4 Tween80 dose response curves.

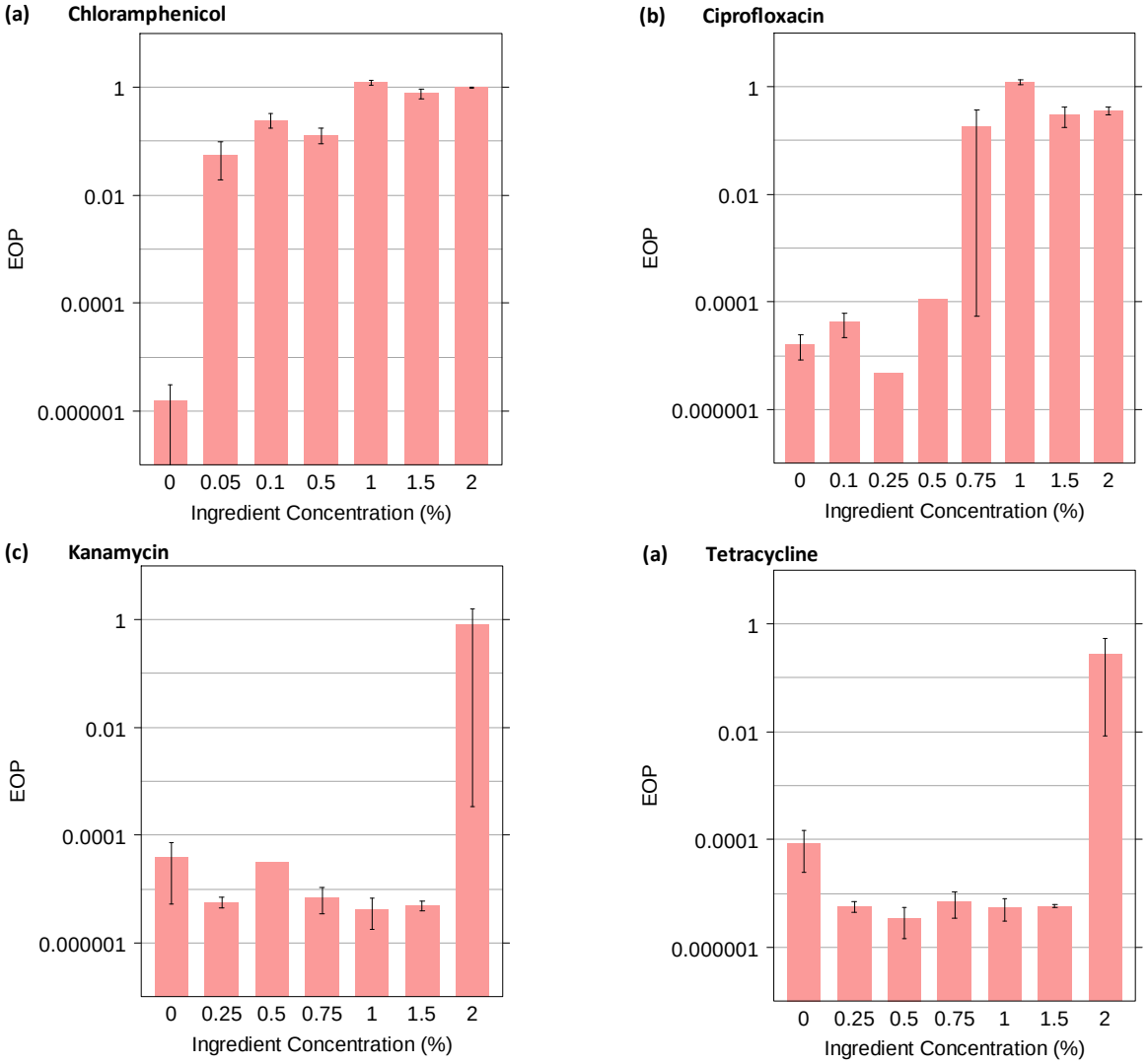

Figure A4: Survival of *S. Typhimurium* on (a) chloramphenicol, (b) ciprofloxacin, (c) kanamycin and (d) tetracycline in the presence of a range of concentrations of Tween80. Survival is reported as EOP. Error bars are SEM. These curves were used to determine the minimum concentration of each herbicide component necessary to induce a 100-fold change in the EOP that was reported in Chapter 3, section 3.2.3.

A5 CMC dose response curves.

