Herbicides alter the *S. marcescens* response to antibiotics

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Abstract

Exposure to herbicides and antibiotics has been associated with increased honeybee mortality and colony loss on a global scale. The focus of my research is on the recently discovered link between herbicides and the development of antibiotic resistance, which could conflict with efforts to halt honeybee decline and antibiotic stewardship.

In this study, I have tested whether exposure to sublethal herbicides changes antibiotic responses in *Serratia marcescens*, an important opportunistic pathogen responsible for honeybee mortality as well as in-hospital infection. *S. marcescens* was exposed to eighteen combinations chosen from three herbicide formulations and six antibiotics. The pattern of antibiotic response induced by herbicides varied in direction and intensity, which included increase, decrease or no change in antibiotic MIC. The most notable result was the 64-fold increase in ciprofloxacin MIC under exposure of Number 8 (glyphosate).

Two further experiments were conducted to determine the mechanism of the increased antibiotic tolerance induced by herbicides. In one experiment, herbicides were withdrawn from *S. marcescens* colonies with elevated ciprofloxacin MIC. Most of the resulting colonies did not survive under the same amount of ciprofloxacin, which indicate that persistent exposure of herbicides is necessary for the ciprofloxacin resistance phenotype. In the other experiment, a mutation assay using rifampicin as selection marker was performed to identify that none of the three herbicides promote mutation in *S. marcescens*. Combined with results from previous studies, it is likely that the elevated tolerance to antibiotics is physiological response such as efflux-pumps. However, further studies are needed to identify the exact mechanism.

Abbreviation

ESBL	extended-spectrum β-lactamase
HGT	horizontal gene transfer
MIC	minimum inhibitory concentration
MRSA	methicillin-resistant *Staphylococcus. aureus*
PAβN	phenylalanine-arginine beta-naphthylamide
WHO	World Health Organization

Chapter One

Introduction

Since the discovery and introduction of penicillin in mid-20th century, more than 20 classes of antibiotics have been developed, leading to the "antibiotic era" in which infectious disease caused by bacteria could be more effectively treated (Gualerzi, Brandi, Fabbretti, & Pon, 2013). However, antibiotic resistant pathogens emerged during the same period, making many antibiotics ineffective (Avorn et al., 2001). As of 2012, more than 70% of bacteria pathogens were noted as resistant to most commercially available antibiotics while development of novel antibiotics was slow (Bérdy, 2012). Currently, antibiotic resistance is listed by WHO as one of the ten greatest threats to global human health (World Health Organization, 2019).

Similarly, herbicides became popular during mid-20th century as a cost-effective way to kill unwanted plants. They are applied not only in agriculture, but also in cities and in household gardens. Due to intensive usage, herbicide residues appear widely in soil, water and foods (Bai & Ogbourne, 2016; Maqueda, Undabeytia, Villaverde, & Morillo, 2017). While herbicides are often claimed to be safe by the manufacturer, the safety is frequently challenged (Bukowska, 2006; González, Soloneski, & Larramendy, 2006; Williams, Kroes, & Munro, 2000). Sold to kill plants, they also could have off-target effects on bacteria. Previous studies in our laboratory group showed that various pesticides change the antibiotic response of some human pathogens (Jun et al., 2019; Kurenbach et al., 2017; Kurenbach et al., 2015), which could promote evolution of antibiotic resistance(Kurenbach, Hill, Godsoe, van Hamelsveld, & Heinemann, 2018). However, their implication on other pathogens is unclear. To fill in the knowledge gap, I intend to expand the scope of previous research by using other model systems.

Previous studies show that pesticides affect antibiotic resistance only when the

exposure is in conjunction with antibiotics (Kurenbach et al., 2018). In my work I sought a model system where dual pesticide and antibiotic exposure was routine. That led me to the honey bee. Honey bees are valuable pollinators that contribute largely to the agricultural economy. Honey bees are frequently exposed to herbicides and antibiotics during common bee-keeping process, making them an exposure pathway for combination of herbicides and antibiotics (Herbert, Vazquez, Arenas, & Farina, 2014; Raymann, Shaffer, & Moran, 2017). We hypothesize that herbicides can affect antibiotic response in *Serratia marcescens*, an opportunistic pathogen hosted by honey bees. For this thesis, I investigated the effects of herbicides on the antibiotic response of *S. marcescens*, whether the effects promote antibiotic resistance in *S. marcescens*, and the implications on bee-keeping practice and potentially also human health.

1.1 Antibiotics and resistance

Antibiotics are medicines used to prevent or treat bacterial infections (World Health Organization, 2018). Traditionally, they are defined as a class of antibacterial agents that are naturally produced by microorganisms as opposed to synthetic agents. In this thesis, the distinction between natural and synthetic agent is not relevant. Any antibacterial agent that may be used therapeutically are referred to as an antibiotic in this paper.

Antibiotics are commonly used to treat or prevent infectious diseases in people, farm animals and crops (Williams-Nguyen et al., 2016). Consumption of antibiotics is on the rise globally. Annual antibiotics consumption in New Zealand increased by 49% between 2006 and 2014 (D. Williamson, Roos, & Verrall, 2016). As a result, pathogens become increasingly resistant to wider range of antibiotics, posing serious risks to public health. The World Health Organization identified nine bacterial pathogens of international concern, three of which are of particular concern in New Zealand, namely *Enterobacteriaceae spp.*, *Staphylococcus aureus* and *Neisseria gonorrhoeae*. (WHO, 2015). By 2016, all of the three pathogens have developed resistance to their relevant

antibiotics (Pullon et al., 2016). In 2012, there were 4,000 cases of infections by *Enterobacteriaceae* producing extended-spectrum β-lactamase (ESBL), which confers resistance to almost all β-lactamases (D. A. Williamson & Heffernan, 2014). Bloodstream isolates from that year included almost double the rate of ESBL-producers than those from 2009 (D. A. Williamson & Heffernan, 2014). A survey in 2014 showed that 8.9% of *S. aureus* infections were caused by methicillin-resistant *S. aureus* (MRSA), which is one important cause of child infection and related mortality in the Western Pacific region (Heffernan, Bakker, Woodhouse, Dyet, & Williamson, 2015). Another survey in 2014 found that antibiotic resistant *N. gonorrhoeae* are highly prevalent, with 98% of isolates resistant to penicillin, 32% of isolates resistant to ciprofloxacin, and 68% of isolates resistant to tetracycline (Lee et al., 2018).

Tetracycline is a bacteriostatic antibiotic that inhibits protein synthesis in bacteria by reversibly binding to the 30S ribosomal subunit (Chopra & Roberts, 2001). Drugs of the tetracycline class are commonly applied to livestock and crops (Marilyn C Roberts & Schwarz, 2016). In honey bee keeping, tetracycline is frequently used as an important drug against American foulbrood (Evans, 2003). For that reason, tetracycline resistance is commonly found in honey bee pathogens (Evans, 2003).

Streptomycin and gentamicin are bactericidal antibiotics from the aminoglycoside family (McManus, Stockwell, Sundin, & Jones, 2002). They bind to the 30S ribosomal subunit and cause mistranslation of protein, leading to eventual cell death (Kotra, Haddad, & Mobashery, 2000). Streptomycin is often used against foulbrood diseases in combination with tetracycline, giving direct exposure to honey bees (Al-Waili, Salom, Al-Ghamdi, & Ansari, 2012). In addition, both streptomycin and gentamicin are commonly used in agriculture to treat or prevent plant diseases such as fire blight in apple and pears (McManus et al., 2002). Antibiotic residue on pollen or bees exposed during application could serve as an alternative pathway of exposure in countries where antibiotic treatment in bee hives is banned (Al-Waili et al., 2012).

Chloramphenicol is a bacteriostatic antibiotic that inhibit protein synthesis by reversibly binding to the 50S subunit of the bacterial ribosome (Schwarz, Kehrenberg, Doublet, & Cloeckaert, 2004). While it is not commonly prescribed as veterinary drug, usage of chloramphenicol in honey bee treatment is not uncommon as seen in the frequent detection of chloramphenicol residue in honey products (Al-Waili et al., 2012).

Ciprofloxacin is a fluoroquinolone antibiotic that targets type II topoisomerases, DNA gyrase and topoisomerase IV of bacteria, inhibiting DNA replication (Aldred, Kerns, & Osheroff, 2014). It acts as a bacteriostatic antibiotic at low concentration and bactericidal at high concentration (Hawkey, 2003). Ciprofloxacin is mainly used as a human medicine, but there are cases where ciprofloxacin is used in treatment of honey bees (Lombardo-Agüí, García-Campaña, Gámiz-Gracia, & Cruces-Blanco, 2012).

Ampicillin is a β-lactam class antibiotic that inhibit synthesis of the peptidoglycan layer of the bacteria cell wall, eventually resulting in cell death (Yao, Kahne, & Kishony, 2012). In agriculture, ampicillin is most commonly used in livestock and poultry (Yassin et al., 2017). While not commonly used on honey bees, there are cases where ampicillin residue is detected in honey, suggesting there are possible exposure pathways from the environment (Solomon, Santhi, & Jayaraj, 2006).

While different types of antibiotics have different mechanisms of action, bacteria also have different ways to cope with them. Antibiotic resistances are commonly divided into three categories: intrinsic, adaptive and acquired resistance (Gualerzi et al., 2013).

Intrinsic resistance is the characteristic of a given species depending on its own biology (Giedraitiene, Vitkauskiene, Naginiene, & Pavilonis, 2011). A common example is that Gram-negative bacteria are often unaffected by antibiotics against

Gram-positive bacteria due to their thickened membrane (Cox & Wright, 2013).

Acquired resistance is the phenomenon when susceptible bacteria become resistant through a genetic change, which involves mutation, horizontal gene transfer (HGT) or both (Giedraitiene et al., 2011). Mutations usually result in low-level antibiotic resistance (Levy & Marshall, 2004). Under antibiotic stress, individuals with antibiotic resistance mutations can accumulate in a population as the susceptible ones die out, which eventually results in uniformed antibiotic resistance in that population (Gualerzi et al., 2013). HGT is the reproduction of genetic material that is independent from reproduction of organisms, and it is usually observed as the lateral movement of genes between organisms (Heinemann & Kurenbach, 2014). When resistant genes travel among bacteria population, especially pathogens, it could turn into a health risk.

