MACQUARIE University
SYDNEY·AUSTRALIA

Sub-inhibitory Concentrations of Antibiotics and Bacterial Evolution

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This thesis is presented as a partial fulfilment to the requirements for the degree of Doctor of Philosophy

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May 2019

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Statement of Candidature

I certify that the work in this thesis entitled “Sub-inhibitory Concentrations of Antibiotics and Bacterial Evolution” has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie university.

I also certify that this is an original piece of research and it has been written by me. And help and assistance that I have received in my research work and the preparation of the thesis itself has been appropriately acknowledged.

In addition, I certify that all information sources and literature used are indicated in the thesis.

Louise Chow

May 2019

Acknowledgements

I wish to express my deep gratitude to my supervisor, Distinguished Professor Michael Gillings. His guidance helped me throughout my research and writing of this thesis. I could not imagine having a better advisor and mentor for my PhD.

Nothing would have been possible without the endless support, encouragement and understanding from my family; my parents Beverley and Alex Chow; my sister Jessica Hyland and my brother-in-law Luke Hyland.

I owe many thanks to the members of the EMMA lab and the biology department, Timothy Ghaly, Ryan Nevatte, Liette Vandine, Marita Holley, Sasha Tetu, Anahit Penesyan and Amy Asher.

I am forever grateful to my friends who provided constant encouragement throughout not only my studies, but over the many hurdles of the past few years, Timothy Ghaly, Mary Ghaly, Elizabeth Hagan-Lawson, Rachael Woods, Jessica Thompson, Jennalee Clark and my partner Philip Kelso. You have all been an endless source of laughter, inspiration and compassion.

This project was supported by Macquarie University and I express my gratitude to the university for giving me this opportunity.

Thesis Summary

Antibiotic resistance is one of the greatest threats to human health in the 21st century. It is predicted that by 2050 antibiotic resistant infections will account for 10 million deaths annually. The mechanisms by which therapeutic concentrations of antibiotics select for resistance mutations and the lateral transfer of resistance genes is well known. However, the effect of lower concentrations of antibiotics, particularly when these are environmental contaminants, is relatively unknown. Antibiotics can enter the environment through human waste streams, agricultural run-off and pharmaceutical effluent. They can then persist in the environment at low concentrations. These low levels of antibiotics can induce the SOS response, a general response to DNA damage. Amongst the various effects of the SOS response are an increase in mutation rates driven by expression of error prone DNA polymerases, and a general increase in rates of recombination, transposition, conjugation and transformation. All these effects increase the likelihood of cells becoming antibiotic resistant.

In this thesis, I collated data on the concentrations of clinically relevant antibiotics that have been reported from diverse environmental compartments. I then used these findings to design experiments that simulated the likely concentrations experienced by environmental bacteria. Once the environmental concentrations of antibiotics had been established, I carried out experimental evolution experiments, exposing bacteria to appropriate concentrations, by performing serial plating across multiple generations. Concentrations of antibiotics equivalent to 1/10 the minimum inhibitory concentrations promoted resistance after as little as 15 single colony passages on media. To identify the mechanisms of resistance, whole genome sequencing was performed. Point mutations were identified in relevant genes from all the lines with increased resistance. In ciprofloxacin treated lines, the relevant mutation occurred in *gyrA,* and was identical to a resistance mutation described in clinical pathogens. In

kanamycin treated lines, a point mutation in *fusA* was detected. Again, this mutation and gene have previously been implicated in kanamycin resistance. To determine the role of the SOS response in the fixation of these mutations I performed similar experiments using RecA knockout mutants.

Most environmental bacteria are not planktonic cells, but grow in biofilms. Consequently, to better mimic the likely effects of antibiotic pollution on environmental bacteria, I investigated the effects of environmental concentrations of antibiotics on biofilm bacteria. To study biofilms, I first had to modify protocols used for liquid and plate experiments, since biofilms display significantly higher resistance to antibiotics compared to their planktonic counterparts. This resulted in a novel method to determine the minimal inhibitory concentration of antibiotics in biofilms. I then exposed bacterial biofilms to environmentally relevant concentrations of antibiotics and using whole genome sequencing identified point mutations known to be associated with antibiotic resistance.

The results of this work have clear implications. Antibiotics persist in the environment at low, but biologically relevant concentrations where they can have significant impacts on normal microbial processes. These low concentrations up-regulate mutation rates and generate increasing bacterial resistance to antibiotics amongst all bacteria, not just those of clinical concern. We expect that the phenomena I describe under experimental conditions are mirrored in the general environment. Acquisition of resistance is essentially stochastic, relying on rare events at a single point in time, coincident with relevant selection pressures that allow newly resistant lineages to compete and increase in abundance. Widespread pollution with antibiotics enhances the rates at which key mutational events are likely to occur, while simultaneously providing the selection regime to promote survival of newly resistant cells. The potential is clear for environmental organisms to acquire resistance, which could then be disseminated globally through horizontal gene transfer or become significant

pathogens in their own right. Antibiotic pollution joins overuse and misuse as a significant threat to human health and the preservation of the efficacy of antibiotics.

Abbreviations

Abbreviations	Definition
AMX	Amoxicillin
AZM	Azithromycin
CEX	Cefalexin
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLI	Clindamycin
CLR	Clarithromycin
CTC	Chlortetracycline
DDD	Defined daily dose
DNA	Deoxyribonucleic acid
DOX	Doxycycline
ENO	Enoxacin
ENR	Enrofloxacin
ERY	Erythromycin
ERY-H20	Erythromycin-H20
FFC	Florfenicol
FLU	Flumequine
HPLC	High Performance Liquid Chromatography
LB	Lysogeny broth
LIN	Lincomycin
LMX	Lomefloxacin
LVX	Levofloxacin
MIC	Minimum inhibitory concentration
MSC	Minimum selective concentration
NAL	Nalidixic acid
NAR	Narasin
NOR	Norfloxacin
OFX	Ofloxacin
OTC	Oxytetracycline
OXA	Oxolinic acid
PCR	Polymerase Chain Reaction
PCU	Population corrected unit
PEF	Pefloxacin
PEN	Penicillin
PNEC	Predicted no effect concentration
QRDR	Quinolone-resistance determining region
ROX	Roxithromycin
SAR	Sarafloxacin
SDI	sulfadimidine
SDM	Sulfadimethoxine
SDZ	Sulfadiazine
SFMr	Sulfamerazine
SMP	Sulfamethoxypyridazine
SMT	Sulfamethazine

SMX	Sulfamethoxazole
SP	sulfapyridine
SPI	Spiramycin
SQX	sulfaquinoxaline
SZ	Sulfathiazole
TAP	Thiamphenicol
TET	Tetracycline
TMP	Trimethoprim
TYL	Tylosin
UV	Ultra Violet
VAN	Vancomycin
VIR	Virginiamycin
WHO	World Health Organisation

Chapter 1: Introduction

This chapter examines the problem of antibiotic resistance and the threat it poses to human health. The use of antibiotics themselves also poses a threat to the environment, since antibiotics are excreted from humans and animals undergoing therapy. This unintentional release is only beginning to be explored, with the fundamental dynamics of release and half-life being largely unknown. Finally, the chapter highlights the gaps in knowledge surrounding this area and the main questions that need to be addressed.

The problem of antibiotic resistance

The discovery of antibiotics in the 20th century revolutionised medicine. Not only could once fatal infections be treated, but procedures that were previously too risky to perform due to the risk of infection could now be safely carried out with the prophylactic use of antibiotics. Unfortunately, bacterial evolution, often driven by the over-use and incorrect use of antibiotics has resulted in the generation of antibiotic resistant bacterial strains. Approximately 70% of nosocomial infections are resistant to at least one type of antibiotic (Zhang et al., 2011) and this proportion is expected to increase as bacteria acquire resistance genes that confer increasingly higher levels of resistance to multiple classes of antimicrobials. The efficacy of antibiotics is being compromised, and many people are concerned we could be entering a post-antibiotic era (Alanis, 2005).

Antibiotic resistance is one of the greatest threats to human health for the 21st century. It is estimated that by 2050, antibiotic resistant infections will account for 10 million deaths per year globally, compared to the current annual death toll of 700,000. This will eclipse cancer as the number one cause of death (Figure 1) (O'Neill, 2014, WHO, 2014).

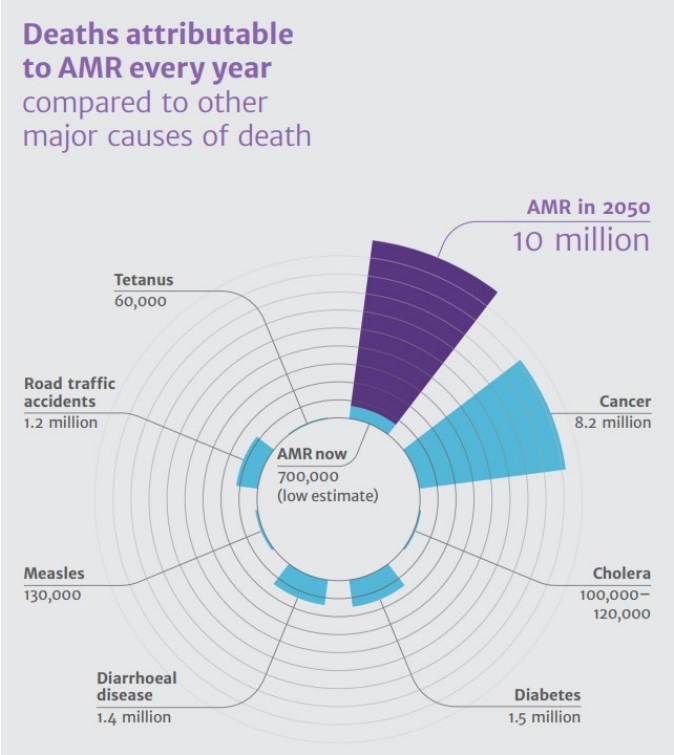

Figure 1: Estimates of annual deaths due to various causes. Antibiotic resistant infections currently account for 700,000 deaths annually. It is predicted that this will rise to 10 million deaths annually by 2050. Figure from O'Neill 2014

Antibiotic resistant infections will also place a significant burden on health services and hospitals, and this burden will be most felt in low income countries. The impact of antibiotic resistance is expected to have serious effects on the world's economy, on a level equivalent to the global financial crisis of 2008, and could see an extra 28 million people fall into poverty (Adeyi, 2017). While some countries have employed antibiotic monitoring systems and regulations around antibiotic use, it is clear this is not enough to control resistance. Antibiotic use increased 65% between 2000 and 2015, expressed as defined daily doses. Based on this trajectory, antibiotic use is expected to increase 200% from 2018 to 2030 (Klein et al., 2018). Much of this growth was driven by low and middle-income countries (Figure 2)(Klein et al., 2018).

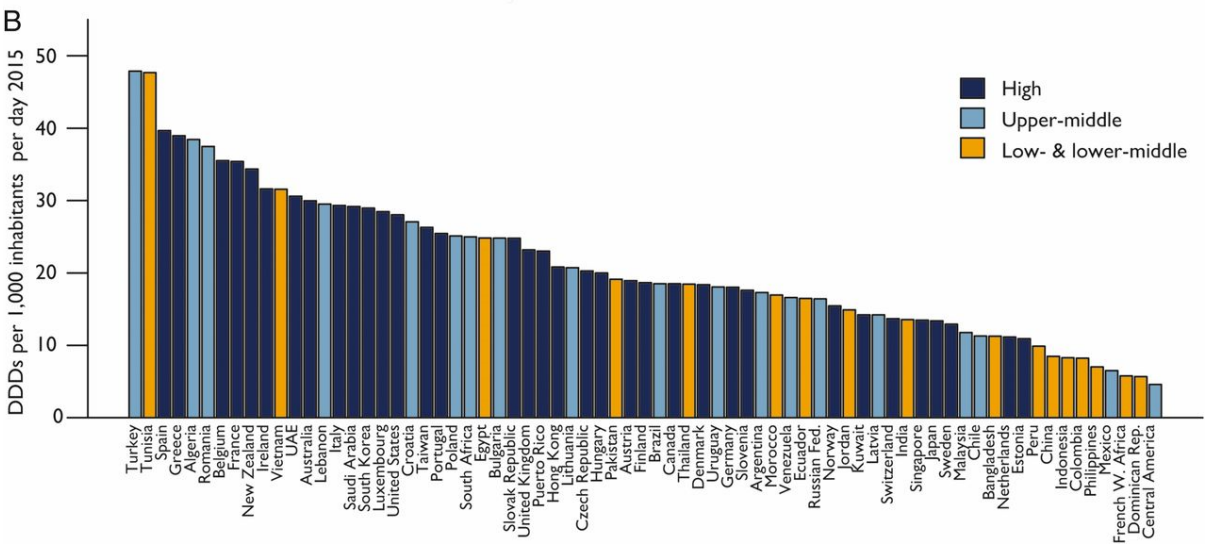

Figure 2: Daily doses of antibiotics per day in different countries. There is significant variation in antibiotic use between countries. In 2015, high income countries generally used more antibiotics per inhabitant per day when compared to low income countries, although there are exceptions to this trend. Figure from Klein et al. 2018

Of further concern is the increase in use of last-resort antibiotics. Last-resort antibiotics should be reserved for when all other appropriate antibiotics have been tried. Unfortunately, there are several strains of bacteria that are resistant to all known antibiotics, including last-resort antibiotics. These include pan-drug resistant *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Falagas and Bliziotis, 2007, Fernandes et al., 2016, Souli et al., 2008). This is partly due to the fact that there has been little research into new antimicrobials and no new classes of antibiotics have been developed in the past 30 years. This is due to the low economic benefit of developing new antimicrobials as they will likely, rapidly become obsolete (Ventola, 2015, Projan, 2003, Norrby et al., 2005, Bartlett et al., 2013). This can be seen in the rapid emergence of antibiotic resistant bacteria that closely follows the use of new antibiotics (Figure 3).

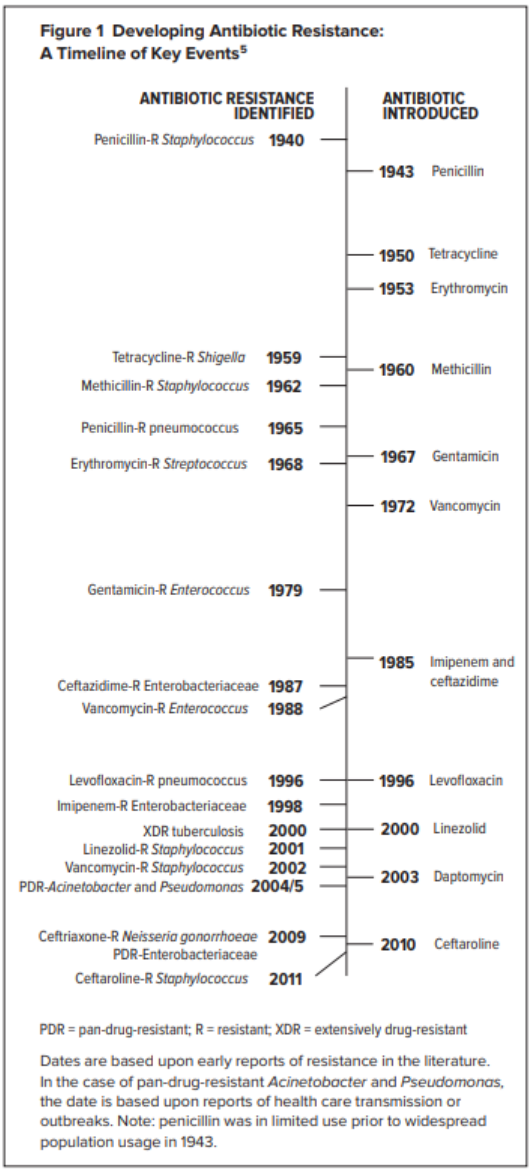

Figure 3: A timeline of antibiotic resistance. Antibiotic resistant infections arise soon after an antibiotic is introduced. This highlights how rapidly resistance can arise and why there is often little commercial benefit in developing new antibiotics. Figure from Ventola 2015.

How bacteria become antibiotic resistant

Bacteria can either be intrinsically resistant to antibiotics, or they can acquire resistance. Antibiotics are often naturally occurring molecules produced by bacteria or fungi and are present in all natural environments (Allen et al., 2010). While their exact function is not

known, it is thought that they are signaling molecules (Martínez, 2008). So, it follows that some bacteria will display resistance to some types of antibiotics. This is known as intrinsic resistance, which is potentially the origin of many of the resistance genes that we see today (Davies and Davies, 2010). However, use of antibiotic compounds at unnaturally high concentrations places significant selective pressure on bacteria, fixing those cells that can acquire resistance.

Bacteria can acquire resistance in two ways, via mutations or horizontal gene transfer. Huge selective pressures are placed on microbial communities as a result of antibiotic use in human medicine, agriculture, aquaculture and veterinary medicine. Selection takes place at the treatment site, where selection pressure is high enough to make acquisition of resistance necessary for survival. The mechanisms involved in acquisition or generation of resistance under these circumstances are well understood (Blair et al., 2014, Levy and Marshall, 2004, Davies and Davies, 2010).

However, there is increasing interest in the potential for sub-inhibitory levels of antibiotics to promote resistance (Andersson and Hughes, 2012). Antibiotics used in medicine and animal husbandry are often poorly metabolized, resulting in up to 90% of the dose being excreted unchanged (Berge et al., 2005, Kümmerer and Henninger, 2003). Antibiotics are not removed by standard waste management processes, but are released into the environment via human waste effluent or via manuring of crops with animal waste (Chee-Sanford et al., 2009, Heuer et al., 2011). Antibiotics also enter the environment directly via crop spraying (McManus, 2014), landfill leachate (Chung et al., 2018), and pharmaceutical factory run off (Larsson, 2014, Tahrani et al., 2016). Consequently, antibiotics are increasingly being viewed as an emerging contaminant (Milic et al., 2013).

It is becoming clear that sub-clinical levels of antibiotics still have significant biological effects. In particular, they affect processes involved in the acquisition or generation of resistance, including mutation, recombination and horizontal gene transfer (Chow et al., 2015, Mesak et al., 2008a, Davies et al., 2006). But what concentrations of antibiotics are needed to stimulate these effects, and are these concentrations found in environmental compartments?

Antibiotic Usage

Antibiotic usage increased 65% between 2000 and 2015, expressed as defined daily doses (DDD). While high income countries still have higher overall use of antibiotics, it is expected that rising average income will boost antibiotic usage in low income countries to equal or exceed current usage in high income countries. The DDD of antibiotics used per 1000 inhabitants per day varies significantly between countries, with less than 10 DDD in Central America to over 40 in Turkey and Tunisia (Klein et al., 2018).

Of all antibiotics manufactured globally, approximately 70-80% are used in agriculture (Rushton and Stärk). This use is mainly in mass animal husbandry (Dibner and Richards, 2005), aquaculture (Cabello, 2006) and fruit spraying (McManus et al., 2002). In animal husbandry, the Population-Corrected Unit (PCU) is defined as milligrams of total antibiotic used per kilogram of meat production. This varies significantly between countries, with countries such as Iceland, Estonia, Latvia and Slovenia being well below 500 PCU and other countries such as France, UK and Spain being above 6000 PCU (Klein et al., 2018).

This variation in antibiotic consumption in both human medicine and animal husbandry suggests that antibiotic usage could be reduced dramatically without negative health or economic consequences (Kummerer and Henninger, 2003).

Methods of entry into the environment

In all these uses, the low absorption rate means that some 70-90% of the antibiotic dose can be excreted unchanged (Lipsitch et al., 2002, Berge et al., 2005). Secondary metabolites of many antibiotics are also still active antimicrobials (Homem and Santos, 2011). For human waste, antibiotics pass into waste treatment facilities, or directly into the environment where waste is not treated. Due to their small molecular size, and relative stability, conventional waste treatment methods are unable to remove them, and they survive treatment (Michael et al., 2013). They are then released into the environment along with waste water effluent or sewage sludge. In agriculture, antibiotics are often directly released into the environment, through disposal of animal waste and fertilization of crops with manure from animals treated with antibiotics (Haller et al., 2002). In aquaculture, antibiotics are directly introduced into the environment as antibiotic-supplemented fish feed (Cabello, 2006).