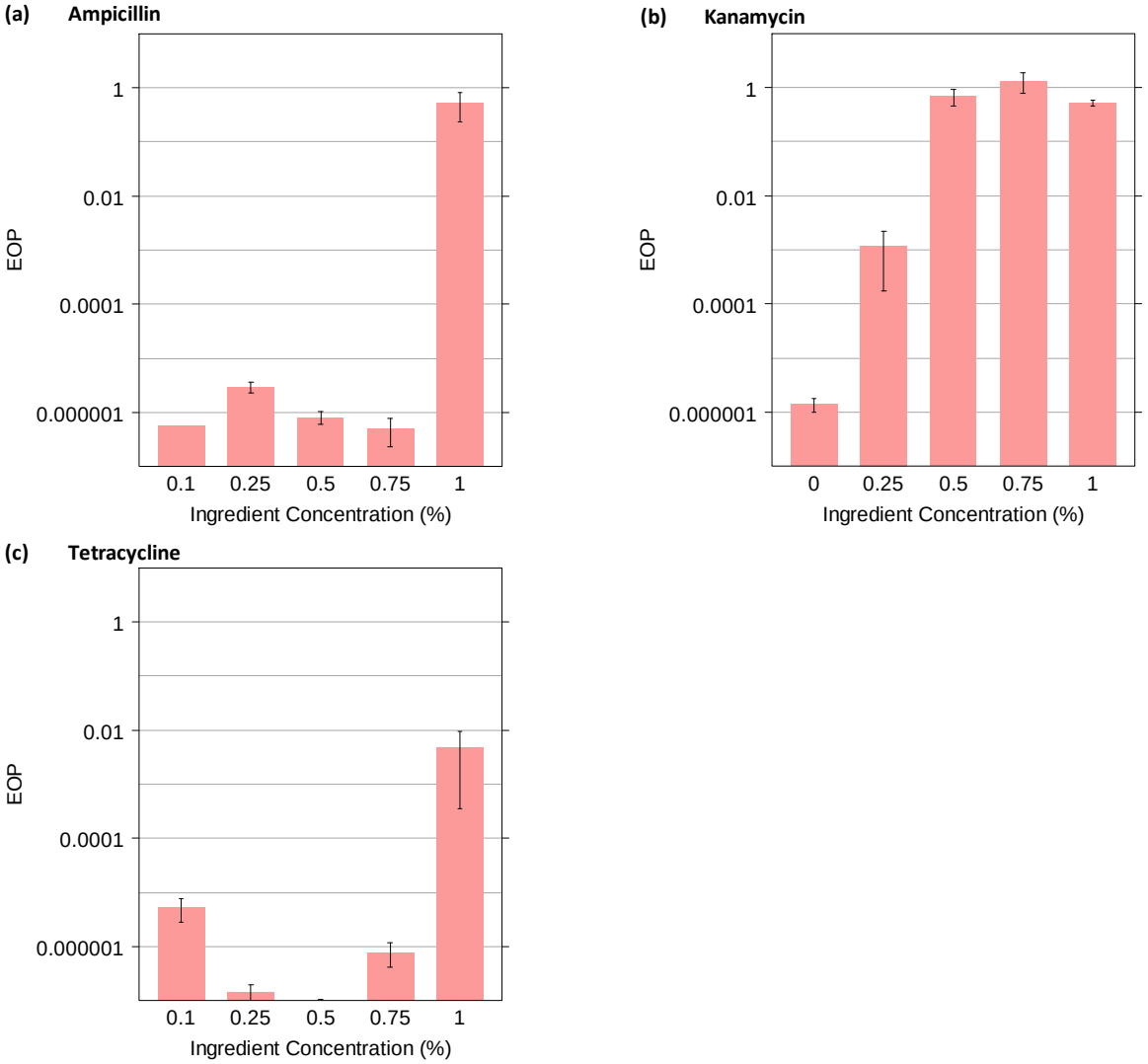

Figure A5: Survival of *S. Typhimurium* on (a) ampicillin, (b) kanamycin and (c) tetracycline in the presence of a range of concentrations of CMC. Survival is reported as EOP. Error bars are SEM. These curves were used to determine the minimum concentration of each herbicide component necessary to induce a 100-fold change in the EOP that was reported in Chapter 3, section 3.2.3.

Appendix B: Summary of results for knockout strains

Table B1: Statistical significance and fold-change in MIC induced by exposure to the herbicides for each combination of antibiotic, herbicide and knockout strain for the data presented in chapter 4 section 4.2.3. NS is not significant, * indicates 0.01 < p < 0.001, ** indicates 0.001 < p < 0.0001 and *** indicates 0.0001 < p. The fold-change of the increases or decreases of the MIC in response to the presence of the herbicide is indicative of the magnitude of the change and is provided in parentheses.

		wt	*ΔacrA*	*ΔacrB*	*ΔacrD*	*ΔtolC*	*ΔompF*
Kamba	Cip	*** (3.0)	*** (1.3)	NS	*** (2.1)	*** (2.0)	*** (2.1)
	Str	*** (1.3)	*** (5.3)	*** (8.0)	*** (2.0)	*** (8.0)	*** (6.7)
	Tet	*** (2.0)	*** (2.0)	* (1.0)	*** (2.7)	*** (1.0)	*** (2.9)
Roundup	Cip	*** (5.0)	* (1.2)	*** (1.3)	*** (2.3)	NS	*** (4.5)
	Str	*** (16.7)	* (1.3)	*** (1.2)	*** (4.0)	*** (2.7)	*** (2.3)
	Tet	*** (2.0)	NS	*** (1.3)	*** (2.0)	*** (1.0)	*** (1.5)