Adaptive resistance is the physiological reaction of bacteria, often involving regulation of gene expression. Mechanisms of adaptive resistance are triggered upon presence of antibiotics, and they are usually reversed after removal of antibiotics (Fernández, Breidenstein, & Hancock, 2011). One common mechanism is biofilm formation. Some bacteria form biofilm under presence of antibiotics, which block antibiotics from entering the cytoplasm and take effect (Romero, Traxler, López, & Kolter, 2011). Up-regulation of efflux pumps is also common mechanism for keeping antibiotic molecules out of the cells (Blair, Richmond, & Piddock, 2014). While these physiological reactions do not involve changes in DNA sequences, they do allow the bacteria to survive longer under antibiotic pressure. This often gives bacteria enough time to have a chance to acquire genotypical resistance through mutation or HGT (Heinemann, 1999).

1.2 Herbicides

Herbicides are substances used to control weeds. As an effective and efficient tool for weed control, herbicides contributed largely to reduced crop losses from

competition with weed species and decreased manual labor (Heap, 2014). Since agriculture expanded and became more reliant on herbicide as the simplest weed control strategy, herbicide consumption has increased dramatically. In New Zealand, total pesticide consumption is estimated to have increased by an order of magnitude from 1960 to 2006, nearly 70% of which are herbicides (MacLeod & Moller, 2006). Herbicides being used widely in large quantity lead to pollution in neighboring environments as they move away from the application area and accumulate elsewhere (Chang, Simcik, & Capel, 2011). Herbicides commonly used in New Zealand are based on 2,4-d, dicamba and glyphosate in various commercial formulations (Harrington & James, 2005; Popay, 2008).

Currently, glyphosate is the most widely used herbicide in the world, including New Zealand (Ministry for Primary Industries, 2019). Due to its non-selective, broad-spectrum, post-emergence properties, glyphosate-based herbicides are popular for weed control in the crop fields, public parks, and private gardens (Beckie, 2011). Glyphosate inhibits the 5-enolpyruvyl-shikimate-3-phostphate synthase (EPSPS) enzyme in the shikimate pathway, which in turn stops synthesis of key amino acids and leads to plant death (Sammons & Gaines, 2014). However, the shikimate pathway is also present in bacteria and fungi, making glyphosate toxic to many bacteria species in the same way (Schulz, Krüper, & Amrhein, 1985). In addition, wide usage of glyphosate lead to emergence of glyphosate-resistant weeds, leading to usage of older herbicides like dicamba and 2,4-d again (Beckie, 2011). Both of these herbicides are synthetic auxins that regulate plant growth, which target dicotyledonous weeds for their higher sensitivity to plant hormones (Green, 2014). All the above herbicides are water soluble, making them likely to leach through the ground and runoff into the environment, causing potential hazards (Borggaard & Gimsing, 2008; Cojocaru et al., 2013; Hermosín et al., 2006).

The potential adverse effects of herbicides on people and the environment have

been discussed intensively. One primary concern is the increasing occurrence and distribution of herbicide-resistant weeds (Heap, 2014). Short-term and long-term toxicity of herbicides and their degradation products is another primary concern for human health and other vertebrates (Bukowska, 2006; González et al., 2006). However, their off-target effect on bacteria, especially pathogenic ones, is often not included in those discussions (Kurenbach et al., 2015).

Previous studies showed that bacteria can physiologically adapt to the relatively low toxicity of herbicides through changes in gene expression, which could subsequently change their response to antibiotics (Kurenbach et al., 2017). Depending on the species of the bacteria and the herbicide, exposed bacteria may exhibit increased tolerance, decreased tolerance, or no change against some antibiotics (Kurenbach et al., 2017). In many cases of increased resistance, it is revealed that expression of efflux pumps is responsible (Kurenbach et al., 2017). This mechanism effectively reduces the concentration of toxic molecules down to sublethal levels, allowing bacteria to survive higher concentrations of antibiotics and giving them more time to potentially acquire antibiotic resistance (Kurenbach et al., 2018). In the case of reduced resistance, antibiotics have a higher selective pressure on bacteria and thus less antibiotic is needed to select for resistant strains (Kurenbach et al., 2018). Either way, exposure of herbicides could accelerate the evolution of antibiotic resistance in bacteria. While herbicides are most often used in an agriculture setting, many antibiotics are used as both human medicine and agricultural drugs. For this reason, resistant pathogens that rise from agricultural could be relevant to human health (Marilyn C Roberts & Schwarz, 2016).

1.3 Honeybees and *S. marcescens*

The honey bee is a valuable animal for human society. They provide pollination service for various crops, contributing to the agricultural economy. They also provide honey and other products, giving additional economic value. In New Zealand, the honey

bee is a $5 billion business with 924,973 hives and 9,217 beekeepers registered by June 2019 (Apiculture New Zealand, 2019). At the same time, honey bees are frequently exposed to a variety of chemicals. Bee hives are often treated with antibiotics to treat or prevent foul-brood diseases, exposing honey bees to medical concentrations of antibiotics (Al-Waili et al., 2012). Pollen and nectar from plants often contain chemical residues, including insecticides, fungicides and herbicides (Colwell, Williams, Evans, & Shutler, 2017). When honey bees forage for pollen or nectar in treated fields, herbicides are transferred from plants to bees to hives (Bogdanov, 2006). These exposures bring adverse effects on honey bees. Compromised immunity caused by exposure to antibiotics or herbicide often results in higher susceptibility to pathogens, allowing opportunistic pathogens to infect the hosts, then leading to elevated mortality and loss of colonies (J. H. Li et al., 2017; Pettis, Kochansky, & Feldlaufer, 2004; Raymann et al., 2017). In addition, these chemicals contaminate bee products such as honey, beeswax and royal jelly, which negatively affect their economic value (Adams, Fussell, Dickinson, Wilkins, & Sharman, 2009; Bogdanov, 2006; Martel, Zeggane, Drajnudel, Faucon, & Aubert, 2006).

While honey bees are exposed to environmental chemicals, so is the bacteria community living inside honey bees. Among those in this community, *S. marcescens* has a potentially large impact on honey bee health, the environment, and human health. While it has recently been shown to be a pathogen of honey bees, *S. marcescens* has long been known to cause infection in human patients and various animals (Raymann, Coon, Shaffer, Salisbury, & Moran, 2018). As an opportunist pathogen, *S. marcescens* usually do not show pathogenicity until the host is immune compromised. Honey bees usual show symptoms of infection only after antibiotics or herbicides disturb the gut microbiota (Raymann, Coon, et al., 2018; Raymann et al., 2017). In human patients, they are commonly seen as a cause of in-hospital infection (Iguchi et al., 2014).

S. marcescens has been a subject of concern due to its high tolerance to hazardous

environments, as seen in its ability to grow in medical chemicals including disinfectants and antiseptics (Hejazi & Falkiner, 1997). *S. marcescens* is also considered as a common antibiotic resistant pathogen, as seen in frequent reports of ESBL producing strains, multi-drug resistance strains and general increase of antibiotic resistance of this species through 2007 to 2015 (Iguchi et al., 2014; Lin et al., 2016; Parente, Rebouças, Santos, Barbosa, & Zanin, 2016; Perry et al., 2017). While it is not a major pathogen of concern, *S. marcescens* is a pathogen that deserves attention. In New Zealand, there have been cases of transplant infection and infant infection caused by *S. marcescens* (Mouat et al., 2018; Wake, Lees, & Cull, 1986). If *S. marcescens* can acquire antibiotic resistance following exposure to herbicides, it will be difficult or even impossible to treat, posing great threat to human health.

1.4 Objectives

The aim of this study was to:

1. investigate if herbicides affected the antibiotic response of *S. marcescens* as observed in other bacteria.

2. investigate if the effects of herbicides on *S. marcescens* cause an adaptive response or select for acquired resistance to antibiotics

3. investigate if the effects of herbicides affect evolution of antibiotic resistance of *S. marcescens*

Chapter Two

Testing susceptibility change of *S. marcescens* under herbicide exposure

2.1 Introduction

It is reported that minimum inhibitory concentration (MIC) of *S. marcescens* to many common antibiotics has been increasing (Fusté et al., 2012), making it more dangerous over time. *S. marcescens* is exposed to various antibiotics used in agriculture or medicine. Among those antibiotics, *S. marcescens* with resistance to ciprofloxacin or gentamicin especially complicate treatment (Cannon, Partridge, Boden, Townsend, & Stockley, 2014; Mouat et al., 2018). Likewise, its habitats include those exposed to herbicides. Therefore, I focussed on the question of whether herbicides can help *S. marcescens* to develop resistance to those important antibiotics. This knowledge could help to prevent resistant strains from emerging.

Previous work has shown that various Gram-negative bacteria evolve resistance to antibiotics faster when exposed to herbicides (Kurenbach et al., 2018). It is therefore likely that *S. marcescens* will also be affected by such exposures. However, the nature of the effect and whether it increases or decreases resistance was not predictable because it varied by species, type of herbicide and kind of antibiotic. The aim of this chapter was to investigate how three types of herbicides effect *S. marcescens'* responses to various antibiotics.

2.2 Methods

2.2.1 Bacteria strains, culture conditions and chemicals

Three different strains of *S. marcescens* were used. B76 strain from School of Biological Sciences (University of Canterbury) was the primary strain used in this study.

The DB10 and DB11 strains isolated from *Drosophila* were also used in this study (Flyg, Kenne, & Boman, 1980). The strains were stored long-term at -80°C in LB medium supplemented with 15% glycerol. Bacteria were maintained for up to one week on Luria-Bertani broth (LB) Agar, grown at 37°C overnight and subsequently stored at 4°C. Luria-Bertani base (Lennox-L-Broth Base, Invitrogen, USA) and agar (Bacteriological Agar No.1, Oxoid, UK) were used. Bacteria were cultured at 37°C with rotation for aeration before use in experiments.