Furthermore, in the gut of humans and animals, some bacteria will be able to persist during antibiotic therapy due to possession of resistance determinants. These bacteria will be shed in feces, and consequently, humans and animals are should be thought of as releasing both antibiotics and bacteria that carry genes conferring resistance to these antibiotics (Gillings, 2018).

Persistence in the environment

It is clear that antibiotics can persist in the environment, but the length of time an antibiotic can persist in the environment varies, depending on the type of antibiotic and the environmental conditions. Furthermore, the metabolites of many antibiotics are still effective antimicrobials and therefore still have an impact on microbial functions (Kümmerer, 2009, García-Galán et al., 2008). It would help remediation of antibiotic pollution if the mechanisms by which bacteria displayed resistance were mainly via degradation of the

antibiotic itself. Unfortunately, the most common resistance mechanisms are efflux pumps and alterations to target sites, meaning that the antibiotics are still active.

There is considerable variation in the stability of antibiotics in the environment, and factors such as heat, oxidation, ultra violet light, and fungal degradation can affect antibiotic stability. Some antibiotics have high sorption into soil, and while this removes them from water sources, they are still active in sediment, where they may be protected from oxidation and UV degradation (Girardi et al., 2011, Alder et al., 2004). Sorption of antibiotics to soil reduces surface and ground water contamination, but it increases the exposure of soil dwelling microorganisms to antibiotics.

In general, the half-life of antibiotics in manure has been estimated at between 2 and100 days (Boxall et al., 2004), allowing ample time for compounds to mix with soil or be transported via run-off. Biodegradability of antibiotics can be measured *in vitro* using a Closed Bottle Test. This has been done for several antibiotics, none of which were found to be readily biodegradable, defined as greater than 60% degradation within 28 days (Alexy et al., 2004, Kummerer et al., 2000).

Effect of environmental concentrations of antibiotics

Antibiotics can persist for significant periods of time (Schlüsener and Bester, 2006), and even low concentrations of antibiotics have significant biological effects (Andersson and Hughes, 2012). Sub-inhibitory concentrations of antibiotics induce the bacterial SOS response, triggered by DNA damage. The SOS response upregulates expression of error-prone DNA polymerase, and increases rates of transposition and recombination. There are approximately 40 genes involved in the SOS response, several of which are translesion DNA polymerases which allow replication machinery to bypass damaged regions of DNA. This maintains chromosomal integrity but also significantly increases the likelihood of base substitutions

(Baharoglu and Mazel, 2014, Mesak et al., 2008a, Cirz et al., 2006). Sub-inhibitory concentrations of several classes of antibiotics (such as aminoglycosides, fluoroquinolones and β-lactams) are well documented to activate the SOS response (Mesak et al., 2008b, Andersson and Hughes, 2014).

The SOS response allows bacteria to quickly respond to changing environments, and this response is further enhanced by an increased rate of horizontal gene transfer. This allows potentially beneficial genetic elements, such as those conferring resistance, to disseminate rapidly through bacterial communities. Consequently, antibiotic pollution has the potential to generate resistance in environmental bacteria. These could then become opportunistic pathogens, or transfer beneficial genes to pathogenic bacteria, either outcome being of significant concern for human health.

Long term exposure to sub-clinical levels of antibiotics is one of the major factors responsible for the generation and transfer of resistance genes (Uslu et al., 2008, Kümmerer, 2004). Sub-clinical concentrations of antibiotics are continuously discharged through sewage effluent, sludge or manure application, providing continual selective pressure. It has been suggested that resistance that evolves in response to clinical levels of antibiotics will be high cost (Andersson and Hughes, 2014), while *de novo* resistance that is generated in response to sub-clinical levels is less likely to have a significant cost on bacterial fitness and will allow these bacteria to out-compete strains in which mutations are costly (Andersson and Hughes, 2014). Consequently, *de novo* resistance can be maintained on chromosomes for longer when generated in response to low levels of antibiotics. This in turn provides a greater opportunity for newly formed resistance genes to be captured by a mobile element via an insertion or recombination event (Ghaly and Gillings, 2018, Lopatkin et al., 2017, Stevenson et al., 2017).

Antibiotic classes and the SOS response

Antibiotics are usually grouped into classes based on their mechanism of action, meaning the way in which they exhibit bactericidal qualities (Table 1) (Kapoor et al., 2017). These mechanisms are: antibiotics that target the cell wall or cell membrane; antibiotics that inhibit protein synthesis; antibiotics that target nucleic acid synthesis or function (Kapoor et al., 2017, McDermott et al., 2003).

The mechanism of action of an antibiotic will dictate the way that a bacteria may evolve resistance to that antibiotic. Antibiotics can trigger the SOS response directly, through DNA damage, as seen in fluoroquinolones, or indirectly, by activating the SOS response via an alternative pathway, for example βeta-lactams (Fajardo and Martínez, 2008). It is important to identify which antibiotics may trigger the SOS response as the SOS response upregulates mutation rates and may therefore increase the likelihood of a beneficial mutation, such as one conferring antibiotic resistance, occurring. Therefore these antibiotics should be identified and monitored accordingly (Baharoglu and Mazel, 2014, Michel, 2005, Úbeda et al., 2005).

Table 1. Antibiotic classes separated into different mechanisms of action; mechanisms of action are listed in column 1. Examples of common antibiotics are given for each class. Antibiotics that act via DNA damage (labelled "Antibiotics that target nucleic acid synthesis or function" in this table), will activate the SOS response in bacteria that exhibit an SOS response. This is because the SOS response follows DNA damage. Antibiotics that have different mechanisms of action may also induce the SOS response via another activation pathway.

Mechanism of Action	Antibiotic class	Examples
Antibiotics targeting the cell wall or cell membrane	βeta lactams	Penicillin Cephalosporins Carbapenems Monobactams Amoxicillin
	Glycopeptides	Vancomycin Dalbavancin Oritavancin Telavancin
	Cephalosporins	Cephalexin Cefaclor Ceftazidime Ceftriaxone
Antibiotics that inhibit protein synthesis – 50S subunit	Macrolides	Azithromycin Clarithromycin Erythromycin
	Lincosamides	Clindamycin Lincomycin
	Streptogramins	Quinupristin/dalfopristin Pristinamycin Virginiamycin
	Amphenicols	Chloramphenicol Thiamphenicol Florfenicol
Antibiotics that inhibit protein synthesis – 30S subunit	Aminoglycosides	Kanamycin Streptomycin Gentamicin Tobramycin
	Tetracyclines	Doxycycline Demeclocyclin Eravacycline Tetracycline
	Glycylcyclines	Tigecycline
Antibiotics that target nucleic acid synthesis or function	Quinolones/fluoroquinolones	Ciprofloxacin Levofloxacin Moxifloxacin
	Sulfonamides	Sulfamethoxazole Trimethoprim Sulfasalazine

Aims of the work presented in this thesis

It is clear that there is much we still do not know about environmental levels of antibiotics and the effect that these concentrations might have on bacterial acquisition of antibiotic resistance. Establishing the range of concentrations of antibiotics in the environment is an immediate concern. Having determined what concentrations of antibiotics are present in the environment, we then need to determine whether these concentrations are biologically relevant. In particular, it is important to understand how environmentally relevant concentrations of antibiotics up-regulate mechanisms that might generate resistance. These responses might include increased rates of mutation, recombination, transposition and lateral gene transfer. We also need to distinguish how these different effects might play out for planktonic cells and for cells in biofilms.

In this thesis, I address these questions. First, I survey the antibiotic concentrations reported from various environments, and compare these with the known minimum selective concentrations for diverse species. Using environmentally relevant concentrations, I then perform experimental evolution assays to determine how quickly resistance arises in the presence of sub-inhibitory concentrations of antibiotics. After initial experiments involving serial plating, I then examine effects of antibiotics on biofilms. I establish the change in minimum inhibitory concentrations afforded by the biofilm lifestyle, and then examine mutation rates in biofilms exposed to increasing concentrations of antibiotics.

The general conclusion from this work is that concentrations of the antibiotics found as pollutants in environmental compartments are sufficiently high to have significant effects on environmental bacteria. While it is not clear if antibiotic resistance in environmental microorganisms will have ecosystem effects, it does seem likely that this phenomenon will affect microbial ecology and interactions. It is certainly likely that resistance generated in

environmental compartments has the potential to affect human well-being, either through the appearance of new opportunistic pathogens, or through lateral transfer of novel resistance determinants into clinically important species.

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Chapter 1: A survey of sub-inhibitory concentrations of antibiotics in the environment

In this chapter we surveyed the literature reporting environmental concentrations of antibiotics. We then determined whether these concentrations were biologically relevant by comparing them to the minimum selective concentrations reported by Gullberg *et al*. 2011. These concentrations are usually defined as between 1/4 and 1/230 of the minimum inhibitory concentration.

We found that environmental concentrations of antibiotics often fell into the range where they are likely to be influencing microbial ecology, and to be driving the selection of antibiotic resistant bacteria. These findings help set a benchmark for limiting the concentrations of antibiotics in the environment as they provide information on the realistic ranges of antibiotic concentrations in these environmental compartments. This allowed us to design experiments to determine if these environmental levels could have an effect on the generation of antibiotic resistant bacteria.

This chapter is the product of a working collaboration between myself, Timothy Ghaly and Michael Gillings. I planned and proposed the study, performed literature searches with assistance from my co-authors, and collected and tabulated concentration data. Timothy Ghaly and I performed statistical analyses. All three authors were involved in the interpretation of the data. I wrote initial drafts of the manuscript, which were then edited and expanded by the other authors.

Detailed Contributions

	Louise Chow	Michael Gillings	Timothy Ghaly
Design	80%	20%	-
Collection of data	80%	20%	-
Analysis	40%	-	60%
Writing	80%	10%	10%

This chapter has been prepared for publication in *Environmental Science and Technology*. The formatting, referencing and word limits adhere to their author guidelines. Figures have been embedded in the text for the purpose of this thesis.

A survey of sub-inhibitory concentrations of antibiotics in the environment

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Keywords: antimicrobial; pollution; evolution; microorganism, ecology, antibiotic resistance

Abstract

Antibiotics are often poorly metabolized, and can enter the environment via human waste streams, agricultural run-off and pharmaceutical effluent. We consequently expect to see a concentration gradient of antibiotic compounds radiating from areas of human population. Such antibiotics should be thought of as pollutants, as they can accumulate, and have biological effects. These low levels of antibiotics also have the ability to increase rates of mutation and lateral transfer events. Here, we conducted a survey of the literature reporting environmental concentrations of antibiotics. We then determined whether these concentrations were biologically relevant by comparing them to their minimum selective concentrations, usually defined as between 1/4 and 1/230 of the minimum inhibitory concentration. Environmental concentrations of antibiotics often fall into this range and are likely to be influencing microbial ecology, and to be driving the selection of antibiotic resistant bacteria.

Introduction

Antibiotic resistance is one of the greatest threats to human health in the 21st century. Approximately 70% of nosocomial infections are resistant to at least one type of antibiotic [1] and the breadth of resistance is expected to increase as bacteria acquire resistance genes that confer increasingly higher levels of resistance to diverse classes of antimicrobials. It is estimated that by 2050, antibiotic resistant infections will account for 10 million deaths annually, increasing from 700,000 deaths currently [2]. Furthermore, antibiotic resistance leads to longer hospital stays and an overall economic burden that is most felt in low-income nations. On its current trajectory, the effect of antimicrobial resistant infections could damage the global economy to an extent level similar to the Global Financial Crisis in 2008, and could see a further 28.3 million people enter into extreme poverty [3].

Following the World Health Organisation's Global Action Plan on antimicrobial resistance, there has been an increase in research on antibiotic resistance and increased implementation of monitoring and surveillance strategies [4]. Until recently, the method often used to manage antibiotic resistant infections was to replace an antibiotic with either a higher concentration of the same antibiotic, or more commonly, a different antibiotic class. This is not a sustainable solution, as little development of new antibiotics has occurred in in recent years, largely due to the marginal commercial benefit arising from antibiotic development [5].

Furthermore, it is clear that current management and control strategies are not working. Between 2000 and 2015 antibiotic usage increased by 65%, as measured by defined daily doses (DDD), with a concerning increase in last-line of defense antibiotics. Based on this trajectory, antibiotic usage is expected to increase 200% by 2030 [6]. While many countries have implemented national antibiotic usage surveillance plans to monitor and control use of

antibiotics, a unified global response is needed to adequately address the growing problem of antibiotic resistance.

Bacteria may be intrinsically resistant to one or more antibiotics, or they may acquire resistance. Antibiotics are often naturally occurring molecules produced by bacteria or fungi and are present in all natural environments [7]. While it is not known exactly what the original function of these antimicrobials might have been, it follows that some bacteria exhibit resistance to naturally occurring antibiotics. This is known as intrinsic resistance, which is likely the origin of many resistance genes that we see today [8]. However, use of antibiotic compounds at unnaturally high concentrations places significant selective pressure on bacteria, killing most cells and fixing just those cells that can acquire resistance.

Bacteria acquire resistance in two ways, via mutations or horizontal gene transfer. Huge selective pressures are placed on microbial communities as a result of antibiotic use in human medicine, agriculture, aquaculture, and veterinary medicine. Selection takes place at the treatment site, where selection pressure is high enough to make acquisition of resistance necessary for survival. The mechanisms involved in acquisition or generation of resistance under these circumstances are well understood [9, 10].

However, there is increasing interest in the potential for sub-inhibitory levels of antibiotics to promote resistance [11]. Antibiotics used in medicine and animal husbandry are often poorly metabolized, with the result that up to 90% of the therapeutic dose can be excreted unchanged [12, 13]. Antibiotics are not then removed by standard waste management processes, but are released into the environment via human waste effluent, or via manuring of crops with animal waste [14, 15]. Antibiotics also enter the environment directly via crop spraying [16], landfill leachate [17], and pharmaceutical factory run off [18, 19]. Consequently, antibiotics are increasingly being viewed as an emerging contaminant [20].

81 It is becoming increasingly clear that sub-clinical levels of antibiotics still have
82 significant biological effects. In particular, they affect the very processes involved in the
83 acquisition or generation of resistance, including mutation, recombination and lateral gene
84 transfer [21]. But what concentrations of antibiotics are needed to stimulate these effects, and
85 are these concentrations found in environmental compartments? Here we gather the available
86 information on environmental levels of antibiotics, and determine whether these
87 concentrations are biologically relevant.

88

89 **Antibiotic usage**

90 Between 2000 and 2015, antibiotic usage increased 65%, with the increase in usage mainly
91 being seen in low income countries[6]. While high income countries still have higher overall
92 use of antibiotics, it is expected that rising average income will boost antibiotic usage in low
93 income countries to equal or exceed current usage in high income countries. The defined
94 daily dose (DDD) of antibiotics used per 1000 inhabitants per day varies significantly
95 between countries, with less than 10 DDD in Central America to over 40 in Turkey and
96 Tunisia [6].

97 Of all antibiotics manufactured globally, approximately 70-80% are used in
98 agriculture [22]. In animal husbandry, the Population-Corrected Unit (PCU) is defined as
99 milligrams of total antibiotic used per kilogram of meat production. This varies significantly
100 between socially and economically comparable countries, with countries such as Iceland,
101 Estonia, Latvia and Slovenia being well below 500 PCU and other countries such as France,
102 UK and Spain being above 6000 PCU [6].

103 This variation in antibiotic consumption suggests that antibiotic usage could be
104 reduced dramatically without negative health consequences for either human medicine or

105 animal husbandry [23]. This is also an example of the importance of antibiotic monitoring
106 systems, since they allow comparisons of antibiotic usage. Unfortunately, monitoring or
107 surveillance systems for antibiotic use and production are not uniform between countries. The
108 availability of antibiotics without prescription, and the widespread lack of regulation for
109 antibiotic usage in animal husbandry makes accurate estimates difficult. There are also
110 differences in usage patterns, for instance streptomycin is widely used for fruit spraying in
111 the USA, while this use is banned in much of Europe[24].

112 In human medicine, the β-lactams, including penicillin, are most widely used,
113 accounting for 50-70% of antibiotic consumption [23, 25]. Tetracyclines and fluoroquinolones
114 are the main classes of antibiotics used in animal husbandry, however, this use varies
115 significantly, since some classes of antibiotics are banned from use in livestock in some
116 countries [22, 26]. Generally, heavy usage of antibiotics is followed by the emergence of
117 resistant bacterial strains. For example, use of colistin in Chinese agriculture has been
118 associated with the appearance of plasmid mediated colistin resistance [27]. The global
119 consumption of last resort antibiotics, such as carbapenems and colistin, has increased [6]. As
120 this use of antibiotics increases, there will be an increasing influx of antibiotic pollution into
121 the environment.

122

123 **Concentrations in the environment**

124 Theoretically, it could be possible to predict environmental levels of antibiotics by examining
125 consumption, excretion, and effluent volume [23, 28]. However, little is known about rates of
126 dissemination and degradation for different antibiotic classes, or how different environmental
127 conditions might affect these rates. Antibiotic concentrations in environmental samples can
128 be measured directly using analytical methods such as high-performance liquid

chromatography (HPLC), or low-pressure liquid chromatography (LPLC). Antibiotics can be detected in aquatic and sediment samples, allowing detection of antibiotics that are soluble in water, and those which exhibit sorption to soil.

Using these techniques, antibiotics can be detected in hospital or pharmaceutical plant effluent at or above the minimal inhibitory concentration (MIC), and can also be detected at ng/L concentrations in a variety of environments close to human influence. These low antibiotic concentrations do not provide the same selective pressure as clinical levels of antibiotics, but can still significantly promote the acquisition of antibiotic resistance genes [11]. Because waste streams can contain both antibiotics and the resistance genes being selected for, diverse resistance determinants can be acquired by an array of bacterial species beyond those of current clinical concern.

There is a gradient of antibiotic concentration radiating from areas of dense human population and around agricultural operations [29, 30]. The key questions that remain unanswered are: What are the standing concentrations of various antibiotic classes along these urban-environmental gradients?; and Are these concentrations above the threshold predicted to exert selective pressure?

To help answer these questions, we collated data from literature reporting measurements of antibiotic concentrations in diverse environments. Literature was collected in 2018 from scholarly databases (Google Scholar ®, PubMed) using a combination of the following search terms; “antibiotic concentration”, “HPLC”, “antibiotics in the environment”, “distribution of antibiotics in the environment”, “levels of antibiotics in the environment”, “release of antibiotics” and “antibiotic pollution”. Papers were also retrieved by examining previous review papers. All papers were original research, peer-reviewed scientific literature. From the search results, research papers reporting concentrations of antibiotics in the

153 environment were retained and used as data sources. Forty papers were used as sources of
154 primary data, covering the period 1999 to 2018.

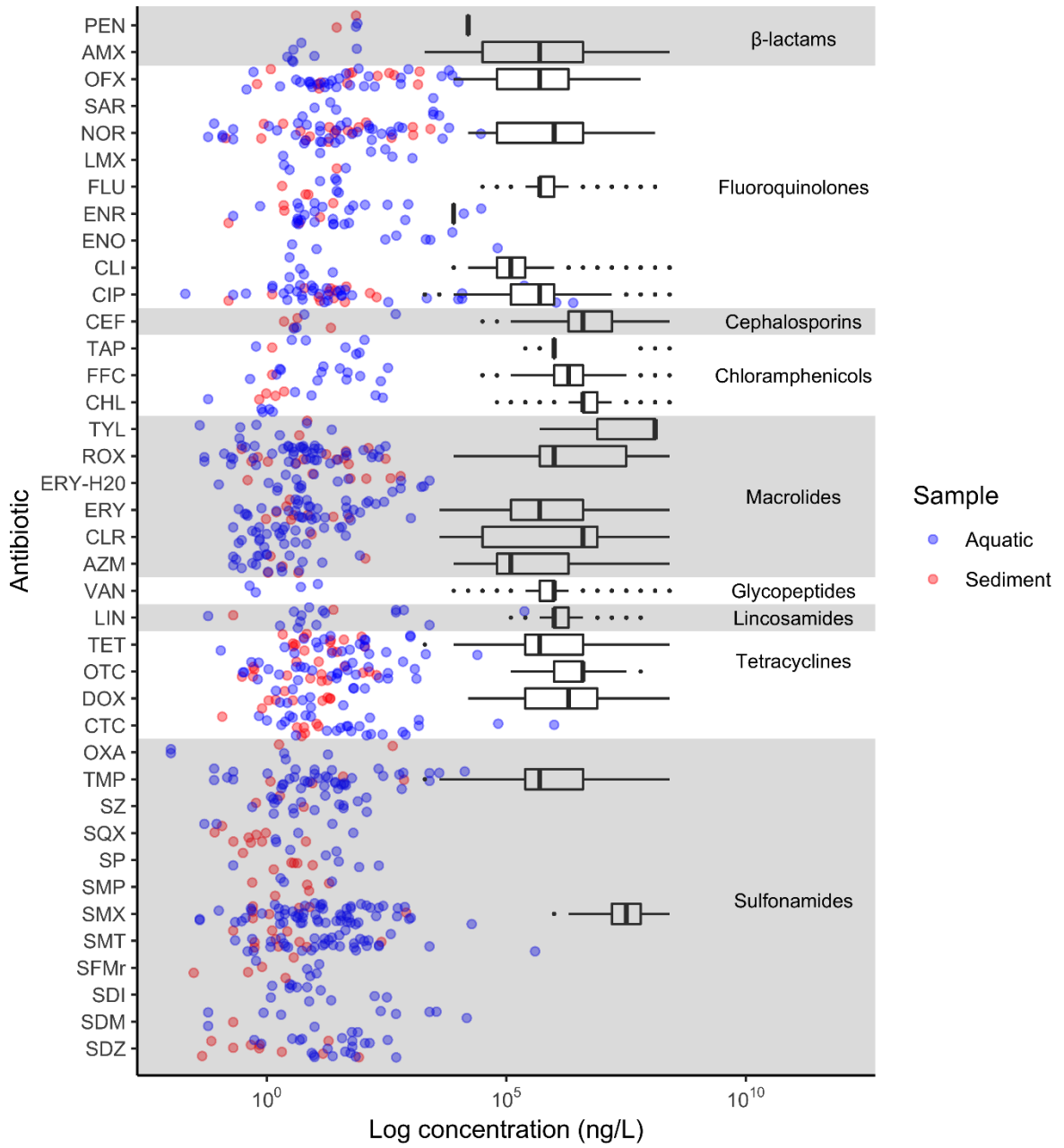
155 Reported concentrations of antibiotics were recorded, and where possible, the
156 minimum, maximum, mean or median concentration was recorded. The sample number,
157 frequency of detection, location, environment type, detection method and reference were
158 recorded alongside the concentration (Table S1). Almost 900 environmental antibiotic
159 concentrations were recorded of which 212 were from sediment and 675 from aquatic
160 environments, encompassing Europe, Asia and North America.

161 Environmental concentrations of antibiotics were compared with their MIC
162 distributions for wild-type bacteria, these data being directly obtained from EUCAST
163 (http://www.eucast.org/). The distributions of MIC measurements were based on collated
164 data from 1,892,215 MIC measurements. The range of MICs for each antibiotic, for all
165 available organisms, was collated. (Antibiotic concentrations and pooled MIC are available
166 as a CSV file File S1 & File S2)

167 **Summary of data**

168 Environmental concentration data were collected for 39 different antibiotics belonging to 9
169 different antibiotic classes. Absolute concentrations ranged from 10^{6} ng/L to 10^{-2} ng/L. MIC
170 values recorded in the EUCAST database were retrieved for as many antibiotic types as
171 possible. These data covered 24 of the antibiotics for which environmental concentrations
172 were available. Antibiotic concentration data were plotted by antibiotic class and type as
173 scatterplots (Figure 1). These were overlain with box and whisker plots of the reported MIC
174 data extracted from EUCAST.

175



176

177 **Figure 1: Concentrations of antibiotics detected in the environment.** Measurements were

178 derived from 675 aquatic (blue) and 212 sediment (red) samples. Box and whisker plots

179 indicate MIC distributions (n = 1,892,215) for all wild type bacteria available from EUCAST.

180 Antibiotics are grouped according to class. For standard antibiotic abbreviations, see Table

181 S1.

182

183 Examining these plots, approximately 2% of antibiotic concentrations in
184 environmental samples have measured antibiotic concentrations that overlap the range of
185 MICs observed for a diverse range of organisms. At these concentrations, the growth of a
186 significant number of environmental bacteria is likely to be inhibited, and cells will be under
187 strong selection for antibiotic resistance.

188 A significant number of measured environmental concentrations fall within values
189 thought to be above the minimum selective concentration (MSC), usually estimated to lie
190 between 1/4 and 1/230 of the MIC [31, 32]. We can expect that these environmental antibiotic
191 concentrations have significant biological effects, including effects on transcription [33] and on
192 rates of recombination, mutation and lateral gene transfer events [34, 35].

193 Generation of *de novo* resistance need only arise once under such selective pressure to
194 fix in a bacterial lineage, and then rapidly spread to other species and locations [36-39].Many
195 records in the dataset are concentrations that are sufficient to select for *de novo* resistance.
196 The majority of measured antibiotic concentrations do fall below the MSC, into the range of
197 the predicted no effect concentration (PNEC), defined as concentrations below the lower
198 range of the MSC (<1/230 MIC) [31].

199 In general, the antibiotic concentrations recorded in aquatic and sediment samples
200 were similar (Figure S1). This might be expected, given that antibiotics are most likely to be
201 transported into the environment via water, and some of these antibiotics are then sequestered
202 into sediment. The dynamics of how antibiotics bind to sediment are not completely known,
203 and the standing concentration in sediment should be an interaction between absorption from
204 the surrounding water and degradation within the sediment. Consequently, we need to know
205 the rate at which antibiotics are being shed into the environment, the absorption rate of
206 antibiotics into sediment, and the half-life of antibiotics in both water and sediment. If the

rate of degradation of antibiotic is slower than the rate that antibiotics are being released into the environment, we expect to see accumulation of antibiotics within environmental compartments.

Limitations of the data

This meta-analysis provides a useful overview of antibiotics in aquatic environments and sediment. In some cases, antibiotics with high usage rates do not appear in the Table. For example, penicillin, a commonly used antibiotic might be expected to be found at high concentrations. However, it is not represented in the data, because it is rapidly degraded in the environment [25]. This demonstrates that direct analysis of environmental samples is important, since it detects antibiotic concentrations that result from the dynamic interaction between rate of release and environmental half-life of particular antibiotics.

For every analytical tool, there are limits of detection. For some of the antibiotics, the minimum concentration required for detection is higher than the concentration where biological effects are predicted to occur (1/4 -1/230 the MIC) [32]. This means that these antibiotics could be present in the environment at undetectable, but biologically relevant concentrations [40]. For example, the MIC of ciprofloxacin for 82 bacterial species falls between 0.002 and 4mg/L, and for amoxicillin it falls between 0.002 and 16mg/L for 30 bacterial species [41]. However, the limit of detection (LOD) for both these antibiotics is 0.005mg/L by high-performance liquid chromatography (HPLC-MS/MS) [42]. This value is sometimes higher than the lowest measured MIC, and is often significantly higher than sub-MIC concentrations that have been suggested to select for resistance (1/4 - 1/230 the MIC). Consequently, low, but relevant concentrations of antibiotics present in the environment might not be detected via commonly used analytical techniques. Furthermore, some

antibiotics may not be detected in water samples because they bind strongly to sludge or sediment, for example tetracyclines and fluoroquinolones [43]. This is why sampling of both aquatic and sediment samples is necessary.

There is a need for accurate and accessible testing methods that are able to detect low levels of antibiotics in liquid and sediment samples. HPLC is an accurate tool for measuring antibiotics in the laboratory, but there is an increasing demand for in-field measurement equipment. This would increase accessibility of environmental antibiotic measurement. [44] The question arises, how can we best determine the concentration of antibiotics in the environment? It would be possible to predict environmental levels of antibiotics by taking into account consumption, metabolic rates, efflux and half-life [23]. However, none of these values are known with any certainty.

Predicted effects of environmental concentrations of antibiotics

In the environment, sub-inhibitory concentrations of antibiotics can upregulate the rate of mutation and gene transfer, ultimately increasing the prevalence of antibiotic resistance [45]. The microbiomes of humans and livestock contain diverse resistance genes [46, 47], therefore, humans and livestock should be thought of as both a source of antibiotics (excreted during treatment) and of antibiotic resistance genes (from the endemic resistome and carriage of clinically relevant resistance genes). Indeed, resistance genes are now being regarded as a new form of pollutant: one that has the ability to replicate [48].

The problem of antibiotic resistance is compounded by the ability of bacteria to acquire genes from their environment and from other bacteria, regardless of species, via horizontal gene transfer. The majority of known antibiotic resistance genes are carried by mobile genetic elements, such as transposons, integrative-conjugative elements and plasmids

[7, 49]. This means that a resistance gene could arise in a single bacterium but be rapidly disseminated around the globe [50]. One well documented example of this is the colistin resistance gene, *mcr-1*, which spread globally following a single *de novo* mutation event. It is likely that this occurred in the Shandong province in China, driven by the heavy agricultural use of colistin in swine farms. Following the initial mobilization event, the *mcr-1* gene was rapidly distributed and has now been detected in five continents in both humans and in livestock [36-38].

Sub-inhibitory concentrations of antibiotics increase rates of mutation and conjugation via the SOS response [34]. The SOS response is a general response to DNA damage, such as the damage inflicted by some antibiotics. There are approximately 40 genes involved in the SOS response, several of which are translesion DNA polymerases which allow the replication machinery to bypass damaged regions of DNA. This maintains chromosomal integrity but also significantly increases the likelihood of base substitutions [51] [35] [52]. Sub-inhibitory concentrations of several classes of antibiotics (such as aminoglycosides, fluoroquinolones and β-lactams) are well documented to activate the SOS response [34, 53].

Long term exposure to sub-clinical levels of antibiotics could be a major factor in the generation and transfer of resistance genes [54, 55]. Sub-clinical concentrations of antibiotics are continuously discharged through sewage effluent, and sludge or manure application, providing continual selective pressure. It has been documented that resistance that evolves in response to clinical levels of antibiotics will be high cost[34], whereas *de novo* resistance that is generated at sub-clinical levels is less likely to have a significant cost on bacterial fitness, and can allow these bacteria to out-compete strains in which mutations are costly [34]. Consequently, *de novo* resistance may be maintained on chromosomes for longer when generated in environmental settings. This in turn provides a greater opportunity for newly formed resistance genes to be captured by a mobile element via an insertion or recombination

event. Once mobilized, however, the cost of a resistance gene to its bacterial host does not necessarily affect its capacity to persist [56-58].

Degradation of antibiotics in the environment

It is clear that antibiotics persist in the environment, but the length of time an antibiotic can persist in the environment varies depending on the type of antibiotic and the environmental conditions. For many antibiotics, the degradation products are still effective antimicrobials and therefore still have an impact on microbial function [30, 59]. There is huge variation in stability of antibiotics, for example, some antibiotics have high sorption into soil, making them able to persist for significant lengths of time, while some antibiotics rapidly degrade under ultraviolet light. Although sorption of antibiotics into sediment removes them from water sources, they are still active in sediment, and here they may be protected from oxidization and UV degradation. [60, 61]. While sorption of antibiotics to soil reduces surface and ground water contamination, it increases the exposure of soil-dwelling microorganisms to antibiotics.

In general, the half-life of antibiotics in manure is estimated to lie between 2-100 days [62], allowing ample time for them be applied, mix with the soil and be transported via run-off. Biodegradability of antibiotics can be measured *in vitro* using a Closed Bottle Test. This has been done for several antibiotics, and none were found to be readily biodegradable, defined as greater than 60% degradation within 28 days [63, 64].

Solutions and future research

Currently there are no regulations or environmental limits on antibiotic pollution, in contrast to many other pollutants such as chlorine, oil and grease, heavy metals, sulfates and nitrogen. All of these can be monitored, have reference standards, and if necessary, treatment protocols

304 [65]. Since there are no global guidelines for antibiotic reference standards and treatment of
305 sewage effluent, there is a significant difference between how countries monitor and treat
306 their sewage effluent. Hospital effluent is well documented to have high concentrations of
307 antibiotics [66], however, in the majority of countries, hospital effluent is classed as "domestic"
308 waste and enters the municipal sewage system. Only a few countries treat this effluent
309 separately before it enters the municipal sewage system [65]. It has been suggested that the
310 minimum selective concentration (MSC) would be more useful than the MIC when proposing
311 acceptable limits of antibiotics in the environment [67, 68]. The minimum selective concentration
312 is the minimum concentration of an antibiotic that provides resistant strains a growth
313 advantage over susceptible strains. The MSC varies between 1/4 and 1/230 of the MIC
314 depending on the antibiotic [32]. For example, the MSC of ciprofloxacin is between 8.6×10^{-6}
315 and 1 mg/L. Whist this is a large range, it gives an indication of what the upper limits of
316 environmental concentrations of ciprofloxacin should be and shows that the levels
317 highlighted in this review are biologically relevant. We should better regulate antibiotic
318 pollution and maintain environmental antibiotic levels below the range of MSC. Antibiotics
319 need to be recognized governing bodies as pollutants and need to have regulatory status.
320 There should be global guidelines for the reference standards and treatment protocols for
321 antibiotic pollution in human waste streams.

322 Treatment of human sewage and livestock waste is necessary in order to prevent
323 antibiotics from entering the environment in the quantities we see here. Generally, antibiotics
324 have long half-lives, and if soluble, are highly mobile and exhibit strong bacteriostatic
325 qualities. Chemical treatment of waste to remove antibiotics is uncommon, as this risks
326 contamination of water with the treatment chemical. Furthermore, the metabolites of
327 antibiotics are generally still active antimicrobials. Removal of antibiotics would be ideally
328 done via physical methods such as reverse osmosis membranes which can remove

329 approximately 90% of antibiotics [69]. The use of sorbents to remove antibiotics is also a viable
330 option as many antibiotics strongly bind with sediment, as long as the sediment is then
331 disposed of in such a way that it does not enter the environment, for example, disposal in
332 lined landfills prevent environmental contamination [69]. Photo-degradation of antibiotics is
333 one of the most common and effective ways to remove antibiotics, however, this process
334 takes time and requires space [70]. Once antibiotics have regulatory status, it would be easier to
335 enforce treatment of waste and more research into effective removal methods would follow.

336 We should not only look to better usage and treatment of antibiotics and waste but
337 also to conditions that can reduce the need for antibiotic treatment. Practices that reduce
338 antibiotic consumption, such as vaccines or hygiene systems, particularly in low-income
339 countries, can be highly effective. For example, when clean water and basic sanitation are
340 available, diarrheal diseases decrease, [71] and effective use of vaccines can reduce future
341 antibiotic needs [72]. New antimicrobials will be ineffective in solving the resistance problem in
342 the long term if these novel drugs are then used in the same way that antibiotics have been
343 used previously. Likewise, antibiotics that are important in human health must be preserved
344 and not used in agriculture. The World Health Organization publishes a list of antibiotics
345 essential to human health. We would argue that there should be a global ban on use of these
346 antimicrobials in agriculture in order to conserve their effectiveness in treating human
347 diseases.

348 Antibiotics enter the environment, where they can persist at biologically relevant
349 concentrations for significant periods of time. When exposed to these levels of antibiotics,
350 there is an upregulation of mutation and DNA transfer which can lead to bacteria acquiring
351 antibiotic resistance genes. This poses significant threat to human health. We acknowledge
352 that antibiotic usage is ingrained into every step of modern medicine, and that mass food
353 production might not be possible without prophylactic usage of antibiotics. However, there

needs to be a shift in antibiotic monitoring, usage and control at every level. The true extent of antibiotic use must be known in order to form workable solutions. Current antibiotic usage is unsustainable and will set the conditions for loss of human life, decreased livestock production and huge economic costs.

The fact that antibiotics are losing their effectiveness after decades of misuse cannot be ignored. Common infectious diseases like tuberculosis, pneumonia, sexually transmitted infections and diarrheal infections are becoming untreatable due to the rise of drug resistant strains. The spread of antibiotic resistance is a global phenomenon. Although resistance genes may arise in one location they can rapidly spread to all parts of the globe. Addressing the problem of antibiotic resistance requires a rapid, and unified global response to preserve antibiotic effectiveness.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

Work was funded by The Faculty of Science and Engineering, Macquarie University and the Australian Government Research Training Program (RTP).

Acknowledgements

The authors would like to acknowledge the members of the EMMA lab, Philip Kelso, and Sasha Tetu for their invaluable contributions to this research.

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562 **Supporting Information**

563 Table S1: Antibiotic concentration data with references

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Acetyl-sulfamethoxazole	Sulfonamides	<50	<50	<50	<50			0		LC- MSMS	United Kingdom	River water	(Ashton et al., 2004)	Table 6, pg. 175
Acetyl-sulfamethoxazole	Sulfonamides	<50	239	<50	70			38		LC- MSMS	United Kingdom	River water	(Ashton et al., 2004)	Table 6, pg. 175
Amoxicillin	Penicillins	n.d.	76				2008	90%	20	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Minh et al., 2009)	Table 5, pg 1058
Amoxicillin	Penicillins	n.d.	5.3		2.85	Winter			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Sediment	(Kafaei et al., 2018)	Table S5
Amoxicillin	Penicillins	n.d.	2.7		1.67	Summer			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Sediment	(Kafaei et al., 2018)	Table S5
Amoxicillin	Penicillins	n.d.	3.4		2.7	Winter			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Seawater	(Kafaei et al., 2018)	Table S5
Amoxicillin	Penicillins	n.d.	3.6		1.63	Summer			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Seawater	(Kafaei et al., 2018)	Table S5
Amoxicillin	Penicillins	0	0		0	March	2005	0%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
Amoxicillin	Penicillins	0	0		0	June	2005	0%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
Amoxicillin	Penicillins	0	0		0	Dec	2004	0%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
Amoxicillin	Penicillins	0	0		0	Feb	2005	0%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
Amoxicillin	Penicillins	3.57	9.91		5.7		2006/7	100%	4	HPLC-MS-MS	River Arno, Italy	River water	(Zuccato et al., 2010)	Table 4, pg 1047
Amoxicillin	Penicillins	n.d.	n.d.		n.d.		2006/7	0%	4	HPLC-MS-MS	River Po, Italy	River water	(Zuccato et al., 2010)	Table 3, pg 1046
Azithromycin	macrolides		1				2001-2002			HPLC-MS/MS.	Emmer, Germany	River, surface water	(Christian et al., 2003)	Table 3
Azithromycin	macrolides		2				2001-2002			HPLC-MS/MS.	Rotterbach, Germany	River, surface water	(Christian et al., 2003)	Table 3
Azithromycin	macrolides		3				2001-2002			HPLC-MS/MS.	Weser, Germany	River, surface water	(Christian et al., 2003)	Table 3
Azithromycin	macrolides		1				2001-2002			HPLC-MS/MS.	Wormkebach, Germany	River, surface water	(Christian et al., 2003)	Table 3
Azithromycin	Macrolides	n.d.	n.d.		n.d.	Winter	2007/8	0%	10	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Azithromycin	Macrolides	n.d.	n.d.		n.d.	Spring	2007/8	0%	4	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Azithromycin	Macrolides	n.d.	n.d.		n.d.	Winter	2007/8	0%	10	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Azithromycin	Macrolides	n.d.	n.d.		n.d.	Spring	2007/8	0%	4	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Azithromycin	Macrolides	n.d.	n.d.		n.d.	Winter	2007/8	0%	10	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Azithromycin	Macrolides	n.d.	n.d.		n.d.	Spring	2007/8	0%	4	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Azithromycin	macrolides		114.7		72.1	Spring	2010	100		UPLC MS/MS	Mar Menor lagoon, Spain	Sediment	(Moreno-González et al., 2015)	Table 2
Azithromycin	macrolides	1.07	2.15		1.4	Winter			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Sediment	(Kafaei et al., 2018)	Table S5
Azithromycin	macrolides	1.2	4.58		1.99	Summer			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Sediment	(Kafaei et al., 2018)	Table S5
Azithromycin	macrolides	1.07	14.45		3.97	Winter			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Seawater	(Kafaei et al., 2018)	Table S5
Azithromycin	macrolides	0.23	4.75		1.21	Summer			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Seawater	(Kafaei et al., 2018)	Table S5
Azithromycin	Macrolides	0.6	2.1			Autumn	2011	100	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Azithromycin	Macrolides	0.3	5.6			Spring	2014	100	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Azithromycin	Macrolides	0.2	0.7			Autumn	2011	100	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Azithromycin	Macrolides	0.2	0.5			Spring	2014	100	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Azithromycin	Macrolides	n.d.	88		22.3	September	2009	91%	23	HPLC-ESI-MS-MS	Laizhou Bay, China	River Water	(Zhang et al., 2012)	Table 1, pg 210
Azithromycin	macrolides	n.d.	0.64			october	2010		35	HPLC-ESI-MS-MS	Beibu Gulf	Seawater	(Zheng et al., 2012)	Table 1. pg 28
Carbadox	Macrolides	10	10					4%	27	HPLC-MS	Han River, South Korea	River Water	(Kim et al., 2016)	Table 3, pg 350
Carbadox	Macrolides	n.d.	n.d.					0%	37	HPLC-MS	Kyungahn Stream	River Water	(Kim et al., 2016)	Table 3, pg 350
Carbodox	Macrolides		n.d.	n.d.			1999-2000	0%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
Carbodox	Macrolides		n.d.					0%	115	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Cefalexin	B-lactams	3.7	21.9	10.8	12.6			100%	15	UHPLC-MS/MS	Dongjiang River, China	Sediment	(Chen et al., 2018)	Table 1
Cefalexin	B-lactams	6.1	493				2008	100%	20	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Minh et al., 2009)	Table 5, pg 1058
Cefalexin	B-lactams	n.d.	4.4		3.34	Winter			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Sediment	(Kafaei et al., 2018)	Table S5
Cefalexin	B-lactams	n.d.	2.3		1.49	Summer			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Sediment	(Kafaei et al., 2018)	Table S5