The antibiotics used in these experiments were: ampicillin (stock concentration 100 mg/mL), chloramphenicol (20 mg/mL), ciprofloxacin (5 mg/mL), tetracycline (12 mg/mL) and gentamicin (10 mg/ml). Stock solutions were stored at -20°C and diluted appropriately for experiments. Ampicillin sodium salt was purchased from Applichem (Germany), ciprofloxacin hydrochloride from Pentex (USA), chloramphenicol, tetracycline hydrochloride, streptomycin sulfate salt and gentamicin sulfate salt from Sigma-Aldrich (USA).

Three commercial herbicide formulations were used in this study: Kamba, Number 8, and 2,4-D. Kamba^{500} (Nufarm, New Zealand) contains the active ingredient dicamba as a dimethylamine salt (500 g/L). Number 8 (Mitre 10, New Zealand) has the active ingredient glyphosate as an isopropylamine salt (360 g/L). 2,4-D Amine 800 WSG (Agpro, NZ) contains the active ingredient 2,4-dichlorophenoxyacetic acid (2,4-d) as a dimethylamine salt (800 g/kg). Concentrations of herbicides were converted to parts per million acid equivalent (ppm ae) of active ingredient for comparability to other formulations and studies. The 2,4-D pellets were dissolved in sterile water to give a solution with a final active ingredient concentration of 66.24 g/L (300 mM). Herbicides were stored at room temperature and added to the growth medium at the appropriate concentration when required.

2.2.2 Determination of the MIC of herbicides and antibiotics

MIC is conventionally defined as the lowest concentration of an antibacterial substance that inhibits bacterial growth following incubation, usually measured after 12 or more hours (Wiegand, Hilpert, & Hancock, 2008). For consistency with previous studies, the MIC in this study was defined as the concentration that caused a 1000-fold reduction in EoP compared to the control group. MICs were determined for the six antibiotics and three herbicides which were used as supplements in solid LB media. *S. marcescens* culture was grown in liquid LB until it reached an optical density (OD_{600}) of around 1, measured using a spectrofluorometer (Novaspec III-Abs 600 nm). The culture was serially diluted 10-fold in LB and the various dilutions were applied in 10 µl droplets ('spot plating') to plates with increasing concentrations of antibiotic or herbicide. The plates were freshly made and dried for at least 40 minutes in a fume hood. The chosen antibiotic and herbicide concentrations ranged from dilutions that allow bacteria to grow to dilutions that inhibit growth. The final culture dilutions plated (10^2 to 10^8) were in triplicate and the plates were incubated at 37°C for 16–20 hours. The lowest concentration of antibiotic or herbicide that inhibited visible bacterial growth after 16 hours incubation was taken as the MIC. Bacterial colonies that grew on a plate without any antibiotic or herbicide was used as the control. All LB plates were poured within 24 hour and dried in a laminar flow cabinet for at least an hour before inoculation.

Figure 2.1 Formula used to calculate Efficiency of Plating (EoP).

$$\text{EoP} = \frac{\text{cfu/ml on plates}}{\text{cfu/ml on control plates}}$$

2.2.3 Testing effects of herbicides on antibiotic resistance

The change in the EoP was measured when the bacteria were simultaneously exposed to both a herbicide and an antibiotic. A single *S. marcescens* colony was

transferred into liquid LB medium. Then the liquid medium was incubated with aeration at 37°C until the culture reached an optical density (OD_{600nm}) above 1. Afterwards, the culture was diluted, and the series of concentrations was applied to LB plates (spot plating, see above, to a final dilution of 10^{-8}~10^{-9} depending on the culture concentration), LB supplemented with either a herbicide or an antibiotic, or LB supplemented with both a herbicide and an antibiotic. The control LB supplemented with herbicide was included to ensure the herbicide alone had no effect on survival. After the droplets had dried, plates were incubated at 37°C and examined daily for up to four days. Individual colonies were counted and colony-forming units per mL (cfu/mL) calculated based on the dilution where growth was observed. The cfu/mL count for each treatment plate was compared to the control plate to calculate the EoP. The EoP is a measure of survival used to normalise for variations in culture density between replicates (Figure 2.1). Each assay was performed three times using independent cultures of bacteria.

2.2.4 Statistical analysis

R was used for all statistical analysis (R Core Team, 2019). For the EoP, a multifactor analysis of variance (ANOVA) was performed on the log-transformed EoP scores to test for synergistic effects of herbicides and antibiotics. For this analysis, plots of residuals were utilized to test for any violations of the assumptions of normality and equality of variance. Contrasts across herbicide concentrations when antibiotic levels were fixed were used to determine the antibiotic concentrations with significant differences among herbicide treatments. The testInteractions function in the phia package in R was used to evaluate the contrasts (De Rosario-Martinez, 2015), and a Bonferroni correction was performed within each experiment (Crawley, 2007). Log-transformed EoP scores were used for the statistical analysis, calculation of standard error of the mean (SEM) and creation of graphs. Graphs are created using Graphpad Prism 6.

2.3 Results

2.3.1 Minimum inhibitory concentration

S. marcescens B76 was exposed to three herbicides and six antibiotics. As shown in Table 2.2, B76 strain was resistant to ampicillin, tetracycline and streptomycin, but was susceptible to ciprofloxacin, gentamicin and chloramphenicol according to the CLSI standard (Table 2.1) (CLSI, 2019).

Table 2.1 S, I, R table of *S. marcescens*

Antibiotics	**S (μg/ml)**	**I (μg/ml)**	**R (μg/ml)**
Ampicillin	≤8	16	≥32
Streptomycin*	≤8	16	≥32
Tetracycline	≤4	8	≥16
Ciprofloxacin	≤0.25	0.5	≥1
Chloramphenicol	≤8	16	≥32
Gentamicin	≤4	8	≥16

*The S, I, R category is not given in current CLSI standard. The number shown in the table was taken from other references (Stock, Burak, Sherwood, Grüger, & Wiedemann, 2003).
S: susceptible
I: intermediate
R: resistant

Table 2.2 MIC table of *S. marcescens* B76.

a)

Antibiotics	**MIC (μg/ml)**
Ampicillin	≤50
Streptomycin	≤100
Tetracycline	≤100
Ciprofloxacin	≤0.125
Chloramphenicol	≤12
Gentamicin	≤4

b)

Herbicide formulation (active ingredients)	**MIC (ppm ae)**
Number 8 (glyphosate)	≤13340
Kamba (dicamba)	≤10390
2,4-D (2,4-d)	≤6630

a) Working MIC of antibiotics. b) Working MIC of commercial herbicide formulation calculated in parts per million acid equivalent (ppm ae) of the respective active ingredients. Values are rounded to the nearest 10 ppm ae. DB10 and DB11 strains have same MIC shown in this table.

Table 2.3 Antibiotics MIC fold-change of *S. marcescens* under herbicide exposure

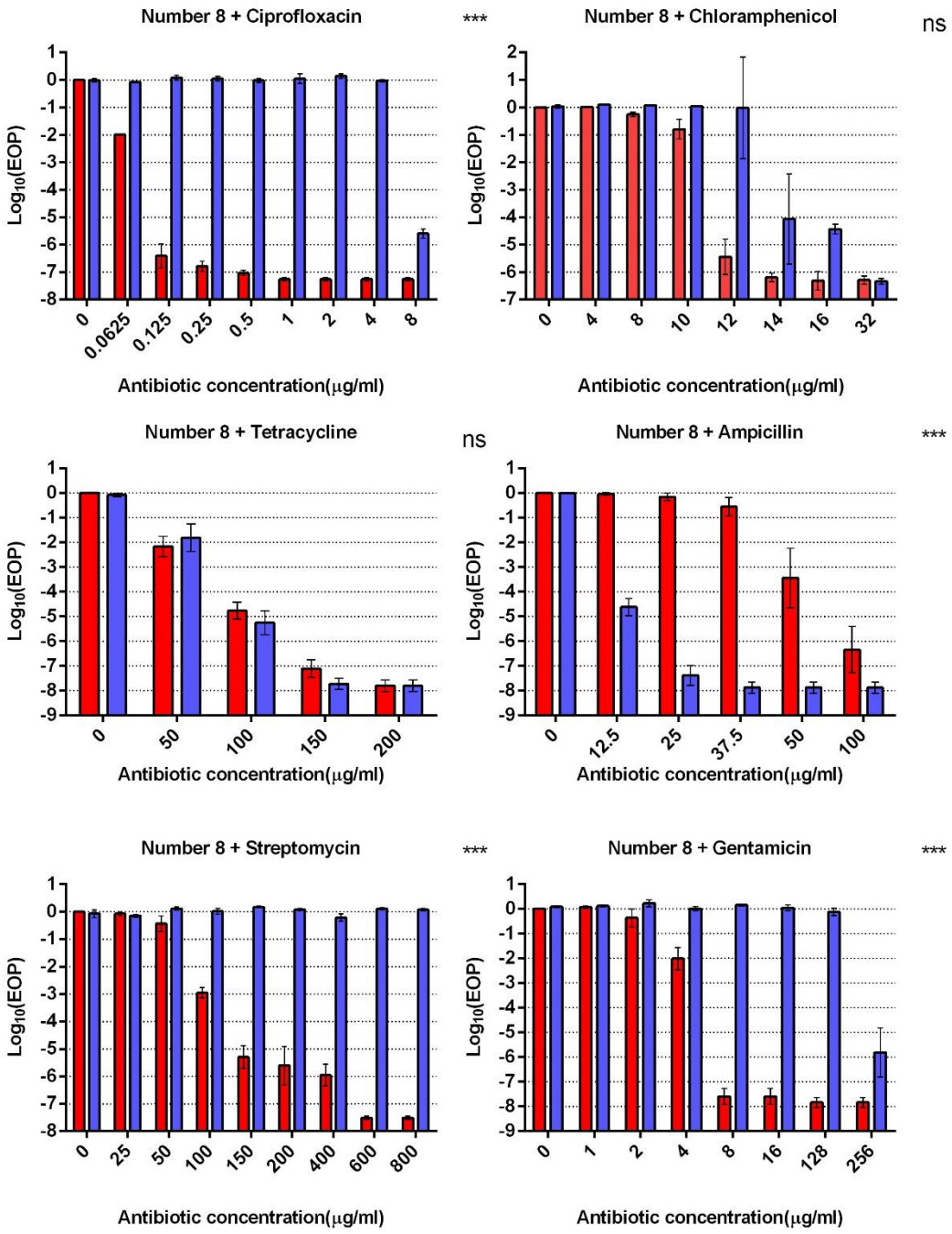
	Number 8 (glyphosate)	**Kamba (dicamba)**	**2,4-D (2,4-d)**
Ciprofloxacin	64	8	8
Chloramphenicol	ns	ns	0.25
Tetracycline	ns	ns	0.33
Ampicillin	0.25	2	0.5
Streptomycin	higher than 8	ns	2
Gentamicin	32	0.5	ns

Fold changes are calculated as the antibiotic MIC under herbicide exposure divided by MIC without herbicide, rounded to 0.01.

2.3.2 Commercial herbicide formulations alter some antibiotic responses of *S. marcescens*

The Number 8 formulation induced statistically significant responses in four of the six antibiotics tested (Figure 2.2). Chloramphenicol ($p = 0.223$) and tetracycline ($p = 0.609$) were the two antibiotics where the addition of Number 8 had no statistically significant effect on growth or MIC (Table 2.2). Number 8 increased the EoP of *S. marcescens* on ciprofloxacin ($p < 2 \times 10^{-16}$) and gentamicin ($p < 2 \times 10^{-16}$), which corresponded to a 64-fold and 32-fold increase in MIC, respectively (Table 2.2). Number 8 also increased the EoP on streptomycin ($p < 2 \times 10^{-16}$), which increased the MIC to a concentration that was beyond CLSI resistance ranges. Meanwhile, Number 8 decreased the EoP of *S. marcescens* on ampicillin ($p = 1.9 \times 10^{-7}$), corresponding to a 0.25 of the original MIC (Table 2.2).

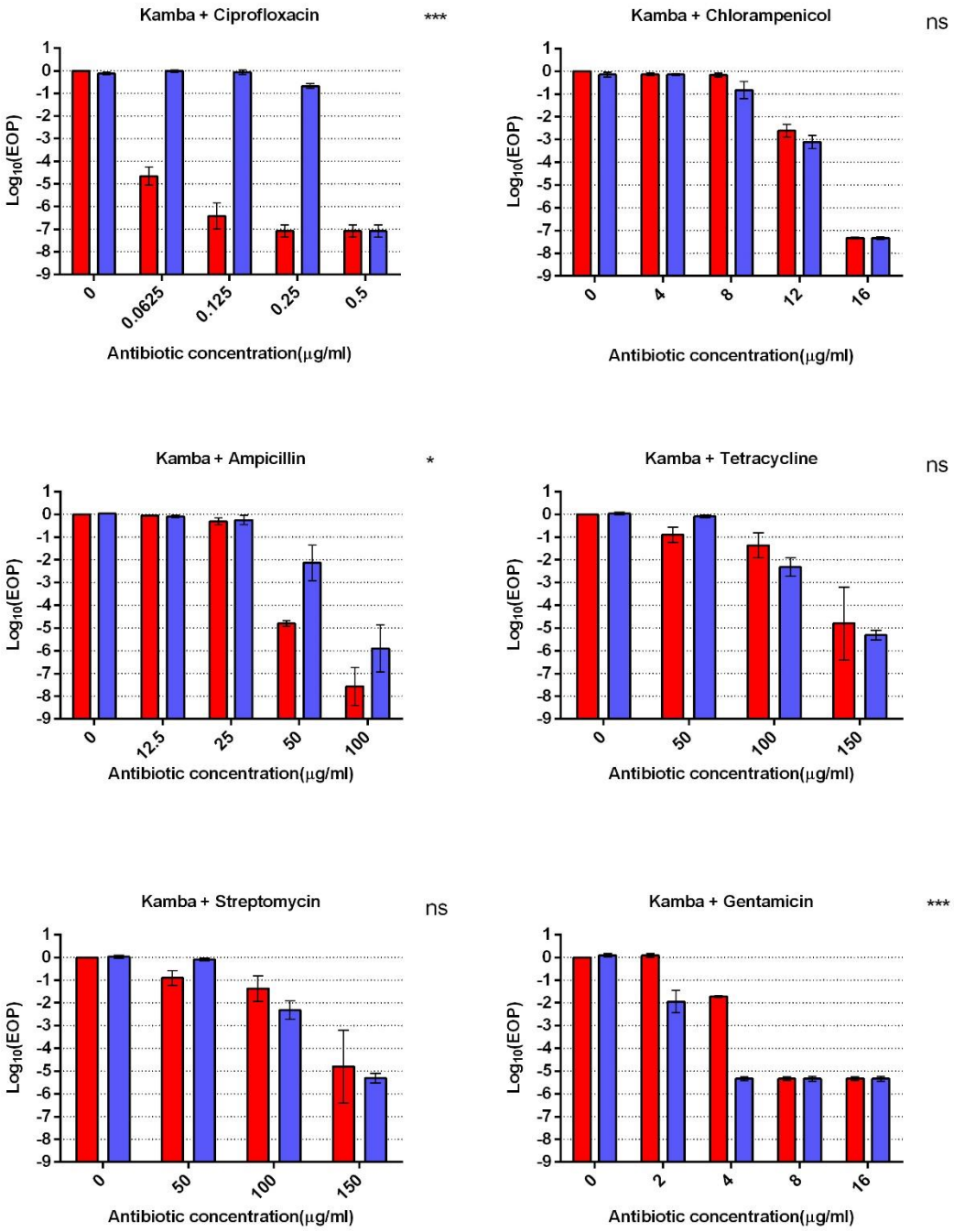
Figure 2.2 Effect of Number 8 on survival of *S. marcescens* on a range of antibiotics



Survival of *S. marcescens* on a range of concentrations of six antibiotics with (blue) and without (red) Number 8 (6670 ppm ae). Survival is reported as log-transformed EoP scores, error bars are SEM (n=3). Asterisks correspond to significance level: (ns) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

The Kamba formulation induced statistically significant responses in four of the six antibiotics tested (Figure 2.3). Similar to the Number 8 formulation, chloramphenicol ($p = 0.2374$) and tetracycline ($p = 0.554$) were the two antibiotics where the addition of Kamba had no statistically significant effect on growth or MIC (Table 2.2). Kamba increased EoP on ciprofloxacin ($p = 1 \times 10^{-11}$) and ampicillin ($p = 0.0385$), resulting in 8-fold and 2-fold increase in MIC respectively (Table 2.2). Statistical analysis showed significance on Kamba-streptomycin combination ($p = 2.45 \times 10^{-4}$), but the change in streptomycin MIC was below 2-fold and fell out of the range of detection. Meanwhile, Kamba decreased EoP of *S. marcescens* on gentamicin ($p = 7.07 \times 10^{-10}$), corresponding to half of original MIC (Table 2.2).

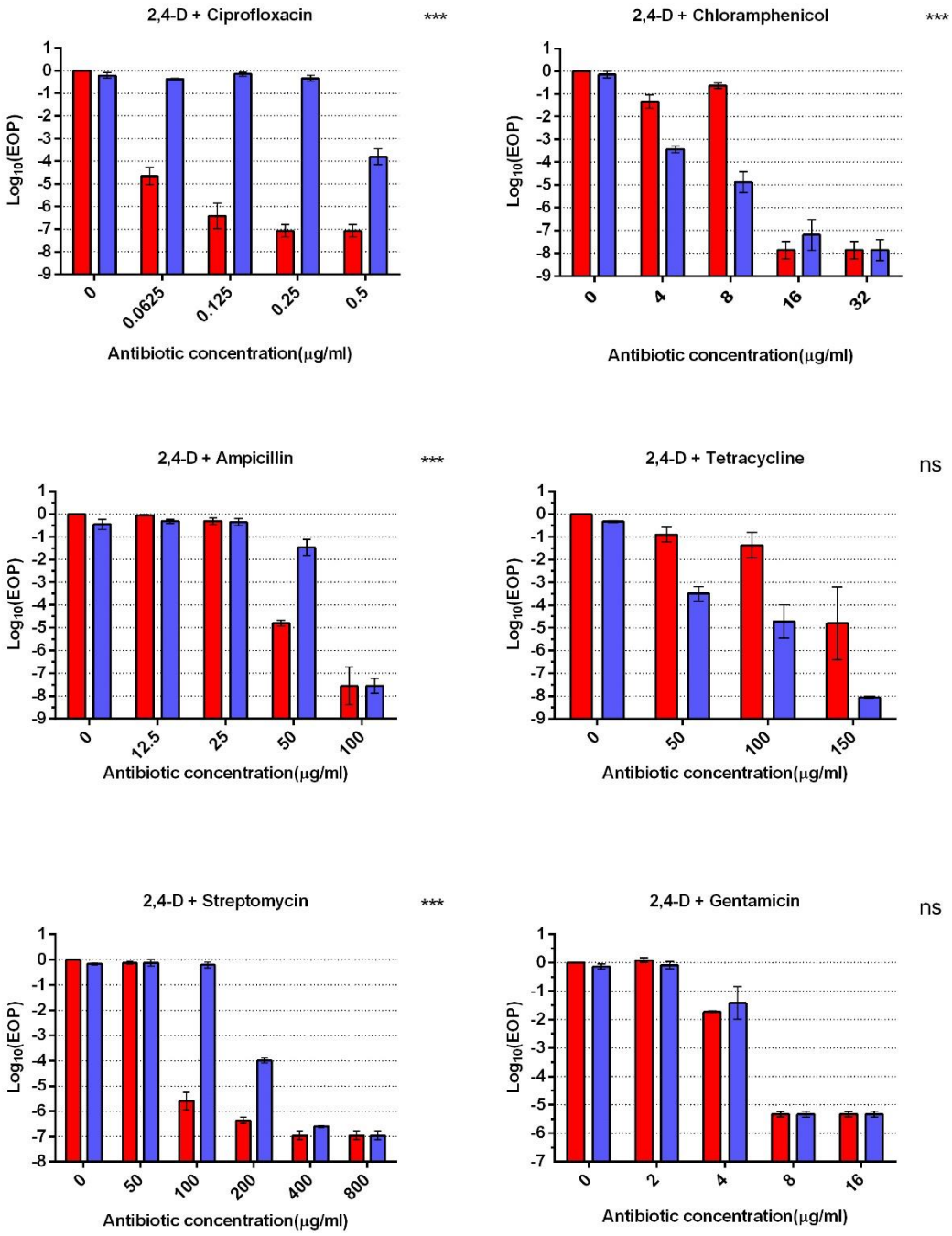
Figure 2.3 Effect of Kamba on survival of *S. marcescens* on a range of antibiotics