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Cefalexin	B-lactams	n.d.	4.2		2.52	Winter			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Seawater	(Kafaei et al., 2018)	Table S5
Cefalexin	B-lactams	n.d.	3.7		2.56	Summer			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Seawater	(Kafaei et al., 2018)	Table S5
Chloramphenicol	Chloramphenicols	n.d.	3.9		0.4	July	2012	39%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
Chloramphenicol	Chloramphenicols	n.d.	0.7		0.3	July	2012	92%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
Chloramphenicol	Chloramphenicols	n.d.	n.d.		n.d.			0%	25	HPLC-MS/MS	Shandong province, China	River water	(Hanna et al., 2018)	Table 1, pg. 135
Chloramphenicol	Chloramphenicols	0.98	1.53		0.356			29%	17	HPLC-MS/MS	Shandong province, China	River sediment	(Hanna et al., 2018)	Table 2, pg. 136
Chloramphenicol	Chloramphenicols	0	1.14		0.33	Summer	2011	30	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
Chloramphenicol	Chloramphenicols	0	2.31		1.02	Summer	2011	45	20	HPLC-MS	Dalian, China	Sediment	(Na et al., 2013)	Table 2, pg 235
Chloramphenicol	Chloramphenicols	n.d.	n.d.	n.d.		July	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Chloramphenicol	Chloramphenicols	n.d.	n.d.	n.d.		October	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Chloramphenicol	Chloramphenicols	n.d.	n.d.	n.d.		January	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Chloramphenicol	Chloramphenicols	n.d.	n.d.	n.d.		May	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Chloramphenicol	Chloramphenicols		0.06	n.d.					52	HPLC	Germany	River water	(Hirsch et al., 1999)	Table 3, pg. 114
Chloramphenicol	Chloramphenicols	-	266		41	March	2005	100%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
Chloramphenicol	Chloramphenicols	-	187		127	June	2005	100%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
Chloramphenicol	Chloramphenicols	0	0		0	Dec	2004	0%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
Chloramphenicol	Chloramphenicols	0	0		0	Feb	2005	0%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
Chloramphenicol	Chloramphenicols	n.d.	0.83	n.d		July	2011	86%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Chloramphenicol	Chloramphenicols	n.d.	0.78	n.d		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Chloramphenicol	Chloramphenicols	n.d.	8.63	3.03		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Chloramphenicol	Chloramphenicols	n.d.	1.34	0.97		May	2012	71%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Chlorotetracycline	Tetracyclines		n.d.	n.d.					14	HPLC	Germany	River water	(Hirsch et al., 1999)	Table 3, pg. 114
Chlortetracycline	Tetracyclines	68000	1000000	220000	353400	Oct-Dec	1998	100%	8	LC/ESI-MS	Iowa and Ohio, USA	Lagoon water	(Campagnolo et al., 2002)	Table 2
Chlortetracycline	Tetracyclines	1500	1500	1500	1500	Oct-Dec	1998	14%	7	LC/ESI-MS	Iowa and Ohio, USA	Stream water	(Campagnolo et al., 2002)	Table 2

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Chlortetracycline	Tetracyclines	n.d.	46.7		3.6	July	2012	39%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
Chlortetracycline	Tetracyclines	6	6.3		2.4	July	2012	85%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
Chlortetracycline	Tetracyclines				2.02				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Chlortetracycline	Tetracyclines				1.05				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Chlortetracycline	Tetracyclines	0	18.1			Dry (Dec)	2015	100%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Chlortetracycline	Tetracyclines	0	8.4			Wet (Jun)	2015	29%	18	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Chlortetracycline	Tetracyclines	263	793					7%	27	HPLC-MS	Han River, South Korea	River Water	(Kim et al., 2016)	Table 3, pg 350
Chlortetracycline	Tetracyclines	29	50					16%	37	HPLC-MS	Kyungahn Stream	River Water	(Kim et al., 2016)	Table 3, pg 350
Chlortetracycline	tetracyclines		0.69	0.42				2%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
Chlortetracycline	Tetracyclines		690					2%	84	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Chlortetracycline	Tetracyclines	n.d.	150							LC-MS/MS	Snake Creek, GA, USA	Surface Water	(Lindsey et al., 2001)	Table 4
Chlortetracycline	Tetracyclines	n.d.	6.5		1.79	Dec	2015	50%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Chlortetracycline	Tetracyclines	n.d.	4.08		0.66	Aug	2016	28%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Chlortetracycline	Tetracyclines				6.4				20	HPLC–MS/MS	Austria	Farm soil	(Martínez-Carballo et al., 2007)	Table 3
Chlortetracycline	Tetracyclines	1.01	3		1.49	Summer	2011	100	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
Chlortetracycline	Tetracyclines	n.d.	4.3	0.628		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Chlortetracycline	Tetracyclines	n.d.	11	0.623		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Chlortetracycline	Tetracyclines	n.d.	5.37	0.76		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Chlortetracycline	Tetracyclines	0.118	12	1.3		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Chlortetracycline	Tetracyclines	0	122.3			Autumn	2011	77.8	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Chlortetracycline	Tetracyclines	0	34.8			Spring	2014	41.7	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Chlortetracycline	Tetracyclines	0	86.6			Autumn	2011	72	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Chlortetracycline	Tetracyclines	0	58.1			Spring	2014	52.6	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Chlortetracycline	Tetracyclines	n.d.	142.5		67.9					HPLC–MS/MS	Taihu Lake, China	Surface Water	(Xu et al., 2014)	table 2

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Chlortetracycline	Tetracyclines	n.d.	48.5		19					HPLC–MS/MS	Taihu Lake, China	Sediment	(Xu et al., 2014)	table 2
Chlortetracycline	Tetracyclines	n.d.	2.03	0.3		July	2011	57%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Chlortetracycline	Tetracyclines	n.d.	2.43	n.d		October	2011	43%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Chlortetracycline	Tetracyclines	n.d.	3.5	1.2		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Chlortetracycline	Tetracyclines	n.d.	n.d.	n.d		May	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Ciprofloxacin	Fluoroquinolones	n.d.	34.2		2.7	July	2012	31%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
Ciprofloxacin	Fluoroquinolones	6.1	27.4	18.4	16.4			100%	15	UHPLC-MS/MS	Dongjiang River, China	Sediment	(Chen et al., 2018)	Table 1
Ciprofloxacin	Fluoroquinolones	0	5.6			Normal (Oct)	2014	75%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Ciprofloxacin	Fluoroquinolones	0	5			Dry (Dec)	2014	63%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Ciprofloxacin	Fluoroquinolones	0	8.6			Wet (Jun)	2015	53%	18	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Ciprofloxacin	Fluoroquinolones	2200	236600							LC-MS/MS	Ujjain, Indai	Waste water	(Diwan et al., 2010)	Table 5, Pg 6
Ciprofloxacin	Fluoroquinolones	n.d.	2.95		n.d.	Winter	2007/8	10%	10	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Ciprofloxacin	Fluoroquinolones	n.d.	9.66		n.d.	Spring	2007/8	25%	4	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Ciprofloxacin	Fluoroquinolones	n.d.	1.31		n.d.	Winter	2007/8	10%	10	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Ciprofloxacin	Fluoroquinolones	n.d.	n.d.		n.d.	Spring	2007/8	25%	4	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Ciprofloxacin	Fluoroquinolones	n.d.	0.37		n.d.	Winter	2007/8	10%	10	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Ciprofloxacin	Fluoroquinolones	n.d.	n.d.		n.d.	Spring	2007/8	25%	4	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Ciprofloxacin	Fluoroquinolones	n.d.	19.4					2	50	LC-MS/MS	Minnesota, USA	Lake water	(Ferrey et al., 2015)	Table 3
Ciprofloxacin	Fluoroquinolones	0.2	18.8		0.867			32%	25	HPLC-MS/MS	Shandong province, China	River water	(Hanna et al., 2018)	Table 1, pg. 135
Ciprofloxacin	Fluoroquinolones	0.16	21.74		2.003			76%	17	HPLC-MS/MS	Shandong province, China	River sediment	(Hanna et al., 2018)	Table 2, pg. 136
Ciprofloxacin	Fluoroquinolones	n.d.	60.3	4.55	9.45			67%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Ciprofloxacin	Fluoroquinolones	n.d.	46	n.d.	2.49			11%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Ciprofloxacin	Fluoroquinolones	n.d.	36.17		3.25	Dec	2015	11%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Ciprofloxacin	Fluoroquinolones	n.d.	2.7		0.18	Aug	2016	6%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Ciprofloxacin	Fluoroquinolones	n.d.	13.1	2.61		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Ciprofloxacin	Fluoroquinolones	1.3	12.1	3.93		October	2011	57%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Ciprofloxacin	Fluoroquinolones	n.d.	42.9	2.14		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Ciprofloxacin	Fluoroquinolones	n.d.	20.1	4.75		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Ciprofloxacin	Fluoroquinolones				0.87				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Ciprofloxacin	Fluoroquinolones				0.81				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Ciprofloxacin	Fluoroquinolones		0.02	0.03				3%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
ciprofloxacin	Fluoroquinolones		30					3%	##	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Ciprofloxacin	Fluoroquinolones				2.9		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Ciprofloxacin	Fluoroquinolones				569		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Ciprofloxacin	Fluoroquinolones				157		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Ciprofloxacin	Fluoroquinolones		<10		-		2006	9%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Ciprofloxacin	Fluoroquinolones		<10		-		2006	0%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
Ciprofloxacin	Fluoroquinolones		<10		-		2006	10%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Ciprofloxacin	Fluoroquinolones		<10		-		2006	0%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
Ciprofloxacin	Fluoroquinolones		<10		-		2006	0%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Ciprofloxacin	Fluoroquinolones		9				2001-2002			HPLC-MS/MS.	Rotterbach, Germany	River, surface water	(Christian et al., 2003)	Table 3
Ciprofloxacin	Fluoroquinolones				25				20	HPLC–MS/MS	Austria	Farm soil	(Martínez-Carballo et al., 2007)	Table 3
Ciprofloxacin	Fluoroquinolones		12000						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Ciprofloxacin	Fluoroquinolones		12000						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Ciprofloxacin	Fluoroquinolones		2500000						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Ciprofloxacin	Fluoroquinolones		1100000						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Ciprofloxacin	Fluoroquinolones		10000						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Ciprofloxacin	Fluoroquinolones				n.d.				2	HPLC-FLD	Central Turkey	Farm soil	(Uslu et al., 2008)	Table 6, pg 61
Ciprofloxacin	Fluoroquinolones				0.053				2	HPLC-FLD	North West Turkey	Farm soil	(Uslu et al., 2008)	Table 6, pg 61
Ciprofloxacin	Fluoroquinolones	0	18			Autumn	2011	66.7	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Ciprofloxacin	Fluoroquinolones	0	12.5			Spring	2014	41.7	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Ciprofloxacin	Fluoroquinolones	0	4.3			Autumn	2011	48	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Ciprofloxacin	Fluoroquinolones	0	5.4			Spring	2014	26.3	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Ciprofloxacin	Fluoroquinolones	n.d.	43.6		8.8					HPLC–MS/MS	Taihu Lake, China	Surface Water	(Xu et al., 2014)	table 2
Ciprofloxacin	Fluoroquinolones	n.d.	25.3		9.8					HPLC–MS/MS	Taihu Lake, China	Sediment	(Xu et al., 2014)	table 2
Ciprofloxacin	Fluoroquinolones	n.d.	n.d.	n.d		July	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Ciprofloxacin	Fluoroquinolones	n.d.	n.d.	n.d		October	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Ciprofloxacin	Fluoroquinolones	n.d.	n.d.	n.d		January	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Ciprofloxacin	Fluoroquinolones	n.d.	2.27	n.d		May	2012	43%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Ciprofloxacin	Fluoroquinolones	n.d.	197		472.18	September	2008	50%	14	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430
Ciprofloxacin	Fluoroquinolones	n.d.	143		44.1	February	2009	69%	13	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430
Ciprofloxacin	Fluoroquinolones	n.d.	346		101	September	2009	91%	23	HPLC-ESI-MS-MS	Laizhou Bay, China	River Water	(Zhang et al., 2012)	Table 1, pg 210
Ciprofloxacin	Fluoroquinolones	n.d.	37.5		19		2006/7	75%	4	HPLC-MS-MS	River Arno, Italy	River water	(Zuccato et al., 2010)	Table 4, pg 1047
Ciprofloxacin	Fluoroquinolones	1.32	16		8.8		2006/7	100%	4	HPLC-MS-MS	River Po, Italy	River water	(Zuccato et al., 2010)	Table 3, pg 1047
Clarithromycin	Macrolides				1.07				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Clarithromycin	Macrolides				0.97				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Clarithromycin	Macrolides		4				2001-2002			HPLC-MS/MS.	Boker-Heide-Kanal, Germany	River, surface water	(Christian et al., 2003)	Table 3
Clarithromycin	Macrolides		2				2001-2002			HPLC-MS/MS.	Emmer, Germany	River, surface water	(Christian et al., 2003)	Table 3
Clarithromycin	Macrolides		4				2001-2002			HPLC-MS/MS.	Nethe, Germany	River, surface water	(Christian et al., 2003)	Table 3

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Clarithromycin	Macrolides		1				2001-2002			HPLC-MS/MS.	Pader, Germany	River, surface water	(Christian et al., 2003)	Table 3
Clarithromycin	Macrolides		37				2001-2002			HPLC-MS/MS.	Rotterbach, Germany	River, surface water	(Christian et al., 2003)	Table 3
Clarithromycin	Macrolides		8				2001-2002			HPLC-MS/MS.	Weser, Germany	River, surface water	(Christian et al., 2003)	Table 3
Clarithromycin	Macrolides	n.d.	1.56		0.6	Winter	2007/8	80%	10	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Clarithromycin	Macrolides	n.d.	n.d.		n.d.	Spring	2007/8	0%	4	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Clarithromycin	Macrolides	n.d.	1.83		0.79	Winter	2007/8	80%	10	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Clarithromycin	Macrolides	n.d.	n.d.		n.d.	Spring	2007/8	0%	4	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Clarithromycin	Macrolides	n.d.	2.33		0.74	Winter	2007/8	80%	10	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Clarithromycin	Macrolides	n.d.	n.d.		n.d.	Spring	2007/8	0%	4	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Clarithromycin	Macrolides		0.26	n.d.					33	HPLC	Germany	River water	(Hirsch et al., 1999)	Table 3, pg. 114
Clarithromycin	Macrolides		8.7		5	Spring	2010	100		UPLC MS/MS	Mar Menor lagoon, Spain	Sediment	(Moreno-González et al., 2015)	Table 2
Clarithromycin	Macrolides	0.6	2.4			Autumn	2011	100	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Clarithromycin	Macrolides	0.7	15.8			Spring	2014	100	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Clarithromycin	Macrolides	0.3	0.7			Autumn	2011	100	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Clarithromycin	Macrolides	0.2	0.5			Spring	2014	100	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Clarithromycin	Macrolides	n.d.	32.9		5	September	2009	87%	23	HPLC-ESI-MS-MS	Laizhou Bay, China	River Water	(Zhang et al., 2012)	Table 1, pg 210
Clarithromycin	macrolides	n.d.	0.72			october	2010		35	HPLC-ESI-MS-MS	Beibu Gulf	Seawater	(Zheng et al., 2012)	Table 1. pg 28
Clarithromycin	Macrolides	6.7	44.76		25.4		2006/7	100%	4	HPLC-MS-MS	River Arno, Italy	River water	(Zuccato et al., 2010)	Table 4, pg 1047
Clarithromycin	Macrolides	0.89	2.19		1.7		2006/7	100%	4	HPLC-MS-MS	River Po, Italy	River water	(Zuccato et al., 2010)	Table 3, pg 1049
Clindamycin	Macrolides		3				2001-2002			HPLC-MS/MS.	Boker-Heide-Kanal, Germany	River, surface water	(Christian et al., 2003)	Table 3
Clindamycin	Macrolides		5				2001-2002			HPLC-MS/MS.	Emmer, Germany	River, surface water	(Christian et al., 2003)	Table 3
Clindamycin	Macrolides		3				2001-2002			HPLC-MS/MS.	Nethe, Germany	River, surface water	(Christian et al., 2003)	Table 3

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Clindamycin	Macrolides		24				2001-2002			HPLC-MS/MS.	Rotterbach, Germany	River, surface water	(Christian et al., 2003)	Table 3
Clindamycin	Macrolides		6				2001-2002			HPLC-MS/MS.	Weser, Germany	River, surface water	(Christian et al., 2003)	Table 3
Danofloxacin	Fluoroquinolones				1.15				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Danofloxacin	Fluoroquinolones				1.52				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Danofloxacin	Fluoroquinolones		<10		-		2006	9%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Danofloxacin	Fluoroquinolones		<10		-		2006	0%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
Danofloxacin	Fluoroquinolones		<10		-		2006	0%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Danofloxacin	Fluoroquinolones		19		19		2006	8%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
Danofloxacin	Fluoroquinolones		<10		-		2006	0%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Difloxacin	Fluoroquinolones				0.409				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Difloxacin	Fluoroquinolones				1.04				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
difloxacin	Fluoroquinolones	0	3.1			Normal (Oct)	2014	63%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
difloxacin	Fluoroquinolones	0	2.7			Dry (Dec)	2014	75%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
difloxacin	Fluoroquinolones	0	5.3			Wet (Jun)	2015	88%	18	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Difloxacin	Fluoroquinolones	n.d.	n.d.	n.d.	n.d.			0%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Difloxacin	Fluoroquinolones	n.d.	n.d.	n.d.	n.d.			0%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
difloxacin	Fluoroquinolones		<10		-		2006	9%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
difloxacin	Fluoroquinolones		<10		-		2006	0%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
difloxacin	Fluoroquinolones		<10		-		2006	0%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
difloxacin	Fluoroquinolones		<10		-		2006	8%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
difloxacin	Fluoroquinolones		<10		-		2006	0%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Doxycycline	Tetracyclines	n.d.	112.3		11.3	July	2012	69%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
Doxycycline	Tetracyclines	n.d.	21.3		7	July	2012	85%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Doxycycline	Tetracyclines				2				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Doxycycline	Tetracyclines				1.7				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Doxycycline	Tetracyclines	0.8	20.9	3.1	5.2			100%	15	UHPLC-MS/MS	Dongjiang River, China	Sediment	(Chen et al., 2018)	Table 1
Doxycycline	Tetracyclines	0	39.7			Dry (Dec)	2015	30%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Doxycycline	Tetracyclines	0	8.1			Wet (Jun)	2015	18%	18	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Doxycycline	Tetracyclines	1.9	3.5		0.327			12%	25	HPLC-MS/MS	Shandong province, China	River water	(Hanna et al., 2018)	Table 1, pg. 135
Doxycycline	Tetracyclines	n.d.	n.d.		n.d.			0%	17	HPLC-MS/MS	Shandong province, China	River sediment	(Hanna et al., 2018)	Table 2, pg. 136
Doxycycline	Tetracyclines		n.d.	n.d.					14	HPLC	Germany	River water	(Hirsch et al., 1999)	Table 3, pg. 114
Doxycycline	Tetracyclines		n.d.	n.d.				0%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
doxycycline	Tetracyclines		n.d.					0%	115	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Doxycycline	Tetracyclines	0	1.56		0.26	Summer	2011	20	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
Doxycycline	Tetracyclines	1.1	1.54		1.33	Summer	2011	100	20	HPLC-MS	Dalian, China	Sediment	(Na et al., 2013)	Table 2, pg 235
Doxycycline	Tetracyclines	n.d.	3.53	n		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Doxycycline	Tetracyclines	n.d.	14.6	n		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Doxycycline	Tetracyclines	n.d.	2.37	n		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Doxycycline	Tetracyclines	n.d.	18.6	n		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Doxycycline	Tetracyclines	0	66.5			Autumn	2011	77.8	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Doxycycline	Tetracyclines	0	8.4			Spring	2014	58.3	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Doxycycline	Tetracyclines	0	64.2			Autumn	2011	72	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Doxycycline	Tetracyclines	0	2.7			Spring	2014	10.5	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Doxycycline	Tetracyclines	n.d.	n.d.	n.d		July	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Doxycycline	Tetracyclines	n.d.	n.d.	n.d		October	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Doxycycline	Tetracyclines	n.d.	5.63	n.d		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Doxycycline	Tetracyclines	n.d.	n.d.	n.d		May	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Enoxacin	Fluoroquinolones	0	3.4			Normal (Oct)	2014	100%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Enoxacin	Fluoroquinolones				31		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Enoxacin	Fluoroquinolones				12.8		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Enoxacin	Fluoroquinolones		11				2006	9%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Enoxacin	Fluoroquinolones		<10		-		2006	0%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
Enoxacin	Fluoroquinolones		<10		-		2006	10%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Enoxacin	Fluoroquinolones		<10		-		2006	0%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
Enoxacin	Fluoroquinolones		<10		-		2006	0%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Enoxacin	Fluoroquinolones		7500						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Enoxacin	Fluoroquinolones		2100						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Enoxacin	Fluoroquinolones		66000						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Enoxacin	Fluoroquinolones		2600						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Enoxacin	Fluoroquinolones	n.d.	508		116	September	2009	91%	23	HPLC-ESI-MS-MS	Laizhou Bay, China	River Water	(Zhang et al., 2012)	Table 1, pg 210
Enrofloxacin	Fluoroquinolones	n.d.	14.6		2.8	July	2012	39%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
Enrofloxacin	Fluoroquinolones	n.d.	8.9		3.2	July	2012	92%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
Enrofloxacin	Fluoroquinolones	4.4	5.2			Normal (Oct)	2014	100%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Enrofloxacin	Fluoroquinolones	4.2	4.5			Dry (Dec)	2014	100%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Enrofloxacin	Fluoroquinolones	0.2	52.2		2.744			48%	25	HPLC-MS/MS	Shandong province, China	River water	(Hanna et al., 2018)	Table 1, pg. 135
Enrofloxacin	Fluoroquinolones	0.16	24.42		2.205			94%	17	HPLC-MS/MS	Shandong province, China	River sediment	(Hanna et al., 2018)	Table 2, pg. 136
Enrofloxacin	Fluoroquinolones	10	113					44%	27	HPLC-MS	Han River, South Korea	River Water	(Kim et al., 2016)	Table 3, pg 350
Enrofloxacin	Fluoroquinolones	10	333					43%	37	HPLC-MS	Kyungahn Stream	River Water	(Kim et al., 2016)	Table 3, pg 350
enrofloxacin	Fluoroquinolones	n.d.	4.42	1.31	1.28			67%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
enrofloxacin	Fluoroquinolones	n.d.	13	n.d.	0.46			4%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Enrofloxacin	Fluoroquinolones	n.d.	0.73		0.04	Dec	2015	6%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Enrofloxacin	Fluoroquinolones	n.d.	4.61		0.89	Aug	2016	44%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Enrofloxacin	Fluoroquinolones	n.d.	2.34	1.17		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Enrofloxacin	Fluoroquinolones	n.d.	n.d.	n		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Enrofloxacin	Fluoroquinolones	n.d.	2.26	n		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Enrofloxacin	Fluoroquinolones	n.d.	4.84	n		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Enrofloxacin	Fluoroquinolones				1.37				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Enrofloxacin	Fluoroquinolones				0.75				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
enrofloxacin	Fluoroquinolones		n.d.	n.d.				0%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
enrofloxacin	Fluoroquinolones		n.d.					0%	##	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Enrofloxacin	Fluoroquinolones				51				3	HPLC-ESI-MS-MS	Belgioioso, Italy	Farm soil	(Sturini et al., 2012)	Table 2, pg 132
Enrofloxacin	Fluoroquinolones				50				3	HPLC-ESI-MS-MS	Linarolo, Italy	Farm soil	(Sturini et al., 2012)	Table 2, pg 132
Enrofloxacin	Fluoroquinolones				23				3	HPLC-ESI-MS-MS	Torre d'Isola, Italy	Farm soil	(Sturini et al., 2012)	Table 2, pg 132
Enrofloxacin	Fluoroquinolones				11		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Enrofloxacin	Fluoroquinolones				6.6		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Enrofloxacin	Fluoroquinolones		<10		-		2006	9%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Enrofloxacin	Fluoroquinolones		<10		-		2006	0%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
Enrofloxacin	Fluoroquinolones		<10		-		2006	10%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Enrofloxacin	Fluoroquinolones		<10		-		2006	0%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
Enrofloxacin	Fluoroquinolones		10		-		2006	0%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
enrofloxacin	Fluoroquinolones				24				20	HPLC–MS/MS	Austria	Farm soil	(Martínez-Carballo et al., 2007)	Table 3
Enrofloxacin	Fluoroquinolones		25						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Enrofloxacin	Fluoroquinolones		30000						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Enrofloxacin	Fluoroquinolones		13000						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Enrofloxacin	Fluoroquinolones		64						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Enrofloxacin	Fluoroquinolones				0.035				2	HPLC-FLD	Central Turkey	Farm soil	(Uslu et al., 2008)	Table 6, pg 61
Enrofloxacin	Fluoroquinolones				0.204				2	HPLC-FLD	North West Turkey	Farm soil	(Uslu et al., 2008)	Table 6, pg 61
Enrofloxacin	Fluoroquinolones	0	53.1			Autumn	2011	88.9	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Enrofloxacin	Fluoroquinolones	0	6.2			Spring	2014	25	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Enrofloxacin	Fluoroquinolones	1.8	24.1			Autumn	2011	12	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Enrofloxacin	Fluoroquinolones	0	41.8			Spring	2014	63.2	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Enrofloxacin	Fluoroquinolones	n.d.	n.d.	n.d		July	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Enrofloxacin	Fluoroquinolones	n.d.	n.d.	n.d		October	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Enrofloxacin	Fluoroquinolones	n.d.	n.d.	n.d		January	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Enrofloxacin	Fluoroquinolones	n.d.	4.77	n.d		May	2012	57%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Enrofloxacin	Fluoroquinolones	n.d.	24.6		10.6	September	2009	57%	23	HPLC-ESI-MS-MS	Laizhou Bay, China	River Water	(Zhang et al., 2012)	Table 1, pg 210
Erythromycin	Macrolides	<10	57	<10	<10		2002	17		LC- MSMS	United Kingdom	River water	(Ashton et al., 2004)	Table 6, pg. 175
Erythromycin	Macrolides	<10	1022	<10	159			38		LC- MSMS	United Kingdom	River water	(Ashton et al., 2004)	Table 6, pg. 175
Erythromycin	Macrolides	0.4	6.9		3.9	July	2012	100%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
Erythromycin	Macrolides	1.5	24.6		10.2	July	2012	100%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
Erythromycin	Macrolides		65				2001-2002			HPLC-MS/MS.	Boker-Heide-Kanal, Germany	River, surface water	(Christian et al., 2003)	Table 3
Erythromycin	Macrolides		49				2001-2002			HPLC-MS/MS.	Emmer, Germany	River, surface water	(Christian et al., 2003)	Table 3
Erythromycin	Macrolides		46				2001-2002			HPLC-MS/MS.	Nethe, Germany	River, surface water	(Christian et al., 2003)	Table 3
Erythromycin	Macrolides		4				2001-2002			HPLC-MS/MS.	Niers, Germany	River, surface water	(Christian et al., 2003)	Table 3
Erythromycin	Macrolides		4				2001-2002			HPLC-MS/MS.	Pader, Germany	River, surface water	(Christian et al., 2003)	Table 3

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Erythromycin	Macrolides		190				2001-2002			HPLC-MS/MS.	Rotterbach, Germany	River, surface water	(Christian et al., 2003)	Table 3
Erythromycin	Macrolides		89				2001-2002			HPLC-MS/MS.	Weser, Germany	River, surface water	(Christian et al., 2003)	Table 3
Erythromycin	Macrolides		135				2001-2002			HPLC-MS/MS.	Wormkebach, Germany	River, surface water	(Christian et al., 2003)	Table 3
Erythromycin	Macrolides	0	7.5			Normal (Oct)	2014	100%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Erythromycin	Macrolides	0	10.7			Dry (Dec)	2014	88%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Erythromycin	Macrolides	0	4.7			Dry (Dec)	2015	20%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Erythromycin	Macrolides	0	8.1			Wet (Jun)	2015	100%	18	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Erythromycin	Macrolides	n.d.	121	4.94	19.5			85%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Erythromycin	Macrolides	n.d.	3.04	0.42	0.59			84%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Erythromycin	Macrolides		52.6		9	Spring	2010	72.2		UPLC MS/MS	Mar Menor lagoon, Spain	Sediment	(Moreno-González et al., 2015)	Table 2
Erythromycin	Macrolides	0.176	1.59	0.65		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Erythromycin	Macrolides	0.205	0.966	0.522		October	2011	71%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Erythromycin	Macrolides	0.305	3.61	0.859		January	2012	57%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Erythromycin	Macrolides	n.d.	3.22	0.694		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Erythromycin	Macrolides	1.24	2.57		2.2	Winter			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Sediment	(Kafaei et al., 2018)	Table S5
Erythromycin	Macrolides	1.83	7.26		3.06	Summer			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Sediment	(Kafaei et al., 2018)	Table S5
Erythromycin	Macrolides	1.6	11.43		5.93	Winter			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Seawater	(Kafaei et al., 2018)	Table S5
Erythromycin	Macrolides	0.26	5.32		1.36	Summer			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Seawater	(Kafaei et al., 2018)	Table S5
Erythromycin	Macrolides	n.d.	4.45	0.8		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Erythromycin	Macrolides	0.35	8.75	0.53		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Erythromycin	Macrolides	n.d.	16.5	9.39		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Erythromycin	Macrolides	5.7	45.4	9.68		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
erythromycin	Macrolides	0.38	282		62.1	September	2009	100%	23	HPLC-ESI-MS-MS	Laizhou Bay, China	River Water	(Zhang et al., 2012)	Table 1, pg 210
Erythromycin	Macrolides	2.88	8.12		5.4		2006/7	100%	4	HPLC-MS-MS	River Arno, Italy	River water	(Zuccato et al., 2010)	Table 4, pg 1047
Erythromycin	Macrolides	0.78	4.62		2.9		2006/7	100%	4	HPLC-MS-MS	River Po, Italy	River water	(Zuccato et al., 2010)	Table 3, pg 1050
Erythromycin dehydrate	Macrolides	0	4			Autumn	2011	88.9	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Erythromycin dehydrate	Macrolides	0	381.5			Spring	2014	50	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Erythromycin dehydrate	Macrolides	0	2.3			Autumn	2011	12	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Erythromycin dehydrate	Macrolides	0	377.8			Spring	2014	47.4	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Erythromycin-H20	Macrolides	2500	2500	2500	2500	Oct-Dec	1998	100%	8	LC/ESI-MS	Iowa and Ohio, USA	Lagoon water	(Campagnolo et al., 2002)	Table 2
Erythromycin-H20	Macrolides	0.4	9.1	3	2			100%	15	UHPLC-MS/MS	Dongjiang River, China	Sediment	(Chen et al., 2018)	Table 1
erythromycin-h20	Macrolides		0.1	1.7				22%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
erythromycin-H20	Macrolides		1700					22%	104	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Erythromycin-H20	Macrolides	4.7	1900				2008	100%	20	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Minh et al., 2009)	Table 5, pg 1058
Erythromycin-H20	Macrolides	-	423		30	March	2005	100%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
Erythromycin-H20	Macrolides	-	636		460	June	2005	100%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
Erythromycin-H20	Macrolides	0	5.2		3.3	Dec	2004	20%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
Erythromycin-H20	Macrolides	0	4.2		3.4	Feb	2005	30%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
erythromycin-h20	Macrolides	n.d.	624.8		109.1					HPLC–MS/MS	Taihu Lake, China	Surface Water	(Xu et al., 2014)	table 2
erythromycin-h20	Macrolides	n.d.	120.3		27.7					HPLC–MS/MS	Taihu Lake, China	Sediment	(Xu et al., 2014)	table 2
Erythromycin-H20	Macrolides	n.d.	385		56.4	September	2008	86%	14	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430
Erythromycin-H20	Macrolides	n.d.	55.9		21.78	February	2009	85%	13	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430
Erythromycin-H20	Macrolides	1.1	50.9			october	2010		35	HPLC-ESI-MS-MS	Beibu Gulf	Seawater	(Zheng et al., 2012)	Table 1. pg 28
Erythromycin-H20	Macrolides	9.68	30.52		17.9		2006/7	100%	4	HPLC-MS-MS	River Arno, Italy	River water	(Zuccato et al., 2010)	Table 4, pg 1047