Survival of *S. marcescens* on a range of concentrations of six antibiotics with (blue) and without (red) Kamba (5200 ppm ae). Survival is reported as log-transformed EoP scores, error bars are SEM (n=3). Asterisks correspond to significance level: (ns) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

The 2,4-D formulation induced statistically significant responses in five of the six antibiotics tested (Figure 2.4). For this herbicide, gentamicin ($p = 0.757$) was the only one antibiotic where the addition of 2,4-D had no statistically significant effect on growth or MIC (Table 2.2). 2,4-D increased EoP of *S. marcescens* on ciprofloxacin ($p = 4.34 \times 10^{-10}$), ampicillin ($p = 9.88 \times 10^{-6}$), and streptomycin ($p = 1 \times 10^{-15}$) which corresponded to 8-fold, 2-fold, and 2-fold increase in MIC, respectively (Table 2.2). Meanwhile, 2,4-D decreased EoP of *S. marcescens* on chloramphenicol ($p = 1.79 \times 10^{-6}$), corresponding to half the original MIC (Table 2.2). The effect of the 2,4-D - tetracycline combination was not statistically significant ($p = 0.12954$), but a decrease in EoP was consistently observed.

Figure 2.4 Effect of 2,4-D on survival of *S. marcescens* on a range of antibiotics



Survival of *S. marcescens* on a range of concentrations of six antibiotics with (blue) and without (red) 2,4-D (3320 ppm ae). Survival is reported as log-transformed EoP scores, error bars are SEM (n=3). Asterisks correspond to significance level: (ns) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

2.3.3 Antibiotic MIC and effects of herbicides on other strains of *S. marcescens*

The DB10 and DB11 strains have the same herbicide MIC as B76 (Table 2.1). However, the DB10 strain is different in other ways (Table 2.4). DB10 is susceptible to ciprofloxacin and gentamicin like B76 strain, but the MICs are both one category higher. DB10 is also susceptible to streptomycin and ampicillin, while B76 is resistant to them. DB10 has a lower MIC for tetracycline, but still within the resistant level.

The tested effects of herbicides on antibiotic MIC of DB10 are mostly similar to those of B76 (Figure 2.5). All three herbicides increased ciprofloxacin MIC of DB10 with the same factor as observed in B76 strain ($p < 2 \times 10^{-16}$ for all three). Herbicide Number 8 increased streptomycin MIC from susceptible to highly resistant (16-fold increase in MIC, $p < 2 \times 10^{-16}$). The herbicide Kamba also doubled the MIC ($p = 7.07 \times 10^{-10}$) of gentamicin for both strains. Number 8 decreased ampicillin MIC of DB10 by half ($p = 7.6 \times 10^{-12}$), while it lowered ampicillin MIC of B76 to a quarter of the original MIC. One exception was the 2,4-D and tetracycline combination. The herbicide 2,4-D brought tetracycline of B76 MIC down to 1/3 of the original MIC, while it increased the MIC of DB10 up to double the original MIC ($p = 6.35 \times 10^{-5}$).

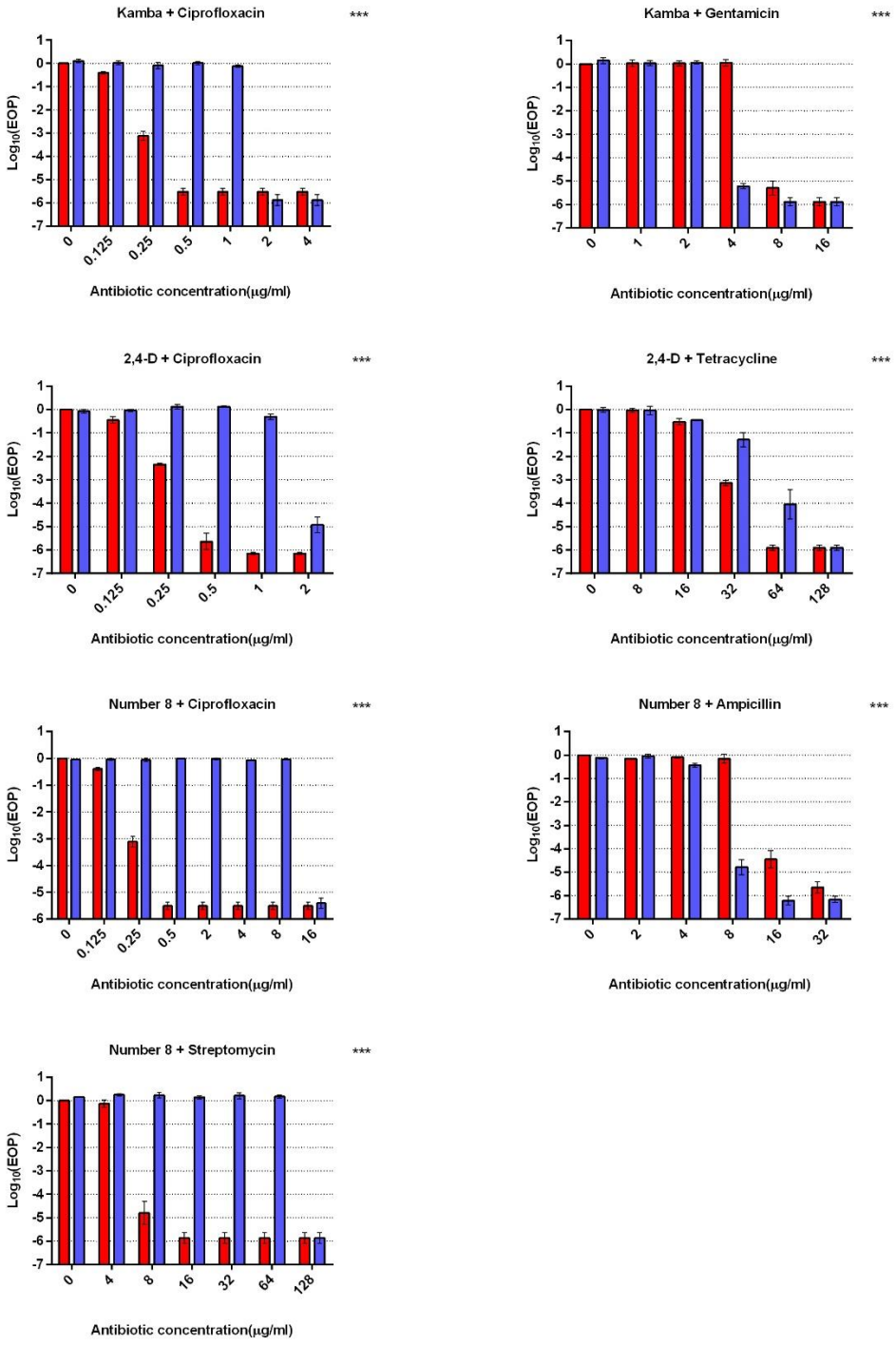
DB11 is described as a streptomycin resistant mutant of DB10. It was verified to be resistant to streptomycin with MIC higher than 1000µg/ml. None of the three herbicides decreased the MIC of streptomycin to or below 1000µg/ml.

Table 2.4 MIC table of *S. marcescens* DB10.

Antibiotics	**MIC (µg/ml)**
Ampicillin	≤16
Streptomycin	≤8
Tetracycline	≤32
Ciprofloxacin	≤0.25
Gentamicin	≤8

Working antibiotic MIC of *S. marcescens* DB10

Figure 2.5 Effect of herbicide and antibiotic combinations on survival of *S. marcescens* DB10



Survival of *S. marcescens* on a range of concentrations of six antibiotics with (blue) and without (red) herbicide. Survival is reported as log-transformed EoP scores, error bars are SEM (n=3). Asterisks correspond to significance level: (ns) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

2.4 Discussion

Herbicides are used in large quantities in urban, rural and conservation environments. Their effects on pathogens of people has been reported previously by my research group. I sought to extend the analysis to pathogens of other species and chose *S. marcescens* because it is a pathogen of an important plant pollinator and can be an opportunistic pathogen of humans. This chapter show that such effect occurs to *S. marcescens*.

2.4.1 MIC of herbicides and antibiotics

S. marcescens is less susceptible to the antimicrobial effects of glyphosate herbicides than reported for *E. coli* and *S. enterica* (Hill, 2017; Kurenbach et al., 2017). This is noteworthy because both *E. coli* and *S. marcescens* possess an EPSPS with the same susceptibility to glyphosate (Schulz et al., 1985). EPSPS inhibition by glyphosate may not be a reliable indicator of MIC because, for example, *S. aureus* is highly susceptible to glyphosate but its EPSPS is insensitive to glyphosate (Hill, 2017; Priestman, Funke, Singh, Crupper, & Schönbrunn, 2005).

I have tested two other formulations of glyphosate under the brand Roundup Weedkiller. The MIC of each formulation was the same (13340 ppm ae). This indicates that toxicity of glyphosate-based herbicide to bacteria is dependent on mechanisms other than EPSPS inhibition. As discussed in previous studies, common co-formulants in herbicides such as carboxymethyl cellulose (CMC) and Tween80 affect antibiotic resistance of bacteria as the active ingredients do (Kurenbach et al., 2017). How the active ingredients and co-formulants interact with each other has been discussed, but it was not tested. This is one possible direction for future research.

Compared with published figures for *E. coli* and *S. enterica* (Kurenbach et al.,

2015), *S. marcescens* was more tolerant of 2,4-D and less tolerant of Kamba. The same trend was also observed for *S. marcescens* compared to *S. aureus* (Hill, 2017). Currently it is unclear how 2,4-D or Kamba alone are toxic to bacteria because there is no study on this topic. Considering that older herbicides such as dicamba are being used again due to emergence of glyphosate-resistant weeds (Beckie, 2011), it is important to resume study on these old herbicides to determine the biochemical causes of toxicity.

In previous studies published by my research group, strains with no resistance to the tested antibiotics were used because resistance mechanisms might affect the herbicide-by-antibiotic interaction (Jun et al., 2019; Kurenbach et al., 2017; Kurenbach et al., 2015). Available to me at the time I began my thesis research was only a strain of *S. marcescens* (B76) that was a multi-drug resistant. It is resistant to high concentrations of ampicillin, tetracycline and streptomycin. Fortunately, later I was able to acquire DB10 which is either classed as susceptible or was susceptible to much lower concentrations of the same antibiotics. For reasons of time constraints, not all the experiments done with B76 were also done with DB10. As both are the same species, the use of DB10 helps to confirm the effects of herbicides observed with B76 (see 2.4.3).

2.4.2 Herbicides change antibiotic response of *S. marcescens* B76

Effects of herbicides on antibiotic resistance varied depending on the herbicide-antibiotic combination. Increases in ciprofloxacin MICs were observed from exposure to each of the three herbicides, similar to what has been observed with other bacteria so far tested (Hill, 2017; Kurenbach et al., 2018; Kurenbach et al., 2015). The greatest increase in MIC of an antibiotic was also observed for ciprofloxacin. It was a 64-fold increase upon exposure to Number 8. The second greatest increase in MIC was to the gentamicin concentration. It was a 32-fold increase upon exposure to Number 8. This indicates that Number 8 potentially facilitates high levels of *S. marcescens* resistance to ciprofloxacin and gentamicin. Considering that ciprofloxacin and gentamicin are

important antibiotics for in clinical treatment of *S. marcescens* implant or transplant infections, *S. marcescens* with resistance to these two antibiotics would be a concern (Mouat et al., 2018).

In two cases, herbicides increased antibiotic MICs by smaller factors. Kamba doubled the ampicillin MIC and 2,4-D doubled the streptomycin MIC. Because B76 is already resistant to ampicillin and streptomycin, the increase in MIC to these antibiotics would unlikely affect clinical outcomes. However, for a strain with an MIC not far below the clinical breakpoint, a doubled MIC can be the difference between clinically treatable and untreatable.

Decreases in MICs were observed in some of the herbicide-antibiotic combinations. In a few cases, herbicides reduced antibiotic MIC from resistant to susceptible. While at first this might appear to be a benefit of herbicide exposure, drawing such conclusions would be naïve. Strains with greater sensitivity to antibiotics are prone to evolve into resistant populations at lower concentrations of the antibiotic, concentrations much more common in the environment than in the hospital (Hill, 2017).

While both Kamba and 2,4-D are based on a synthetic auxin, their effects on antibiotic MIC in *S. marcescens* are usually different. This phenomenon is also observed in previous studies, but not as frequent (Hill, 2017; Kurenbach et al., 2015). This phenomenon could be explained by the two herbicides interacting with antibiotic resistance mechanism differently due to difference in their chemical structure. It could also be the difference in co-formulant in two herbicides.

2.4.3 Comparison of herbicide effects on different strains of *S. marcescens*

As discussed above, I did not test all herbicide-antibiotic combinations on DB10. Instead, I selected a range of representative and significant combinations. Among these

were ones that caused an increase or decrease in antibiotic MIC, and combinations that involved antibiotics to which DB10 is susceptible but B76 is not. Most of the results on DB10 were consistent with results observed using B76. This indicate that the effects of herbicides were not strain-specific in this case. One exception was that 2,4-D decreased the tetracycline MIC of B76 while it increased tetracycline MIC of DB10. Considering that neither strain was fully susceptible to tetracycline but that each tolerated different maximum concentrations, the different results indicate that the response to 2,4-D may differ due to possibly different biochemical resistance mechanisms in these strains.

Three mechanisms can be responsible for tetracycline resistance: tetracycline efflux pump, ribosomal protection protein, and tetracycline inactivation enzyme (Marilyn C. Roberts, 2005). All of the three mechanisms can be found in *Serratia* species (L. Li, Ye, Zhang, & Meng, 2016). For further study, genome sequencing will be necessary to identify what tetracycline resistance genes are present in *S. marcescens* B76 and DB10. Afterwards, studies can be carried out using knock-out strains, specific inhibitor or transcriptome analysis to identify which gene is responsible for the different tetracycline responses.

Chapter Three

Investigating mechanisms of herbicide-induced antibiotic responses

3.1 Introduction

As seen from the results in chapter two, it is confirmed that herbicide exposures can cause *S. marcescens* to respond differently to antibiotics. Depending on the specific combination of antibiotic and herbicide, one of three responses was observed: either an increase, decrease, or no change in the MIC of the antibiotic.

Using *E. coli* as the test organism, the effect of herbicide exposure was found to be initially phenotypic rather than a selection of particular genotypes in the exposed population (Kurenbach et al., 2015). The effect was therefore reversible in the entire population when the herbicide was removed and did not require the outgrowth of genotypically susceptible strains to replace the resistant population. Use of gene knock-out strains also showed that the herbicide induced transcription of efflux pumps (Kurenbach et al., 2017). Moreover, the increased MIC of antibiotics after herbicide exposure returned to normal when the bacteria were exposed to PAβN (phenylalanine-arginine beta-naphthylamide), an efflux pump inhibitor (Kurenbach et al., 2015). Taken together, the results indicate that the initial effect is adaptive: herbicides induce toxin-response pathways in exposed bacteria and this causes a cross-resistance to antibiotics too. Here I intended to test if the antibiotic resistance induced by herbicides were adaptive resistance that can be reversed by removing the herbicides or acquired resistance that cannot revert by simply removing the herbicides.

To test the hypothesis that herbicides cause an adaptive response in *S. marcescens*, I mainly used ciprofloxacin because exposure to all three kinds of herbicides increased *S. marcescens*' resistance to ciprofloxacin, which was true for other bacteria tested in

previous studies as well (Kurenbach et al., 2017; Kurenbach et al., 2015). Taking these studies into consideration, ciprofloxacin resistance is the only common reaction observed from different bacteria species after exposure to the three herbicide formulations.

I also tested if the mutation rate was affected by the herbicides. I used rifampicin as the selection marker for mutants. Rifampicin resistance is caused by a single mutation in *rpo*B, making it a convenient marker to observe changes in mutation rates (Hill, 2017; Miller et al., 1999). Change in frequency of rifampicin resistant mutant reflects change in mutation rate.

3.2 Methods

3.2.1 Bacteria strains, culture conditions and chemicals

S. marcescens B76 was used in this study. The bacteria were stored and grown as described in 2.2.1.

Rifampicin (Duchefa Biochemie, Netherlands), ciprofloxacin and the three commercial herbicides formulations (described in 2.2.1) were used in the following experiments.

3.2.2 Mutagenicity of herbicides

Twelve independent cultures of *S. marcescens* B76 were inoculated from single colonies mixed in 10 mL LB medium. Three of the twelve cultures contained Number 8 (6670 ppm ae), another three contained Kamba (5200 ppm ae), and another three contained 2,4-D (3320 ppm ae). Cultures were incubated at 37°C with rotation providing aeration until their OD_{600} reached approximately 1.0. LB agar plates supplemented with 100 μg/mL rifampicin (Sigma-Aldrich, USA) were inoculated with a 100 μl of each culture, resulting in a 10-fold dilution. In order to determine the starting density of the culture (in cfu/ml), a titre of the culture on LB agar was performed for each saturated culture using the drop plate method as described in 2.2. Plates were incubated at 37°C for 16 hours before colonies were counted. The frequency of rifampicin resistant mutants was then compared between LB only and LB plus herbicide treatments. This assay was adapted from Miller et al (Miller et al., 1999).

3.2.3 Antibiotic response of *S. marcescens* after herbicide withdraw

As described in 2.2.2, a single *S. marcescens* B76 colony was transferred to liquid LB medium and then incubated with aeration at 37°C until the culture reached an optical density (OD_{600nm}) of ~1. Then the cultures were diluted by a factor of 10^6 and 100 μl of each culture was plated on LB medium supplemented with ciprofloxacin and one of the three herbicides. The combinations are 4 ug/ml ciprofloxacin plus Number 8 (6670 ppm ae), 0.25 ug/ml ciprofloxacin plus Kamba (5200 ppm ae), and 0.25 ug/ml ciprofloxacin plus 2,4-D (3320 ppm ae). After around 16 hours of 37°C incubation, the colonies were counted, and the plates were replicated using sterile velvet fixed on a block onto plates supplemented with the same amount of ciprofloxacin but without herbicides. Then the replicas were incubated for up to four days. Surviving colonies were counted and ratio of survival was calculated. Up to ten surviving colonies were also transferred to plates containing ciprofloxacin at the same original concentration to verify if they were resistant. A replication onto plates containing only LB was made at the same time to ensure the technique of transfer has a high success rate.

3.2.4 Statistical analysis

R was used for all statistical analysis (R Core Team, 2019). For the mutagenicity test described in 3.2.2, a two-tailed t-test was used to determine if there was any significant difference in the frequency of rifampicin resistant mutants between the group with LB only and groups with herbicide treatment. For result where the number of mutant was below detection limit (one colony per plate), the value of detection limit was filled in for statistical analysis. Figures are created using Graphpad Prism 6.