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Erythromycin-H20	Macrolides	1.66	5.31		3.7		2006/7	100%	4	HPLC-MS-MS	River Po, Italy	River water	(Zuccato et al., 2010)	Table 3, pg 1051
Erythromycin-H2O	Macrolides				3.51				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Erythromycin-H2O	Macrolides				3.78				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Erythromycin-H2O	Macrolides		1.7	0.15					52	HPLC	Germany	River water	(Hirsch et al., 1999)	Table 3, pg. 114
Fleroxacin	Fluoroquinolones				0.89				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Fleroxacin	Fluoroquinolones				1.49				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Fleroxacin	Fluoroquinolones	n.d.	6.35	2.05	2.29			70%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Fleroxacin	Fluoroquinolones	n.d.	6.35	n.d.	0.15			2%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
florfenicol	Phenicol	n.d.	241.1		116.3	July	2012	92%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
florfenicol	Phenicol	n.d.	1.3		0.5	July	2012	77%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
Florfenicol	Phenicol	1.6	15.3		0.929			16%	25	HPLC-MS/MS	Shandong province, China	River water	(Hanna et al., 2018)	Table 1, pg. 135
Florfenicol	Phenicol	n.d.	n.d.		n.d.			0%	17	HPLC-MS/MS	Shandong province, China	River sediment	(Hanna et al., 2018)	Table 2, pg. 136
florfenicol	Phenicol	49	340					37%	27	HPLC-MS	Han River, South Korea	River Water	(Kim et al., 2016)	Table 3, pg 350
florfenicol	Phenicol	n.d.	n.d.					0%	37	HPLC-MS	Kyungahn Stream	River Water	(Kim et al., 2016)	Table 3, pg 350
florfenicol	Chloramphenicols	0	2.27		0.53	Summer	2011	75	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
florfenicol	Chloramphenicols	0	1.21		0.43	Summer	2011	40	20	HPLC-MS	Dalian, China	Sediment	(Na et al., 2013)	Table 2, pg 235
florfenicol	Phenicol	n.d.	n.d.	n.d.		July	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
florfenicol	Phenicol	n.d.	n.d.	n.d.		October	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
florfenicol	Phenicol	n.d.	n.d.	n.d.		January	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
florfenicol	Phenicol	n.d.	n.d.	n.d.		May	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
florfenicol	Phenicol	1.88	13.9	10.5		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
florfenicol	Phenicol	0.45	33.8	11.7		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
florfenicol	Phenicol	1.98	89.5	46.7		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
florfenicol	Phenicol	11.5	46.3	20.8		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Flucloxacillin	Penicillins		7				2001-2002			HPLC-MS/MS.	Rotterbach, Germany	River, surface water	(Christian et al., 2003)	Table 3
Flumequine	Fluoroquinolones				2.1		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Flumequine	Fluoroquinolones				6.5		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Flumequine	Fluoroquinolones				7.4		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Flumequine	Fluoroquinolones		32		15		2006	100%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Flumequine	Fluoroquinolones		29		12		2006	67%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
Flumequine	Fluoroquinolones		29		18		2006	80%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Flumequine	Fluoroquinolones		27		12		2006	75%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
Flumequine	Fluoroquinolones		13		11		2006	20%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
josamycin	Macrolides	n.d.	0.9	n.d.	0.07			15%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
josamycin	Macrolides	n.d.	n.d.	n.d.	n.d.			0%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Leucomycin	Macrolides				1.59				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Leucomycin	Macrolides				0.96				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
levofloxacin	Fluoroquinolones	5000	8800							LC-MS/MS	Ujjain, Indai	Waste water	(Diwan et al., 2010)	Table 5, Pg 6
Levofloxacin	Fluoroquinolones	0.3	6		0.534			28%	25	HPLC-MS/MS	Shandong province, China	River water	(Hanna et al., 2018)	Table 1, pg. 135
Levofloxacin	Fluoroquinolones	0.82	2.89		0.277			24%	17	HPLC-MS/MS	Shandong province, China	River sediment	(Hanna et al., 2018)	Table 2, pg. 136
L-floxacin/ofloxacin	Quinolones	n.d.	10.88		5		2006/7	75%	4	HPLC-MS-MS	River Arno, Italy	River water	(Zuccato et al., 2010)	Table 4, pg 1047
L-floxacin/ofloxacin	Quinolones	0.65	18.06		10.9		2006/7	100%	4	HPLC-MS-MS	River Po, Italy	River water	(Zuccato et al., 2010)	Table 3, pg 1048
lincomycin	Lincosamides	2500	240000	64000	86500	Oct-Dec	1998	100%	8	LC/ESI-MS	Iowa and Ohio, USA	Lagoon water	(Campagnolo et al., 2002)	Table 2
lincomycin	Lincosamides	500	500	500	500	Oct-Dec	1998	100%	1	LC/ESI-MS	Iowa and Ohio, USA	Stream water	(Campagnolo et al., 2002)	Table 2
lincomycin	Lincosamides	0.2	24.7	6	6.4			100%	15	UHPLC-MS/MS	Dongjiang River, China	Sediment	(Chen et al., 2018)	Table 1
lincomycin	Lincosamides	0	14.9			Normal (Oct)	2014	75%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
lincomycin	Lincosamides	0	7.7			Dry (Dec)	2014	75%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
lincomycin	Lincosamides	5	16.5			Dry (Dec)	2015	100%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
lincomycin	Lincosamides	0	1.9			Wet (Jun)	2015	12%	18	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
lincomycin	Lincosamides		0.06	0.73				19%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
lincomycin	Lincosamides		730					19%	115	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
lincomycin	Lincosamides	5.34	10.92		8.1		2006/7	100%	4	HPLC-MS-MS	River Arno, Italy	River water	(Zuccato et al., 2010)	Table 4, pg 1047
lincomycin	Lincosamides	3.72	4.47		5.7		2006/7	100%	4	HPLC-MS-MS	River Po, Italy	River water	(Zuccato et al., 2010)	Table 3, pg 1052
Lomefloxacin	Fluoroquinolones	n.d.	n.d.	n.d.	n.d.			0%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Lomefloxacin	Fluoroquinolones	n.d.	29	n.d.	0.98			7%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Lomefloxacin	Fluoroquinolones				0.6				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Lomefloxacin	Fluoroquinolones				1.75				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Lomefloxacin	Fluoroquinolones				5.3		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Lomefloxacin	Fluoroquinolones				2.1		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Lomefloxacin	Fluoroquinolones		<10		-		2006	0%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Lomefloxacin	Fluoroquinolones		<10		-		2006	0%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
Lomefloxacin	Fluoroquinolones		<10		-		2006	0%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Lomefloxacin	Fluoroquinolones		<10		-		2006	0%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
Lomefloxacin	Fluoroquinolones		<10		-		2006	0%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Lomefloxacin	Fluoroquinolones		1100						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Lomefloxacin	Fluoroquinolones		420						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Lomefloxacin	Fluoroquinolones		45						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Lomefloxacin	Fluoroquinolones	0	13.1			Autumn	2011	33.3	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Lomefloxacin	Fluoroquinolones	0	3			Spring	2014	25	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Lomefloxacin	Fluoroquinolones	0	2.2			Autumn	2011	20	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Lomefloxacin	Fluoroquinolones	0	2.3			Spring	2014	10.5	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Marbofloxacin	Fluoroquinolones				0.85				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Marbofloxacin	Fluoroquinolones				0.64				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Marbofloxacin	Fluroquinolones				43				3	HPLC-ESI-MS-MS	Belgioioso, Italy	Farm soil	(Sturini et al., 2012)	Table 2, pg 132
Marbofloxacin	Fluroquinolones				15				3	HPLC-ESI-MS-MS	Linarolo, Italy	Farm soil	(Sturini et al., 2012)	Table 2, pg 132
Marbofloxacin	Fluroquinolones				24				3	HPLC-ESI-MS-MS	Torre d'Isola, Italy	Farm soil	(Sturini et al., 2012)	Table 2, pg 132
Methacycline	Tetracyclines				1.34				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Methacycline	Tetracyclines				1.11				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Metronidazole	nitroimidazole	0.4	1.6		0.101			12%	25	HPLC-MS/MS	Shandong province, China	River water	(Hanna et al., 2018)	Table 1, pg. 135
Metronidazole	nitroimidazole	n.d.	n.d.		n.d.			0%	17	HPLC-MS/MS	Shandong province, China	River sediment	(Hanna et al., 2018)	Table 2, pg. 136
Monensin	Ionophores				0.5777				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Monensin	Ionophores				1.42				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Nalidixic acid	Quinolones				18		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Nalidixic acid	Quinolones				51		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Nalidixic acid	Quinolones				77		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Nalidixic acid	Quinolones		<10		-		2006	0%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Nalidixic acid	Quinolones		<10		-		2006	0%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
Nalidixic acid	Quinolones		<10		-		2006	10%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Nalidixic acid	Quinolones		<10		-		2006	8%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
Nalidixic acid	Quinolones		<10		-		2006	0%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Narasin	Ionophores				2.37				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Narasin	Ionophores				50				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Norfloxacin	Fluoroquinolones	n.d.	0.2		2.6	July	2012	39%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
Norfloxacin	Fluoroquinolones				0.97				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Norfloxacin	Fluoroquinolones				0.88				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Norfloxacin	Fluoroquinolones	9.7	132.3	22	30.3			100%	15	UHPLC-MS/MS	Dongjiang River, China	Sediment	(Chen et al., 2018)	Table 1
Norfloxacin	Fluoroquinolones	6400	29600							LC-MS/MS	Ujjain, Indai	Waste water	(Diwan et al., 2010)	Table 5, Pg 6
Norfloxacin	Fluoroquinolones	0.2	78.1		4.359			24%	25	HPLC-MS/MS	Shandong province, China	River water	(Hanna et al., 2018)	Table 1, pg. 135
Norfloxacin	Fluoroquinolones	0.14	2.2		0.455			71%	17	HPLC-MS/MS	Shandong province, China	River sediment	(Hanna et al., 2018)	Table 2, pg. 136
Norfloxacin	Fluoroquinolones		0.12	0.12				1%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
Norfloxacin	Fluoroquinolones	n.d.	156	19.9	28.6			67%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Norfloxacin	Fluoroquinolones	49.4	1140	255	267			100%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Norfloxacin	Fluoroquinolones	n.d.	1.65		0.1	Dec	2015	11%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Norfloxacin	Fluoroquinolones	n.d.	1.11		0.15	Aug	2016	28%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Norfloxacin	Fluoroquinolones	n.d.	27				2008	65%	20	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Minh et al., 2009)	Table 5, pg 1058
Norfloxacin	Fluoroquinolones	0.771	20.2	2.44		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Norfloxacin	Fluoroquinolones	0.87	25.6	8.3		October	2011	71%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Norfloxacin	Fluoroquinolones	n.d.	69.3	3.53		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Norfloxacin	Fluoroquinolones	n.d.	39.6	2.64		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Norfloxacin	Fluoroquinolones		120					90%	##	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Norfloxacin	Fluoroquinolones				1.6		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Norfloxacin	Fluoroquinolones				225		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Norfloxacin	Fluoroquinolones				89		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Norfloxacin	Fluoroquinolones		163		46		2006	36%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Norfloxacin	Fluoroquinolones		13		-		2006	33%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
Norfloxacin	Fluoroquinolones		13		-		2006	40%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Norfloxacin	Fluoroquinolones		60		31		2006	33%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
Norfloxacin	Fluoroquinolones		34		22		2006	40%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Norfloxacin	Fluoroquinolones		680						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Norfloxacin	Fluoroquinolones		4700						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Norfloxacin	Fluoroquinolones		140						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Norfloxacin	Fluoroquinolones	5.04	118.58		25.32	Winter			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Sediment	(Kafaei et al., 2018)	Table S5
Norfloxacin	Fluoroquinolones	7.95	83.53		21.75	Summer			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Sediment	(Kafaei et al., 2018)	Table S5
Norfloxacin	Fluoroquinolones	19.36	63.01		33.92	Winter			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Seawater	(Kafaei et al., 2018)	Table S5
Norfloxacin	Fluoroquinolones	35.04	89.43		51.5	Summer			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Seawater	(Kafaei et al., 2018)	Table S5
Norfloxacin	Fluoroquinolones	0	6.9			Autumn	2011	22.2	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Norfloxacin	Fluoroquinolones	0	134.2			Spring	2014	83.3	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Norfloxacin	Fluoroquinolones	0	4.5			Autumn	2011	64	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Norfloxacin	Fluoroquinolones	0	47.1			Spring	2014	78.9	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Norfloxacin	Fluoroquinolones	0.06	6.06							HPLC	Vietnam	River water	(Le and Munekage, 2004)	Table 23, pg. 926
Norfloxacin	Fluoroquinolones	0.08	4.04							HPLC	Vietnam	River water	(Le and Munekage, 2004)	Table 23, pg. 926
Norfloxacin	Fluoroquinolones	6.51	2615.96							HPLC	Vietnam	River sediment	(Le and Munekage, 2004)	Table 23, pg. 926
Norfloxacin	Fluoroquinolones	0	13		12	March	2005	16%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
Norfloxacin	Fluoroquinolones	-	251		150	June	2005	100%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
Norfloxacin	Fluoroquinolones	0	28.1		9.4	Dec	2004	40%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
Norfloxacin	Fluoroquinolones	0	20.1		12.3	Feb	2005	40%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
Norfloxacin	Fluoroquinolones	n.d.	6.5		4.3					HPLC–MS/MS	Taihu Lake, China	Surface Water	(Xu et al., 2014)	table 2
Norfloxacin	Fluoroquinolones	n.d.	28.4		9.9					HPLC–MS/MS	Taihu Lake, China	Sediment	(Xu et al., 2014)	table 2
Norfloxacin	Fluoroquinolones	n.d.	n.d.	n.d		July	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Norfloxacin	Fluoroquinolones	n.d.	n.d.	n.d		October	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Norfloxacin	Fluoroquinolones	n.d.	n.d.	n.d		January	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Norfloxacin	Fluoroquinolones	n.d.	14.2	n.d		May	2012	43%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Norfloxacin	Fluoroquinolones	n.d.	1120		172.39	September	2008	57%	14	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430
Norfloxacin	Fluoroquinolones	n.d.	403		148.9	February	2009	69%	13	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430
Norfloxacin	Fluoroquinolones	n.d.	572		118	September	2009	83%	23	HPLC-ESI-MS-MS	Laizhou Bay, China	River Water	(Zhang et al., 2012)	Table 1, pg 210
Ofloxacin	Fluoroquinolones	n.d.	28.5		6.5	July	2012	69%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
Ofloxacin	Fluoroquinolones	n.d.	12.4		4.1	July	2012	92%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
Ofloxacin	Fluoroquinolones				0.797				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Ofloxacin	Fluoroquinolones				1.05				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Ofloxacin	Fluroquinolones	n.d.	13.5	5	6.9			93%	15	UHPLC-MS/MS	Dongjiang River, China	Sediment	(Chen et al., 2018)	Table 1
Ofloxacin	Fluroquinolones		20				2001-2002			HPLC-MS/MS.	Rotterbach, Germany	River, surface water	(Christian et al., 2003)	Table 3
Ofloxacin	Fluroquinolones		5				2001-2002			HPLC-MS/MS.	Weser, Germany	River, surface water	(Christian et al., 2003)	Table 3
Ofloxacin	Fluoroquinolones	4500	7500							LC-MS/MS	Ujjain, Indai	Waste water	(Diwan et al., 2010)	Table 5, Pg 6
Ofloxacin	Fluroquinolones	n.d.	8.9					2	50	LC-MS/MS	Minnesota, USA	Lake water	(Ferrey et al., 2015)	Table 3
Ofloxacin	Fluroquinolones	0.38	32.6	6.65	9.23			100%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Ofloxacin	Fluroquinolones	n.d.	362	8.64	21			62%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Ofloxacin	Fluroquinolones	n.d.	0.53		0.09	Dec	2015	22%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Ofloxacin	Fluroquinolones	n.d.	n.d.		n.d.	Aug	2016	0%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Ofloxacin	Fluoroquinolones	8.1	634				2008	100%	20	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Minh et al., 2009)	Table 5, pg 1058
Ofloxacin	Fluoroquinolones	0.63	61	2.78		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Ofloxacin	Fluoroquinolones	1.22	48.1	11.2		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Ofloxacin	Fluoroquinolones	n.d.	458.2	12		January	2012	86%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Ofloxacin	Fluoroquinolones	n.d.	206.3	3.22		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Ofloxacin	Fluoroquinolones				4.9		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Ofloxacin	Fluoroquinolones				498		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Ofloxacin	Fluoroquinolones				603		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Ofloxacin	Fluoroquinolones		910						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Ofloxacin	Fluoroquinolones		180						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Ofloxacin	Fluoroquinolones		10000						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Ofloxacin	Fluoroquinolones		6400						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Ofloxacin	Fluoroquinolones		630						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Ofloxacin	Fluoroquinolones	0	23			Autumn	2011	33.3	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Ofloxacin	Fluoroquinolones	0	135.1			Spring	2014	75	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Ofloxacin	Fluoroquinolones	0	7.6			Autumn	2011	68	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Ofloxacin	Fluoroquinolones	0	1.9			Spring	2014	10.5	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Ofloxacin	Fluoroquinolones	0	16		11	March	2005	16%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
Ofloxacin	Fluoroquinolones	-	108		77	June	2005	100%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
Ofloxacin	Fluoroquinolones	0	8.1		5.2	Dec	2004	50%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
Ofloxacin	Fluoroquinolones	0	16.4		10	Feb	2005	60%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
Ofloxacin	Fluroquinolones	n.d.	82.8		32.2					HPLC–MS/MS	Taihu Lake, China	Surface Water	(Xu et al., 2014)	table 2
Ofloxacin	Fluroquinolones	n.d.	52.8		16.5					HPLC–MS/MS	Taihu Lake, China	Sediment	(Xu et al., 2014)	table 2
Ofloxacin	Fluoroquinolones	n.d.	n.d.	n.d		July	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Ofloxacin	Fluoroquinolones	n.d.	n.d.	n.d		October	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Ofloxacin	Fluoroquinolones	n.d.	4.13	n.d		January	2012	43%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Ofloxacin	Fluoroquinolones	n.d.	12.4	0.85		May	2012	57%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Ofloxacin	Fluoroquinolones	n.d.	1560		234.64	September	2008	57%	14	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430
Ofloxacin	Fluoroquinolones	11.4	1440		385.7	February	2009	77%	13	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Ofloxacin	Fluoroquinolones	n.d.	45.4		9.9	September	2009	39%	23	HPLC-ESI-MS-MS	Laizhou Bay, China	River Water	(Zhang et al., 2012)	Table 1, pg 210
Oleandomycin	Macrolides				2.01				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Oleandomycin	Macrolides				1.55				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Oleandomycin	Macrolides	n.d.	n.d.		n.d.		2006/7	0%	4	HPLC-MS-MS	River Arno, Italy	River water	(Zuccato et al., 2010)	Table 4, pg 1047
Oleandomycin	Macrolides	n.d.	n.d.		n.d.		2006/7	0%	4	HPLC-MS-MS	River Po, Italy	River water	(Zuccato et al., 2010)	Table 3, pg 1056
Olfoxacin	Quinolones		<10		-		2006	9%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Olfoxacin	Quinolones		<10		-		2006	0%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
Olfoxacin	Quinolones		<10		-		2006	20%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Olfoxacin	Quinolones		55		30		2006	17%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
Olfoxacin	Quinolones		<10		-		2006	20%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Ornidazol	Nitro-imidazole		53		-		2006	9%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Ornidazol	Nitro-imidazole		12		-		2006	17%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
Ornidazol	Nitro-imidazole		10		-		2006	20%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Ornidazol	Nitro-imidazole		58		28		2006	33%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
Ornidazol	Nitro-imidazole		<10		-		2006	0%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Oxolinic acid	Quinolones				1.6		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Oxolinic acid	Quinolones				8.1		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Oxolinic acid	Quinolones				3.8		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Oxolinic acid	Diaminopyrimidines		<10		-		2006	18%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Oxolinic acid	Diaminopyrimidines		<10		-		2006	17%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
Oxolinic acid	Diaminopyrimidines		<10		-		2006	10%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Oxolinic acid	Diaminopyrimidines		19		13		2006	17%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
Oxolinic acid	Diaminopyrimidines		<10		-		2006	0%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Oxolinic acid	Diaminopyrimidines	0.01	2.5							HPLC	Vietnam	River water	(Le and Munekage, 2004)	Table 23, pg. 926
Oxolinic acid	Diaminopyrimidines	0.01	2.31							HPLC	Vietnam	River water	(Le and Munekage, 2004)	Table 23, pg. 926

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Oxolinic acid	Diaminopyrimidines	1.81	426.31							HPLC	Vietnam	River sediment	(Le and Munekage, 2004)	Table 23, pg. 926
Oxytetracycline	Tetracyclines	n.d.	219.8		78.3	July	2012	85%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
Oxytetracycline	Tetracyclines	n.d.	18.6		6.9	July	2012	100%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
Oxytetracycline	Tetracyclines				5				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Oxytetracycline	Tetracyclines				1.82				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Oxytetracycline	Tetracyclines	12.2	102.4	44.2	44.6			100%	15	UHPLC-MS/MS	Dongjiang River, China	Sediment	(Chen et al., 2018)	Table 1
Oxytetracycline	Tetracyclines	0	48.7			Dry (Dec)	2015	80%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Oxytetracycline	Tetracyclines	0	8.9			Wet (Jun)	2015	53%	18	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Oxytetracycline	Tetracyclines	n.d.	n.d.		n.d.	Winter	2007/8	0%	10	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Oxytetracycline	Tetracyclines	n.d.	0.65		0.32	Spring	2007/8	50%	4	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Oxytetracycline	Tetracyclines	n.d.	n.d.		n.d.	Winter	2007/8	0%	10	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Oxytetracycline	Tetracyclines	n.d.	0.68		0.28	Spring	2007/8	50%	4	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Oxytetracycline	Tetracyclines	n.d.	n.d.		n.d.	Winter	2007/8	0%	10	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Oxytetracycline	Tetracyclines	n.d.	0.32		0.11	Spring	2007/8	50%	4	LC-MS/MS	Arc River, Aix en Provence France	River water	(Feitosa-Felizzola and Chiron, 2009)	Table 2, pg 54
Oxytetracycline	Tetracyclines		n.d.	n.d.					14	HPLC	Germany	River water	(Hirsch et al., 1999)	Table 3, pg. 114
Oxytetracycline	Tetracyclines	133	1236					7%	27	HPLC-MS	Han River, South Korea	River Water	(Kim et al., 2016)	Table 3, pg 350
Oxytetracycline	Tetracyclines	30	30					3%	37	HPLC-MS	Kyungahn Stream	River Water	(Kim et al., 2016)	Table 3, pg 350
Oxytetracycline	Tetracyclines		0.34	0.34				1%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
Oxytetracycline	Tetracyclines		340					1%	84	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Oxytetracycline	Tetracyclines	700	1340							LC-MS/MS	KS, USA	Surface Water	(Lindsey et al., 2001)	Table 4
Oxytetracycline	Tetracyclines	n.d.	340							LC-MS/MS	Suwannee River, GA	Surface Water	(Lindsey et al., 2001)	Table 4
Oxytetracycline	Tetracyclines	n.d.	n.d.		n.d.	Dec	2015	0%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Oxytetracycline	Tetracyclines	n.d.	n.d.		n.d.	Aug	2016	0%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Oxytetracycline	Tetracyclines	n.d.	44				2008	10%	20	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Minh et al., 2009)	Table 5, pg 1058

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Oxytetracycline	Tetracyclines	1.09	6.28		2.46	Summer	2011	100	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
Oxytetracycline	Tetracyclines	0.52	2.68	0.99		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Oxytetracycline	Tetracyclines	0.525	14	0.951		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Oxytetracycline	Tetracyclines	0.305	8.13	0.765		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Oxytetracycline	Tetracyclines	0.552	13.9	4.02		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Oxytetracycline	Tetracyclines				3.4				20	HPLC–MS/MS	Austria	Farm soil	Martínez-Carballo et al., 2007)	Table 3
Oxytetracycline	Tetracyclines	0	4.5			Autumn	2011	44.4	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Oxytetracycline	Tetracyclines	0	61.8			Spring	2014	33.3	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Oxytetracycline	Tetracyclines	0	4.1			Autumn	2011	8	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Oxytetracycline	Tetracyclines	0	28.7			Spring	2014	57.9	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Oxytetracycline	Tetracyclines	n.d.	72.8		44.2					HPLC–MS/MS	Taihu Lake, China	Surface Water	(Xu et al., 2014)	table 2
Oxytetracycline	Tetracyclines	n.d.	196.7		52.8					HPLC–MS/MS	Taihu Lake, China	Sediment	(Xu et al., 2014)	table 2
Oxytetracycline	Tetracyclines	n.d.	n.d.	n.d		July	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Oxytetracycline	Tetracyclines	n.d.	n.d.	n.d		October	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Oxytetracycline	Tetracyclines	n.d.	0.48	n.d		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Oxytetracycline	Tetracyclines	5.13	22.5	11.6		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Oxytetracycline	Tetracyclines	n.d.	42.19		11.41	September	2008	71%	14	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430
Oxytetracycline	Tetracyclines	4.18	139		63.14	February	2009	92%	13	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430
Oxytetracycline	Tetracyclines	n.d.	n.d.		n.d.		2006/7	0%	4	HPLC-MS-MS	River Arno, Italy	River water	(Zuccato et al., 2010)	Table 4, pg 1047
Oxytetracycline	Tetracyclines	n.d.	1.82		1.1		2006/7	50%	4	HPLC-MS-MS	River Po, Italy	River water	(Zuccato et al., 2010)	Table 3, pg 1057
Pefloxacin	Fluoroquinolones				1.4				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Pefloxacin	Fluoroquinolones				1.37				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
penicillin	Penicillins			73			2004-2005	1	6	HPLC	Wangyang River	River water	(Li et al., 2008)	Table 2
penicillin	Penicillins			78			2004-2006	1	6	HPLC	Wangyang River	River water	(Li et al., 2008)	Table 2
Penicillin G	Penicillins	n.d.	28.9	n.d.	4.8			33%	15	UHPLC-MS/MS	Dongjiang River, China	Sediment	(Chen et al., 2018)	Table 1

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Penicillin G	Penicillins		n.d.	n.d.					14	HPLC	Germany	River water	(Hirsch et al., 1999)	Table 3, pg. 114
Penicillin V	Penicillins	n.d.	73.2	11.3	16.3			80%	15	UHPLC-MS/MS	Dongjiang River, China	Sediment	(Chen et al., 2018)	Table 1
Penicillin V	Penicillins		n.d.	n.d.					14	HPLC	Germany	River water	(Hirsch et al., 1999)	Table 3, pg. 114
Pipemidic acid	pyridopyrimidine				17		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Pipemidic acid	pyridopyrimidine				18		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Pipemidic acid	Diaminopyrimidines		<10		-		2006	0%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Pipemidic acid	Diaminopyrimidines		<10		-		2006	0%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
Pipemidic acid	Diaminopyrimidines		<10		-		2006	0%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Pipemidic acid	Diaminopyrimidines		<10		-		2006	0%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
Pipemidic acid	Diaminopyrimidines		<10		-		2006	0%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Piperacillin	Ureidopenicillin		48				2001-2002			HPLC-MS/MS.	Rotterbach, Germany	River, surface water	(Christian et al., 2003)	Table 3
Roxithromycin	Macrolides	0.2	2.2		0.9	July	2012	100%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
Roxithromycin	Macrolides	0.3	4.1		1.9	July	2012	100%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
Roxithromycin	Macrolides				2.11				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Roxithromycin	Macrolides				1.34				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Roxithromycin	Macrolides		4				2001-2002			HPLC-MS/MS.	Boker-Heide-Kanal, Germany	River, surface water	(Christian et al., 2003)	Table 3
Roxithromycin	Macrolides		6				2001-2002			HPLC-MS/MS.	Emmer, Germany	River, surface water	(Christian et al., 2003)	Table 3
Roxithromycin	Macrolides		5				2001-2002			HPLC-MS/MS.	Nethe, Germany	River, surface water	(Christian et al., 2003)	Table 3
Roxithromycin	Macrolides		14				2001-2002			HPLC-MS/MS.	Rotterbach, Germany	River, surface water	(Christian et al., 2003)	Table 3
Roxithromycin	Macrolides		10				2001-2002			HPLC-MS/MS.	Weser, Germany	River, surface water	(Christian et al., 2003)	Table 3
Roxithromycin	Macrolides		12				2001-2002			HPLC-MS/MS.	Wormkebach, Germany	River, surface water	(Christian et al., 2003)	Table 3

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
roxithromycin	Macrolides	2.8	11.1			Normal (Oct)	2014	100%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
roxithromycin	Macrolides	0	9			Dry (Dec)	2014	88%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
roxithromycin	Macrolides	3.6	10.1			Dry (Dec)	2015	100%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
roxithromycin	Macrolides	0	5.8			Wet (Jun)	2015	100%	18	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Roxithromycin	Macrolides		0.56	n.d.					52	HPLC	Germany	River water	(Hirsch et al., 1999)	Table 3, pg. 114
Roxithromycin	Macrolides		0.05	0.18				5%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
Roxithromycin	Macrolides		180					5%	108	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Roxithromycin	Macrolides	n.d.	155	2.64	27.2			93%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Roxithromycin	Macrolides	n.d.	302	36.4	64.9			93%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Roxithromycin	Macrolides	n.d.	47				2008	35%	20	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Minh et al., 2009)	Table 5, pg 1058
Roxithromycin	Macrolides	1.06	5.84	3.27		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Roxithromycin	Macrolides	0.653	17.5	2.26		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Roxithromycin	Macrolides	n.d.	51.5	6.05		January	2012	86%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Roxithromycin	Macrolides	0.483	28.5	4.64		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Roxithromycin	Macrolides	2.9	9.8			Autumn	2011	100	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Roxithromycin	Macrolides	0.6	6.7			Spring	2014	100	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Roxithromycin	Macrolides	1.3	2.9			Autumn	2011	100	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Roxithromycin	Macrolides	0.3	3.7			Spring	2014	100	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Roxithromycin	Macrolides	0	105		16	March	2005	92%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
Roxithromycin	Macrolides	-	169		66	June	2005	100%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
Roxithromycin	Macrolides	0	21.1		6.1	Dec	2004	30%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
Roxithromycin	Macrolides	0	30.6		5.1	Feb	2005	50%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
Roxithromycin	Macrolides	n.d.	218.3		50.7					HPLC–MS/MS	Taihu Lake, China	Surface Water	(Xu et al., 2014)	table 2

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Roxithromycin	Macrolides	n.d.	45.2		16.9					HPLC–MS/MS	Taihu Lake, China	Sediment	(Xu et al., 2014)	table 2
Roxithromycin	Macrolides	0.18	8.2	0.43		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Roxithromycin	Macrolides	0.13	6.85	1.23		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Roxithromycin	Macrolides	0.05	3.45	1.38		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Roxithromycin	Macrolides	0.36	5.45	0.57		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Roxithromycin	Macrolides	n.d.	133		52.8	September	2008	86%	14	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430
Roxithromycin	Macrolides	n.d.	40.9		14.65	February	2009	85%	13	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430
Roxithromycin	Macrolides	n.d.	227		37.9	September	2009	83%	23	HPLC-ESI-MS-MS	Laizhou Bay, China	River Water	(Zhang et al., 2012)	Table 1, pg 210
Roxithromycin	Macrolides	n.d.	0.53			october	2010		35	HPLC-ESI-MS-MS	Beibu Gulf	Seawater	(Zheng et al., 2012)	Table 1. pg 28
Salinomycin	Ionophores				2.45				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Salinomycin	Ionophores				50				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sarafloxacin	Fluroquinolones	3000	4000	3500	3330	Oct-Dec	1998	42%	7	LC/ESI-MS	Iowa and Ohio, USA	Stream water	(Campagnolo et al., 2002)	Table 2
Sarafloxacin	Fluroquinolones	3000	3000	3000	3000	Oct-Dec	1998	100%	1	LC/ESI-MS	Iowa and Ohio, USA	Stream water	(Campagnolo et al., 2002)	Table 2
Sarafloxacin	Fluroquinolones		n.d.	n.d.				0%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
Sarafloxacin	Fluroquinolones		n.d.					0%	115	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Sarafloxacin	Fluroquinolones	n.d.	28.2	11	9.3			70%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Sarafloxacin	Fluroquinolones	n.d.	n.d.	n.d.	n.d.			0%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Sarafloxacin	Fluroquinolones	n.d.	21.29		4.91	Dec	2015	39%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Sarafloxacin	Fluroquinolones	n.d.	5.59		0.66	Aug	2016	28%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Sarafloxacin	Fluroquinolones		<10		-		2006	9%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Sarafloxacin	Fluroquinolones		<10		-		2006	0%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
Sarafloxacin	Fluroquinolones		<10		-		2006	10%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Sarafloxacin	Fluroquinolones		<10		-		2006	0%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
Sarafloxacin	Fluroquinolones		10		-		2006	0%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
spiramycin	Macrolides	n.d.	2.96	n.d.	0.24			15%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
spiramycin	Macrolides	n.d.	n.d.	n.d.	n.d.			0%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Spiramycin	Macrolides	n.d.	17.92		7.9		2006/7	75%	4	HPLC-MS-MS	River Arno, Italy	River water	(Zuccato et al., 2010)	Table 4, pg 1047
Spiramycin	Macrolides	n.d.	2.35		1.1		2006/7	25%	4	HPLC-MS-MS	River Po, Italy	River water	(Zuccato et al., 2010)	Table 3, pg 1053
Sulfacetamide	Sulfonamides	0	1.51		0.33	Summer	2011	20	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
Sulfacetamide	Sulfonamides	0	1.39		0.13	Summer	2011	10	20	HPLC-MS	Dalian, China	Sediment	(Na et al., 2013)	Table 2, pg 235
sulfachloropyridazine	Sulfonamides	60	60					4%	27	HPLC-MS	Han River, South Korea	River Water	(Kim et al., 2016)	Table 3, pg 350
sulfachloropyridazine	Sulfonamides	n.d.	n.d.					0%	37	HPLC-MS	Kyungahn Stream	River Water	(Kim et al., 2016)	Table 3, pg 350
sulfachloropyridazine	Sulfonamides		n.d.	n.d.				0%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
sulfachloropyridazine	Sulfonamides		n.d.					0%	84	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Sulfachlorpyridazine	Sulfonamides	n.d.	89.4		27.1					HPLC–MS/MS	Taihu Lake, China	Surface Water	(Xu et al., 2014)	table 2
Sulfachlorpyridazine	Sulfonamides	n.d.	15.8		7.3					HPLC–MS/MS	Taihu Lake, China	Sediment	(Xu et al., 2014)	table 2
Sulfadiazine	Sulfonamides	4.9	112.5		53.6	July	2012	100%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
Sulfadiazine	Sulfonamides	0.07	0.71		0.4	July	2012	100%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
Sulfadiazine	Sulfonamides				1.3				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfadiazine	Sulfonamides				0.87				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfadiazine	Sulfonamides	0.7	19.5	3.8	7.6			100%	15	UHPLC-MS/MS	Dongjiang River, China	Sediment	(Chen et al., 2018)	Table 1
Sulfadiazine	Sulfonamides	0	56.2			Dry (Dec)	2015	90%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
sulfadiazine	Sulfonamides	n.d.	505	56	118			100%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
sulfadiazine	Sulfonamides	n.d.	2.07	n.d.	0.41			42%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Sulfadiazine	Sulfonamides	0.77	61.28		24.35	Dec	2015	100%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Sulfadiazine	Sulfonamides	n.d.	8.73		1.07	Aug	2016	78%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Sulfadiazine	Sulfonamides	0.045	0.2	0.12		July	2011	29%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfadiazine	Sulfonamides	n.d.	n.d.	n		October	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Sulfadiazine	Sulfonamides	n.d.	n.d.	n		January	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfadiazine	Sulfonamides	n.d.	0.469	0.452		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfadiazine	Sulfonamides				1.1				20	HPLC–MS/MS	Austria	Farm soil	Martínez-Carballo et al., 2007)	Table 3
Sulfadiazine	Sulfonamides	0	37.4			Autumn	2011	33.3	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Sulfadiazine	Sulfonamides	0	5.5			Spring	2014	75	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Sulfadiazine	Sulfonamides	0	9.6			Spring	2014	68.4	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Sulfadiazine	Sulfonamides	-	141		38	March	2005	100%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
Sulfadiazine	Sulfonamides	-	336		209	June	2005	100%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
Sulfadiazine	Sulfonamides	0	0		0	Dec	2004	0%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
Sulfadiazine	Sulfonamides	0	0		0	Feb	2005	0%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
Sulfadiazine	Sulfonamides	0.55	23.9	13		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfadiazine	Sulfonamides	n.d.	43.5	21.6		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfadiazine	Sulfonamides	n.d.	71.8	32.5		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfadiazine	Sulfonamides	9.97	61.5	21.6		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfadiazine	Sulfonamides	n.d.	15		2.51	September	2008	36%	14	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430
Sulfadiazine	Sulfonamides	n.d.	83.9		18.6	February	2009	69%	13	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430
Sulfadiazine	Sulfonamides	n.d.	18.7		1.7	September	2009	48%	23	HPLC-ESI-MS-MS	Laizhou Bay, China	River Water	(Zhang et al., 2012)	Table 1, pg 210
Sulfadiazine	Sulfonamides	n.d.	3.41			october	2010		35	HPLC-ESI-MS-MS	Beibu Gulf	Seawater	(Zheng et al., 2012)	Table 1. pg 28
Sulfadimethoxine	Sulfonamides	2500	2500	2500	2500	Oct-Dec	1998	100%	8	LC/ESI-MS	Iowa and Ohio, USA	Lagoon water	(Campagnolo et al., 2002)	Table 2
Sulfadimethoxine	Sulfonamides	350	350	350	350	Oct-Dec	1998	14%	7	LC/ESI-MS	Iowa and Ohio, USA	Stream water	(Campagnolo et al., 2002)	Table 2
Sulfadimethoxine	Sulfonamides	500	500	500	500	Oct-Dec	1998	100%	1	LC/ESI-MS	Iowa and Ohio, USA	Stream water	(Campagnolo et al., 2002)	Table 2
Sulfadimethoxine	Sulfonamides				0.8				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfadimethoxine	Sulfonamides				1.21				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfadimethoxine	Sulfonamides	10	80					30%	27	HPLC-MS	Han River, South Korea	River Water	(Kim et al., 2016)	Table 3, pg 350
Sulfadimethoxine	Sulfonamides	n.d.	n.d.					0%	37	HPLC-MS	Kyungahn Stream	River Water	(Kim et al., 2016)	Table 3, pg 350

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Sulfadimethoxine	Sulfonamides		0.06	0.06				1%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
Sulfadimethoxine	Sulfonamides		60					1%	84	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Sulfadimethoxine	Sulfonamides	0.86	n.d.	n.d.	n.d.			0%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Sulfadimethoxine	Sulfonamides	n.d.	0.2	n.d.	0.04			27%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Sulfadimethoxine	Sulfonamides	240	15000							LC-MS/MS	KS, USA	Surface Water	(Lindsey et al., 2001)	Table 4
Sulfadimethoxine	Sulfonamides	n.d.	60							LC-MS/MS	North Dry Creek, Kearny, NE	Surface Water	(Lindsey et al., 2001)	Table 4
Sulfadimethoxine	Sulfonamides	0	2		0.13	Summer	2011	10	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
sulfadimidine	Sulfonamides		7				2001-2002			HPLC-MS/MS.	Emmer, Germany	River, surface water	(Christian et al., 2003)	Table 3
sulfadimidine	Sulfonamides		3				2001-2002			HPLC-MS/MS.	Nethe, Germany	River, surface water	(Christian et al., 2003)	Table 3
sulfadimidine	Sulfonamides		3				2001-2002			HPLC-MS/MS.	Weser, Germany	River, surface water	(Christian et al., 2003)	Table 3
sulfadimidine	Sulfonamides	0	7.7			Normal (Oct)	2014	88%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
sulfadimidine	Sulfonamides	1.22	13.4			Dry (Dec)	2014	100%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
sulfadimidine	Sulfonamides	1.3	22.2			Dry (Dec)	2015	100%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
sulfadimidine	Sulfonamides	0	4.3			Wet (Jun)	2015	88%	18	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
sulfadimidine	Sulfonamides				1				20	HPLC–MS/MS	Austria	Farm soil	Martínez-Carballo et al., 2007)	Table 3
sulfadimidine	Sulfonamides	-	179		67	March	2005	100%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
sulfadimidine	Sulfonamides	-	323		184	June	2005	100%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
sulfadimidine	Sulfonamides	0	0		0	Dec	2004	0%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
sulfadimidine	Sulfonamides	0	0		0	Feb	2005	0%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
sulfadimidine	Sulfonamides	n.d.	3.36			october	2010		35	HPLC-ESI-MS-MS	Beibu Gulf	Seawater	(Zheng et al., 2012)	Table 1. pg 28
Sulfadizine	Sulfonamides	0	2.05		0.19	Summer	2011	15	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
Sulfadizine	Sulfonamides	0	1.68		0.5	Summer	2011	35	20	HPLC-MS	Dalian, China	Sediment	(Na et al., 2013)	Table 2, pg 235

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Sulfadoxine	Sulfonamides				0.98				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfadoxine	Sulfonamides				1.23				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfadoxine	Sulfonamides	0	1.88		0.33	Summer	2011	40	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
Sulfadoxine	Sulfonamides				2.5				20	HPLC–MS/MS	Austria	Farm soil	Martínez-Carballo et al., 2007)	Table 3
Sulfamerazine	Sulfonamides	n.d.	n.d.		n.d.	July	2012	23%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
Sulfamerazine	Sulfonamides	0.03	0.8		0.2	July	2012	100%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
Sulfamerazine	Sulfonamides				1.13				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfamerazine	Sulfonamides				1.15				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfamerazine	Sulfonamides	nd	12.5					4	50	LC-MS/MS	Minnesota, USA	Lake water	(Ferrey et al., 2015)	Table 3
Sulfamerazine	Sulfonamides		n.d.	n.d.				0%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
Sulfamerazine	Sulfonamides		n.d.					0%	104	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Sulfamerazine	Sulfonamides	n.d.	n.d.	n.d.	n.d.			0%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Sulfamerazine	Sulfonamides	n.d.	2.47	n.d.	0.05			2%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Sulfamerazine	Sulfonamides	0	3.67		1.73	Summer	2011	55	20	HPLC-MS	Dalian, China	Sediment	(Na et al., 2013)	Table 2, pg 235
Sulfamerazine	Sulfonamides	n.d.	n.d.	n		July	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfamerazine	Sulfonamides	n.d.	n.d.	n.d		October	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfamerazine	Sulfonamides	n.d.	n.d.	n		January	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfamerazine	Sulfonamides	n.d.	0.408	n.d.		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfamerazine	Sulfonamides	0	11			Autumn	2011	77.8	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Sulfamerazine	Sulfonamides	0	8.1			Spring	2014	66.7	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Sulfamerazine	Sulfonamides	0	7			Autumn	2011	72	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Sulfamerazine	Sulfonamides	0	0.6			Spring	2014	52.6	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Sulfamerazine	Sulfonamides	n.d.	n.d.	n.d		July	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfamerazine	Sulfonamides	n.d.	n.d.	n.d		October	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Sulfamerazine	Sulfonamides	n.d.	n.d.	n.d		January	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfamerazine	Sulfonamides	n.d.	n.d.	n.d		May	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfameter	Sulfonamides				0.63				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfameter	Sulfonamides				0.86				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfameter	Sulfonamides	0	1.92		0.1	Summer	2011	5	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
Sulfameter	Sulfonamides	0	56.65		8.82	Summer	2011	20	20	HPLC-MS	Dalian, China	Sediment	(Na et al., 2013)	Table 2, pg 235
Sulfamethazine	Sulfonamides	2500	400000	100000	149600	Oct-Dec	1998	100%	8	LC/ESI-MS	Iowa and Ohio, USA	Lagoon water	(Campagnolo et al., 2002)	Table 2
Sulfamethazine	Sulfonamides	19.9	389.4		188.9	July	2012	100%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
Sulfamethazine	Sulfonamides	0.2	2.7		1.2	July	2012	100%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
Sulfamethazine	Sulfonamides				1.03				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfamethazine	Sulfonamides				0.99				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfamethazine	Sulfonamides	nd	134					4	50	LC-MS/MS	Minnesota, USA	Lake water	(Ferrey et al., 2015)	Table 3
Sulfamethazine	Sulfonamides		n.d.	n.d.					52	HPLC	Germany	River water	(Hirsch et al., 1999)	Table 3, pg. 114
Sulfamethazine	Sulfonamides	10	67					11%	27	HPLC-MS	Han River, South Korea	River Water	(Kim et al., 2016)	Table 3, pg 350
Sulfamethazine	Sulfonamides	10	123					47%	37	HPLC-MS	Kyungahn Stream	River Water	(Kim et al., 2016)	Table 3, pg 350
sulfamethazine	Sulfonamides		0.22	0.22				1%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
Sulfamethazine	Sulfonamides		220					1%	84	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Sulfamethazine	Sulfonamides	n.d.	16.1	2.68	5.25			85%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Sulfamethazine	Sulfonamides	n.d.	6.92	1.08	1.47			93%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Sulfamethazine	Sulfonamides	n.d.	220							LC-MS/MS	North Dry Creek, Kearny, NE	Surface Water	(Lindsey et al., 2001)	Table 4
Sulfamethazine	Sulfonamides	n.d.	14.88		3.46	Dec	2015	61%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Sulfamethazine	Sulfonamides	n.d.	2.46		0.67	Aug	2016	67%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Sulfamethazine	Sulfonamides	n.d.	8.6				2008	10%	20	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Minh et al., 2009)	Table 5, pg 1058
Sulfamethazine	Sulfonamides	0	2.81		0.34	Summer	2011	15	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
Sulfamethazine	Sulfonamides	0	1.76		0.26	Summer	2011	15	20	HPLC-MS	Dalian, China	Sediment	(Na et al., 2013)	Table 2, pg 235