3.3 Results

3.3.1 Herbicides were not mutagenic

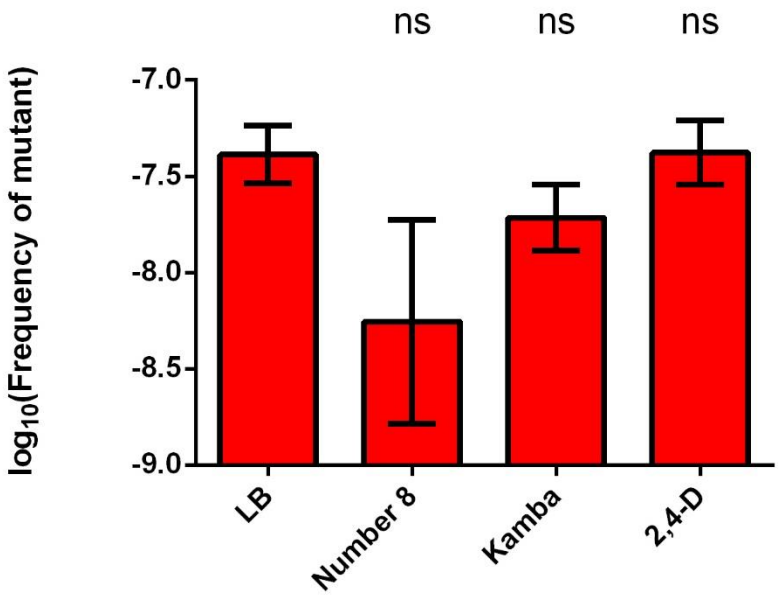
As shown in Table 3.1, exposure to Kamba consistently lowered the number of mutants to below the detection limit (10 cfu/ml). For the group with no herbicide exposure? or the group exposed to 2,4-D, the mutation frequency was either at or below the detection limit. For Number 8 treatment, the number was consistently at or above the detection limit. There was no statistical difference between each group (Figure 3.1).

Table 3.1 *S. marcescens* rifampicin resistant mutants

Group	Mutant count (cfu/ml)	Frequency
LB	10	2.94×10^{-8}
	≤10	4.00×10^{-8}
	≤10	5.88×10^{-8}
Number 8	70	2.26×10^{-8}
	10	2.44×10^{-9}
	10	3.13×10^{-9}
Kamba	≤10	2.70×10^{-8}
	≤10	1.25×10^{-8}
	≤10	2.13×10^{-8}
2,4-D	≤10	5.88×10^{-8}
	≤10	4.55×10^{-8}
	10	2.78×10^{-8}

Colony count and frequency of mutants. For colony count below detection limit (10 cfu/ml), 10 cfu/ml was used to calculate frequency.

Figure 3.1 Frequency of *S. marcescens* rifampicin resistant mutants



Rifampicin resistant mutant of *S. marcescens* B76 raised from three different herbicides. Frequency is reported as the log-transformed score. In the cases when mutant is below detection limit (10 cfu/ml), 10 cfu/ml was used in calculation and statistical analysis. Error bars are SEM (n=3).

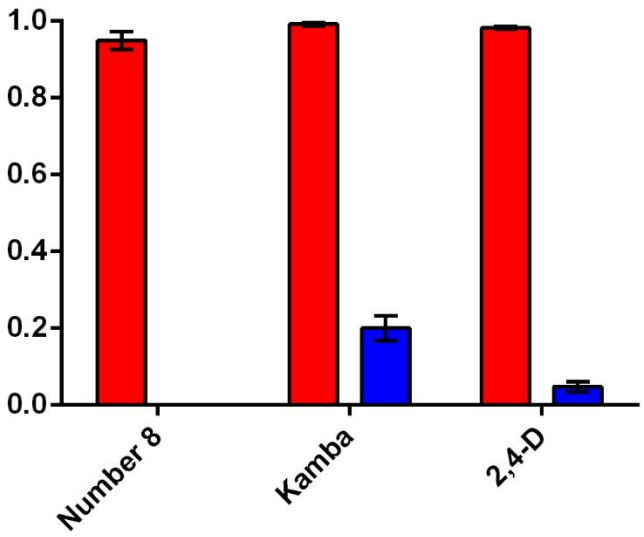
ns: not significant

3.3.2 Ciprofloxacin resistance induced by herbicide exposure was adaptive

As described in 3.2.3, colonies growing on solid medium supplemented with both a herbicide and ciprofloxacin were transferred to a plate with a solid medium supplemented only with the same concentration of ciprofloxacin. If the herbicide induced an adaptive (physiological) change in susceptibility, it was expected that no colonies would transfer and grow on the replica plate. However, if the herbicide was selecting mutants pre-adapted to ciprofloxacin, the colonies would transfer bacteria able to establish new colonies on the replica plate at the frequency of the mutation. To control against false negative results due to a failure to adequately imprint the replica, the replica with ciprofloxacin was followed by a replica on LB plate. If all colonies grew on the second imprinted plate, then biomass was transferred to both replica plates.

As shown in Figure 3.2, the transfer rates were close to 1, which indicated that the transfer was efficient. Nevertheless, no colony treated with Number 8 survived after transferring to solid media supplemented with only ciprofloxacin. This indicated that the ciprofloxacin resistance phenotype induced by Number 8 required persistent presence of Number 8. Withdrawal of Number 8 uniformly restored susceptibility. However, for the Kamba and 2,4-D treatment, some colonies survived on the replica plate with medium supplemented with only ciprofloxacin. Up to ten colonies were from these plates were chosen at random to streak onto plates containing the same amount of ciprofloxacin. This was done to verify if they actually retained elevated tolerance to ciprofloxacin or were false positives. On average, 75% and 77% of the colonies survived the second transfer, respectively. Thus, some were false positives while others appeared to be mutants.

Figure 3.2 Rate of transfer and survival on ciprofloxacin plates



Rate of surviving *S. marcescens* colonies on LB plates (red) compared with original herbicide treatment plates and rate of surviving colonies on ciprofloxacin plates (blue). Error bars are SEM (n=3).

3.4 Discussion

For this chapter, I have performed experiments for initial diagnosis of the mechanisms by which herbicides induce *S. marcescens* ciprofloxacin resistance. In addition, I tested if herbicides were mutagenic at the concentrations and exposure times relevant to my experiments. These experiments provide some insight about how herbicide could affect bacteria under the real-life circumstances.

3.4.1 Herbicides did not promote mutation of *S. marcescens*

Previous studies showed that at concentrations used in these experiments, herbicides do not promote mutation of other bacteria (Hill, 2017). The results in this study agree with previous studies. Three commercial herbicide formulations did not promote mutation of *S. marcescens* at the tested concentrations. Considering the results are mostly at or below the detection limit, the conclusion could be incomplete. Further study is required to test if these herbicides have no effect on mutation rate or if indeed they may suppress the mutation rate.

3.4.2 Ciprofloxacin resistance induced by herbicide exposure was adaptive

The effects of all three herbicides on ciprofloxacin resistance was resistance that was not sustained for populations of bacteria when the herbicides were withdrawn. Most of the *S. marcescens* colonies from herbicide plus ciprofloxacin treatment did not survive after they were transferred to plates containing only the same amount of ciprofloxacin. For the group with Number 8 treatment, none of the colonies survived 4 μg/ml of ciprofloxacin. Due to time limit, I did not test if the MIC dropped from 4 μg/ml to the original susceptible level (0.125 μg/ml) after Number 8 withdraw or a number between the two. However, these results still showed that the heightened ciprofloxacin resistance requires ongoing exposure to Number 8.

For *S. marcescens* treated by Kamba or 2,4-D, most colonies of bacteria were unable to found new colonies when transferred to medium with only ciprofloxacin. Therefore, again, the effect of the herbicide was adaptive. However, a small portion of colonies survived after they were transferred on ciprofloxacin (0.25 µg/ml) medium. Most of these surviving colonies were verified to have increased ciprofloxacin MIC that was acquired. I did not test the actual MIC of those colonies due to time limit, so it is unknown if they were classified as resistant by CLSI standard (CLSI, 2019). However, the results show that *S. marcescens* that were exposed to Kamba or 2,4-D in combination with ciprofloxacin could produce some colonies with higher tolerance by both adaptation and mutation. It is important to know if exposure to the herbicide alone could produce the same effect. This was not the case in previous studies (Kurenbach et al., 2018).

Chapter Four

Future directions

4.1 Further experiments

In this chapter I will describe how the work I did would inform future work.

4.1.1 More on herbicide effects on antibiotic response in *S. marcescens*

In this study, most of the antibiotics used were from different groups. The exceptions were streptomycin and gentamicin, both of which are aminoglycoside antibiotics. Number 8 increased MIC of both antibiotics, while Kamba and 2,4-D caused varied responses. Kamba had no effect on the streptomycin MIC but reduced the gentamicin MIC by half. 2,4-D doubled the streptomycin MIC while it had no effect on the gentamicin MIC. These observations indicate that one herbicide could have different effects on the same class of antibiotic. Bacteria can have different biochemical mechanisms for resistance to different antibiotics from the same group. For example, *Enterobacteriaceae* (including *E. coli* and *S. marcescens*) are known to produce β-lactamases, extended-spectrum β-lactamases (ESBL) or carbapenemase (Wilson & Torok, 2018). Each one of these enzyme has a different spectrum of activity against certain types of β-lactam class antibiotics. While results of herbicides on one antibiotic can be indicative for other members of the group, they are not necessarily the same. For that reason, it is useful to test how each herbicide affected the MIC of other antibiotics even if they are from the same group of tested antibiotics.

In this study, most of the experiments were conducted on B76, which is genotypically resistant to some antibiotics used in this study. Part of the experiments were conducted on DB10, and some of the results are different for the strains. If there

was more time, a full set of experiment should be conducted on DB10 strain so that I can potentially detect more differences between the two strains. If time allowed, testing on other *S. marcescens* strains like honeybee isolates and clinical isolates would be useful.

In previous studies, the minimum inducing concentration of the herbicide was determined (Kurenbach et al., 2015). This experiment would be a good addition to this study. It is especially useful for interpreting results involving Number 8 and Kamba, because the concentrations used in this study were higher than the concentration commonly used in field sprayer (Table 4.1). In addition, the most common pathway of herbicide exposure for insects like honeybees is through environmental residue, which usually has a far lower concentration than the spray (Motta, Raymann, & Moran, 2018). This test is needed to assess relevance to agricultural exposures.

Table 4.1 Range of herbicide application

Herbicide (active ingredient)	**Spray**	**Non-spray usage**	**This study**
Number 8 (glyphosate)	1340~4000	38110~266800	6670
Kamba (dicamba)	280~2500	-	5200
2,4-D (2,4-d)	5000~30000	-	3320

Concentrations of commercial herbicide formulation used in field application and in this study, calculated in parts per million acid equivalent (ppm ae) of the respective active ingredients. Values are rounded to the nearest 10 ppm ae. For Number 8, the values were calculated from label of Roundup weedkiller with the same concentration of active ingredient (Roundup, 2020). For 2,4-D, the values were calculated assuming the product is solved in 100~200L water per hectare (Agpro, 2020).

4.1.2 More study on the mechanisms

Previous studies on *E. coli* using PAβN showed that efflux pumps play a big part in the antibiotic response induced by herbicides (Kurenbach et al., 2015). Other studies suggest that efflux-pumps that can be inhibited by PAβN are important for resistance against fluoroquinolone antibiotics (e.g. ciprofloxacin) in *S. marcescens* (Begic & Worobec, 2007). Future studies using PAβN or other efflux-pump inhibitors would help to confirm the biochemical mechanism of adaptive resistance to ciprofloxacin (Kurenbach et al., 2015; Schumacher et al., 2006).

4.1.3 Long-term effects of herbicide

A short-term evolution of *E. coli* showed that herbicides promote selection of antibiotic resistant bacteria whether the herbicide increased or decreased the antibiotic MIC (Kurenbach et al., 2018). It would be meaningful to do the same experiments on *S. marcescens* to add more pieces of evidence in favour of or against the conclusion. It is also important to know if such effects on evolution occur in *S. marcescens* because *S. marcescens* is commonly found in the clinical environment as a multi-drug resistant pathogen (Iguchi et al., 2014).

As discussed in chapter one, honeybees could be a vector for *S. marcescens* to be exposed to both antibiotics and herbicides (Motta et al., 2018; Raymann et al., 2017). However, I did not test exposures in the environment. Are co-exposures frequent? How frequent would they need to be to cause the effects I observe in the laboratory? Answers to such questions are important to test if exposure to herbicides alone promotes emergence of antibiotic resistant bacteria using the same principle of the short-term evolution experiment.

4.2 Future study

This study provides more evidence on the danger of herbicides in facilitating antibiotic resistance. However, there are more questions waiting to be answered. Future studies are needed to build up the connection between this study, agricultural and clinical practice.

4.2.1 Synergetic effects of herbicides and other chemicals

Previous studies showed that Kamba and salicylic acid have additive effects on increasing chloramphenicol MIC in *S. Typhimurium* (Gibson, 2016). This indicate that different chemicals that induce antibiotic resistance may have synergy among each other. It is highly relevant to agriculture practice, because it is now common to use multiple herbicides at once. Dicamba is often used in addition to other herbicides for different occasions (Nufarm, 2020). Due to emergence of Roundup (glyphosate)-resistant weeds, glyphosate herbicides are also commonly supplemented with other herbicides (Beckie, 2011). The fact that bacteria in the environment can be exposed to multiple kinds of herbicides at once suggests that it is important to know if different herbicides have synergy in inducing antibiotic resistance.

In addition to herbicides, there are other chemicals in the environment, such as insecticides, surfactants, preservatives, plastics, and heavy metals (Karbalaei, Hanachi, Walker, & Cole, 2018; Kirchhof & de Gannes, 2013; Raymann, Motta, et al., 2018). Previous study in my lab group showed that some insecticides can induce tolerance to antibiotic (Jun et al., 2019). There are also studies showing that heavy metals in sublethal amount can induce antibiotic resistance in some bacteria (Chen et al., 2015). These environmental pollutants on their own are concerning enough in promoting antibiotic resistance. Alongside with herbicides, they can be more dangerous if they have additive effects. More studies are needed on this topic.

It is also worth noting that there are many vectors of various environmental chemicals and bacteria. Insects such as honeybees are one kind of vector for bacteria to be exposed to herbicides and antibiotics, which is the reason it is looked into in this study. Meanwhile, there exist other vectors that require attention. Micro plastics have been described as a vector for bacteria communities, which may improve the chance of horizontal gene transfer (Shen et al., 2019). It is problematic because transfer of antibiotic resistance genes is possible between *S. marcescens* and other species (Mo, Sunde, Ilag, Langsrud, & Heir, 2017). In addition, micro plastics can absorb chemicals such as herbicides and antibiotics on their surface, making them a pathway of exposure similar to honeybees (Zhang et al., 2019). Combining these two effects, micro plastics can potentially enhance the effects of herbicides on raising antibiotic resistant bacteria. For this reason, more research into micro plastic as vector is needed.

4.2.2 Effects of bacteria community

During this study, it is observed that effects of 2,4-D on ciprofloxacin resistance in *S. marcescens* were not consistent across different experiments. While using the drop plate method, *S. marcescens* colonies grew normally under combined 2,4-D and ciprofloxacin (up to 0.25 μg/ml) exposure. However, when *S. marcescens* from the same stock was spread on a full plate, fewer appeared to survive. This phenomenon was not observed when using other herbicides. These observations suggest that effects of some herbicides may be associated with community density. In different environments such as the inside of honeybee's gut or in the soil, the density of bacteria can vary. In addition, one bacteria species does not exist as a monoculture. Instead, bacteria of different species form communities and interact with each other (Chodkowski & Shade, 2017). Bacteria community plays a big part in the survival of bacteria within, including the promotion of biofilm formation and antibiotic resistance (Vega & Gore, 2014). It is possible that bacteria community affects how herbicide induce altered response to antibiotic in *S. marcescens*. More studies are needed on this topic in order to link the

lab observations to agricultural practice.

4.2.3 Effects of herbicides on other traits of bacteria

Antibiotic resistance is one important trait for pathogenic bacteria. However, other traits of pathogens cannot be ignored. Biofilm is an important trait in pathogens. It increases resistance against antibiotics, making a pathogen harder to treat (Hall & Mah, 2017). In previous studies, it was established that herbicides are toxic to bacteria, and they can trigger defence mechanisms of bacteria (Kurenbach et al., 2015). A study on *Pseudomonas aeruginosa* shows that glyphosate induced more biofilm formation (Lima, Baumeier, Rosa, Campelo, & Rosa, 2014). Considering *S. marcescens* has ability to form biofilms that make it resistant to antibiotic and non-antibiotic stress, it is possible that biofilm formation is involved in elevated antibiotic tolerance after herbicide exposure (Ray, Shenoy, Orihuela, & González-Juarbe, 2017).

4.3 Policy making

In New Zealand, antibiotic stewardship is described as optimising the use of antimicrobial medicines in human health, animal health and agriculture by maintaining and enhancing the regulation of animal and agriculture antimicrobials (Ministry of Health NZ, 2019). These plans involving governments and medical professionals are important for ongoing efficacy of antibiotics. However, current antibiotic stewardship plans only address usage of *antibiotics*. On the other hand, bacteria are exposed to various chemicals from human activities including but not limited to herbicides, insecticides, heavy metals and preservatives. These chemicals are not included in antibiotic stewardship plans because they are not considered antibiotics. However, they may undermine antibiotic stewardship plans by promoting antibiotic resistance regardless of antibiotic usage. Conclusions from this study along with previous studies are warnings that antibiotic stewardship should involve other part of the society such as agriculture and industry, and it should include non-antibiotic chemicals into

consideration.

Chapter Five

Summary

The work in this study expanded upon previous work on herbicides inducing a response to antibiotics (Jun et al., 2019; Kurenbach et al., 2018; Kurenbach et al., 2015). The range of tested species was expanded to include *S. marcescens*, a pathogen implicated in honeybee decline and an opportunistic infection of humans. Three herbicide formulations used in previous studies were tested along with six antibiotics from five class that are relevant to *S. marcescens*. In total, eighteen herbicide-antibiotic combinations were tested on B76 and seven of them were also tested on DB10. The mutagenicity of three herbicides was tested, and the question of whether the inducing effects of herbicides on antibiotic responses was adaptive was also tested.

As discussed extensively by governments, medical professionals and the general public, antibiotic resistance is a serious threat to human health (World Health Organization, 2018). If left unchecked, the world will likely go into a post-antibiotics era where antibiotics are no longer useful (World Health Organization, 2014). Efforts are being made to avoid that future through regulations on antibiotic usage (Ministry of Health NZ, 2019). However, previous studies in my lab group showed that a variety of chemicals can affect antibiotic tolerance of bacteria and subsequently promote emergence of antibiotic resistance (Jun et al., 2019; Kurenbach et al., 2017; Kurenbach et al., 2018). These studies suggest that chemicals such as herbicides can undermine the efforts made to combat antibiotic resistance. The purpose of this thesis was to further build on potential exposure pathways, in this case created by the treatment of honeybees with antibiotics.

In twelve out of eighteen herbicide-antibiotic combination, the herbicide either

increased or decreased MIC of antibiotics measured using *S. marcescens* B76, which could potentially promote evolution of antibiotic resistance according to previous studies (Kurenbach et al., 2018). Among those combinations, the glyphosate-based Number 8 formulation was especially effective in raising tolerance to ciprofloxacin and gentamicin, which could be highly dangerous in a clinical setting. Future studies are necessary to figure out how much herbicide is required to induce change in antibiotic response in *S. marcescens* so that an assessment of risk can be made in combination with other studies on environmental residues of these herbicides.

In this thesis, the mechanisms of how herbicides affect antibiotic response in *S. marcescens* were partially diagnosed. No mutagenic property was identified for any of the three herbicides. Physiological response was identified as the main mechanism, which probably involves efflux-pumps according to previous studies (Kurenbach et al., 2015). More studies are needed to further identify the exact mechanisms and assess the danger of herbicides promoting antibiotic resistance accordingly. Meanwhile, the results in this thesis should raise awareness of potential dangers of herbicides hampering the efforts to curtail antibiotic resistance. Actions need to be taken to facilitate further studies and to amend current plans of antibiotic stewardship accordingly.

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