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Sulfamethazine	Sulfonamides	n.d.	1.54	0.768		July	2011	29%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfamethazine	Sulfonamides	0.559	0.559	0.559		October	2011	14%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfamethazine	Sulfonamides	n.d.	4.84	2.42		January	2012	43%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfamethazine	Sulfonamides	n.d.	1.3	n.d.		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfamethazine	Sulfonamides		<10		-		2006	0%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Sulfamethazine	Sulfonamides		<10		-		2006	0%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
Sulfamethazine	Sulfonamides		<10		-		2006	0%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Sulfamethazine	Sulfonamides		<10		-		2006	0%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
Sulfamethazine	Sulfonamides		<10		-		2006	40%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Sulfamethazine	Sulfonamides	0	33.8			Autumn	2011	77.8	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Sulfamethazine	Sulfonamides	0.5	16.4			Spring	2014	100	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Sulfamethazine	Sulfonamides	0	0.4			Autumn	2011	8	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Sulfamethazine	Sulfonamides	0	1.2			Spring	2014	63.2	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Sulfamethazine	Sulfonamides		44.08	1.95	6.54	june/august	2013-2015	100%	290	POCIS	Iowa River, USA	River water	(Washington et al., 2018)	Table 2, pg 362
Sulfamethazine	Sulfonamides		32.7	0.9	6.2	june/august	2013-2016	88%	290	POCIS	Iowa River, USA	River water	(Washington et al., 2018)	Table 2, pg 363
Sulfamethazine	Sulfonamides		1.99	0.4	0.72	june/august	2013-2017	54%	290	POCIS	Iowa River, USA	River water	(Washington et al., 2018)	Table 2, pg 364
Sulfamethazine	Sulfonamides		66.92	0.7	5.3	june/august	2013-2018	92%	290	POCIS	Iowa River, USA	River water	(Washington et al., 2018)	Table 2, pg 365
Sulfamethazine	Sulfonamides	2.28	23.1	5.25		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfamethazine	Sulfonamides	0.53	89.1	47.6		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfamethazine	Sulfonamides	1.23	73.3	36.8		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfamethazine	Sulfonamides	5.45	24.4	12.2		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
sulfamethazine	Sulfonamides	n.d.	248		35.7	September	2008	79%	14	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430
sulfamethazine	Sulfonamides	n.d.	154		45.76	February	2009	92%	13	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Sulfamethazine	Sulfonamides	n.d.	108		12.8	September	2009	96%	23	HPLC-ESI-MS-MS	Laizhou Bay, China	River Water	(Zhang et al., 2012)	Table 1, pg 210
Sulfamethizole	Sulfonamides	nd	2.48					2	50	LC-MS/MS	Minnesota, USA	Lake water	(Ferrey et al., 2015)	Table 3
Sulfamethizole	Sulfonamides		0.13	0.13				1%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
Sulfamethizole	Sulfonamides		130					1%	104	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Sulfamethizole	Sulfonamides	0	1.34		0.38	Summer	2011	30	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
sulfamethoazole	Sulfonamides		0.15	1.9				13%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
Sulfamethox	Sulfonamides		94		14	Spring	2010	33		UPLC MS/MS	Mar Menor lagoon, Spain	Sediment	(Moreno-González et al., 2015)	Table 2
Sulfamethoxazole	Sulfonamides	<50	<50	<50	<50			0		LC- MSMS	United Kingdom	River water	(Ashton et al., 2004)	Table 6, pg. 175
Sulfamethoxazole	Sulfonamides	<50	<50	<50	<50			0		LC- MSMS	United Kingdom	River water	(Ashton et al., 2004)	Table 6, pg. 175
Sulfamethoxazole	Sulfonamides	2.2	764.9		259.6	July	2012	100%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
Sulfamethoxazole	Sulfonamides	0.05	0.6		0.2	July	2012	100%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
Sulfamethoxazole	Sulfonamides				0.98				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfamethoxazole	Sulfonamides				1.34				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfamethoxazole	Sulfonamides		15				2001-2002			HPLC-MS/MS.	Boker-Heide-Kanal, Germany	River, surface water	(Christian et al., 2003)	Table 3
Sulfamethoxazole	Sulfonamides		19				2001-2002			HPLC-MS/MS.	Emmer, Germany	River, surface water	(Christian et al., 2003)	Table 3
Sulfamethoxazole	Sulfonamides		34				2001-2002			HPLC-MS/MS.	Nethe, Germany	River, surface water	(Christian et al., 2003)	Table 3
Sulfamethoxazole	Sulfonamides		4				2001-2002			HPLC-MS/MS.	Pader, Germany	River, surface water	(Christian et al., 2003)	Table 3
Sulfamethoxazole	Sulfonamides		52				2001-2002			HPLC-MS/MS.	Rotterbach, Germany	River, surface water	(Christian et al., 2003)	Table 3
Sulfamethoxazole	Sulfonamides		35				2001-2002			HPLC-MS/MS.	Weser, Germany	River, surface water	(Christian et al., 2003)	Table 3
Sulfamethoxazole	Sulfonamides		12				2001-2002			HPLC-MS/MS.	Wormkebach, Germany	River, surface water	(Christian et al., 2003)	Table 3
Sulfamethoxazole	Sulfonamides	0	14.5			Normal (Oct)	2014	88%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Sulfamethoxazole	Sulfonamides	0	6.3			Dry (Dec)	2014	88%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Sulfamethoxazole	Sulfonamides	0	4.7			Dry (Dec)	2015	90%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Sulfamethoxazole	Sulfonamides	0	5.1			Wet (Jun)	2015	41%	18	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Sulfamethoxazole	Sulfonamides				7.2		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Sulfamethoxazole	Sulfonamides				2.5		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Sulfamethoxazole	Sulfonamides	n.d.	57.1					2	50	LC-MS/MS	Minnesota, USA	Lake water	(Ferrey et al., 2015)	Table 3
Sulfamethoxazole	Sulfonamides	nd	57.1					2	50	LC-MS/MS	Minnesota, USA	Lake water	(Ferrey et al., 2015)	Table 3
Sulfamethoxazole	Sulfonamides	0.3	13		1.301			52%	25	HPLC-MS/MS	Shandong province, China	River water	(Hanna et al., 2018)	Table 1, pg. 135
Sulfamethoxazole	Sulfonamides	n.d.	n.d.		n.d.			0%	17	HPLC-MS/MS	Shandong province, China	River sediment	(Hanna et al., 2018)	Table 2, pg. 136
Sulfamethoxazole	Sulfonamides		0.48	0.03					52	HPLC	Germany	River water	(Hirsch et al., 1999)	Table 3, pg. 114
Sulfamethoxazole	Sulfonamides	17	270					44%	27	HPLC-MS	Han River, South Korea	River Water	(Kim et al., 2016)	Table 3, pg 350
Sulfamethoxazole	Sulfonamides	10	147					47%	37	HPLC-MS	Kyungahn Stream	River Water	(Kim et al., 2016)	Table 3, pg 350
Sulfamethoxazole	Sulfonamides		1900					13%	104	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
sulfamethoxazole	Sulfonamides	n.d.	940	121	240			96%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
sulfamethoxazole	Sulfonamides	n.d.	7.86	n.d.	0.28			25%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Sulfamethoxazole	Sulfonamides	n.d.	1020							LC-MS/MS	Cuyahoga River, Steele, OH	Surface Water	(Lindsey et al., 2001)	Table 4
Sulfamethoxazole	Sulfonamides	n.d.	220							LC-MS/MS	WA, USA	Groundwater	(Lindsey et al., 2001)	Table 4
Sulfamethoxazole	Sulfonamides	0.47	47.41		11.8	Dec	2015	100%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Sulfamethoxazole	Sulfonamides	n.d.	5.63		1.36	Aug	2016	83%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Sulfamethoxazole	Sulfonamides	n.d.	47				2008	40%	20	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Minh et al., 2009)	Table 5, pg 1058
Sulfamethoxazole	Sulfonamides	0	2.23		1.28	Summer	2011	90	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
Sulfamethoxazole	Sulfonamides	0.515	0.515	0.515		July	2011	14%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfamethoxazole	Sulfonamides	n.d.	n.d.	n.d		October	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Sulfamethoxazole	Sulfonamides	n.d.	1.13	0.515		January	2012	29%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfamethoxazole	Sulfonamides	n.d.	0.516	n.d.		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfamethoxazole	Sulfonamides				1.5				20	HPLC–MS/MS	Austria	Farm soil	Martínez-Carballo et al., 2007)	Table 3
Sulfamethoxazole	Sulfonamides		121		54		2006	100%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Sulfamethoxazole	Sulfonamides		544		140		2006	100%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
Sulfamethoxazole	Sulfonamides		72		44		2006	100%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Sulfamethoxazole	Sulfonamides		93		40		2006	100%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
Sulfamethoxazole	Sulfonamides		82		37		2006	100%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Sulfamethoxazole	Sulfonamides	0	5.9			Autumn	2011	88.9	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Sulfamethoxazole	Sulfonamides	0	13.4			Spring	2014	75	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Sulfamethoxazole	Sulfonamides	0	0.1			Autumn	2011	4	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Sulfamethoxazole	Sulfonamides	0	0.8			Spring	2014	42.1	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Sulfamethoxazole	Sulfonamides	0.04	2.39							HPLC	Vietnam	River water	(Le and Munekage, 2004)	Table 23, pg. 926
Sulfamethoxazole	Sulfonamides	0.04	5.57							HPLC	Vietnam	River water	(Le and Munekage, 2004)	Table 23, pg. 926
Sulfamethoxazole	Sulfonamides	4.77	820.49							HPLC	Vietnam	River sediment	(Le and Munekage, 2004)	Table 23, pg. 926
Sulfamethoxazole	Sulfonamides	-	165		37	March	2005	100%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
Sulfamethoxazole	Sulfonamides	-	193		134	June	2005	100%	12	HPLC-MS-MS	Pearl River, Guangzhou	River water	(Xu et al., 2007)	Table 3, pg 675
Sulfamethoxazole	Sulfonamides	0	0		0	Dec	2004	0%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
Sulfamethoxazole	Sulfonamides	0	0		0	Feb	2005	0%	10	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Xu et al., 2007)	Table 3, pg 675
Sulfamethoxazole	Sulfonamides	1.48	28.5	17.6		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfamethoxazole	Sulfonamides	n.d.	36.2	30.3		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfamethoxazole	Sulfonamides	3.49	56.8	43.8		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfamethoxazole	Sulfonamides	4.34	46.5	22		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfamethoxazole	Sulfonamides	0.36	527		62.8	September	2009	100%	23	HPLC-ESI-MS-MS	Laizhou Bay, China	River Water	(Zhang et al., 2012)	Table 1, pg 210
Sulfamethoxazole	Sulfonamides	n.d.	10.4			october	2010		35	HPLC-ESI-MS-MS	Beibu Gulf	Seawater	(Zheng et al., 2012)	Table 1. pg 28

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Sulfamethoxazole	Sulfonamides	1.79	11.4		5.3		2006/7	100%	4	HPLC-MS-MS	River Arno, Italy	River water	(Zuccato et al., 2010)	Table 4, pg 1047
Sulfamethoxazole	Sulfonamides	1.83	2.39		2.1		2006/7	100%	4	HPLC-MS-MS	River Po, Italy	River water	(Zuccato et al., 2010)	Table 3, pg 1054
Sulfamethoxypyridazine	Sulfonamides	0	1.95		0.23	Summer	2011	15	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
Sulfamethoxypyridazine	Sulfonamides	0	7.67		1.49	Summer	2011	25	20	HPLC-MS	Dalian, China	Sediment	(Na et al., 2013)	Table 2, pg 235
Sulfamonomethoxine	Sulfonamides				0.56				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfamonomethoxine	Sulfonamides				1.15				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfamonomethoxine	Sulfonamides	1.5	20.1	5.5	7.8			100%	15	UHPLC-MS/MS	Dongjiang River, China	Sediment	(Chen et al., 2018)	Table 1
Sulfamonomethoxine	Sulfonamides	n.d.	23.1	5.4	6.92			96%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Sulfamonomethoxine	Sulfonamides	n.d.	0.5	n.d.	0.06			27%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Sulfamonomethoxine	Sulfonamides	0	2.28		0.39	Summer	2011	25	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
Sulfamonomethoxine	Sulfonamides	0	7		1.63	Summer	2011	30	20	HPLC-MS	Dalian, China	Sediment	(Na et al., 2013)	Table 2, pg 235
sulfapyridine	Sulfonamides	n.d.	103.1		24.1	July	2012	92%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
sulfapyridine	Sulfonamides	n.d.	6.6		1.7	July	2012	92%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
Sulfapyridine	Sulfonamides				0.75				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfapyridine	Sulfonamides				0.93				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
sulfapyridine	Sulfonamides	0	17.2			Dry (Dec)	2015	10%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Sulfapyridine	Sulfonamides	0.2	3.1		0.135			13%	25	HPLC-MS/MS	Shandong province, China	River water	(Hanna et al., 2018)	Table 1, pg. 135
Sulfapyridine	Sulfonamides	n.d.	n.d.		n.d.			0%	17	HPLC-MS/MS	Shandong province, China	River sediment	(Hanna et al., 2018)	Table 2, pg. 136
Sulfapyridine	Sulfonamides	n.d.	85	2.58	13			63%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Sulfapyridine	Sulfonamides	n.d.	1.4	n.d.	0.16			25%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
sulfapyridine	Sulfonamides	0.32	3.71	1.36		July	2011	57%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
sulfapyridine	Sulfonamides	n.d.	4.38	0.602		October	2011	43%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
sulfapyridine	Sulfonamides	n.d.	3.28	0.759		January	2012	71%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
sulfapyridine	Sulfonamides	n.d.	9.12	n.d.		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
sulfapyridine	Sulfonamides	n.d.	28.6	1.7		July	2011	86%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
sulfapyridine	Sulfonamides	n.d.	31.9	2.43		October	2011	71%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
sulfapyridine	Sulfonamides	n.d.	219	1.98		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
sulfapyridine	Sulfonamides	n.d.	17.2	3.26		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
sulfaquinoxaline	Sulfonamides	n.d.	64.2		21.5	July	2012	92%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
sulfaquinoxaline	Sulfonamides	0.08	0.9		0.4	July	2012	100%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
Sulfaquinoxaline	Sulfonamides				0.85				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfaquinoxaline	Sulfonamides				1.56				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfaquinoxaline	Sulfonamides	0.8	6.6	1.9	2.8			100%	15	UHPLC-MS/MS	Dongjiang River, China	Sediment	(Chen et al., 2018)	Table 1
sulfaquinoxaline	Sulfonamides	0.202	0.469	0.269		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
sulfaquinoxaline	Sulfonamides	0.082	0.608	0.373		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
sulfaquinoxaline	Sulfonamides	0.42	0.959	0.427		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
sulfaquinoxaline	Sulfonamides	n.d.	0.117	0.04		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
sulfaquinoxaline	Sulfonamides	n.d.	1.58	0.23		July	2011	86%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
sulfaquinoxaline	Sulfonamides	n.d.	4.55	0.5		October	2011	71%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
sulfaquinoxaline	Sulfonamides	0.05	23.5	0.78		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
sulfaquinoxaline	Sulfonamides	0.09	1.53	0.21		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfathiazole	Sulfonamides	n.d.	121.1		34.1	July	2012	92%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
Sulfathiazole	Sulfonamides	n.d.	0.6		0.2	July	2012	100%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
Sulfathiazole	Sulfonamides				1.11				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfathiazole	Sulfonamides				0.97				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfathiazole	Sulfonamides	0	16.7			Dry (Dec)	2015	10%	10	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Sulfathiazole	Sulfonamides	nd	32.8					10	50	LC-MS/MS	Minnesota, USA	Lake water	(Ferrey et al., 2015)	Table 3
Sulfathiazole	Sulfonamides	40	40					4%	27	HPLC-MS	Han River, South Korea	River Water	(Kim et al., 2016)	Table 3, pg 350

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Sulfathiazole	Sulfonamides	10	123					24%	37	HPLC-MS	Kyungahn Stream	River Water	(Kim et al., 2016)	Table 3, pg 350
Sulfathiazole	Sulfonamides		n.d.	n.d.				0%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
Sulfathiazole	Sulfonamides		n.d.					0%	104	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Sulfathiazole	Sulfonamides	n.d.	1.38	n.d.	0.08			7%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Sulfathiazole	Sulfonamides	n.d.	5.94	0.57	0.64			76%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Sulfathiazole	Sulfonamides	n.d.	80							LC-MS/MS	KS, USA	Surface Water	(Lindsey et al., 2001)	Table 4
Sulfathiazole	Sulfonamides	0	1.24		0.34	Summer	2011	35	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
Sulfathiazole	Sulfonamides	0	1.89		0.09	Summer	2011	5	20	HPLC-MS	Dalian, China	Sediment	(Na et al., 2013)	Table 2, pg 235
Sulfathiazole	Sulfonamides	n.d.	n.d.	n		July	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfathiazole	Sulfonamides	n.d.	n.d.	n.d		October	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfathiazole	Sulfonamides	n.d.	n.d.	n		January	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfathiazole	Sulfonamides	n.d.	n.d.	n.d.		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Sulfathiazole	Sulfonamides				3.9				20	HPLC–MS/MS	Austria	Farm soil	Martínez-Carballo et al., 2007)	Table 3
Sulfathiazole	Sulfonamides	0	0.5			Autumn	2011	11.1	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Sulfathiazole	Sulfonamides	0	3.7			Spring	2014	75	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Sulfathiazole	Sulfonamides	0	1.4			Spring	2014	52.6	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Sulfathiazole	Sulfonamides	n.d.	1.45	n.d		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfathiazole	Sulfonamides	n.d.	1.68	n.d		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfathiazole	Sulfonamides	n.d.	5.23	n.d		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfathiazole	Sulfonamides	n.d.	3.57	n.d		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Sulfisoxazole	Sulfonamides				1.02				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfisoxazole	Sulfonamides				1.63				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Sulfisoxazole	Sulfonamides	n.d.	n.d.	n.d.	n.d.			0%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Sulfisoxazole	Sulfonamides	n.d.	1.71	0.81	0.71			65%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Sulfisoxazole	Sulfonamides	0	2.01		0.23	Summer	2011	20	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
sulphadimethoxine	Sulfonamides	n.d.	43.3		11.9					HPLC–MS/MS	Taihu Lake, China	Surface Water	(Xu et al., 2014)	table 2
sulphadimethoxine	Sulfonamides	n.d.	15.7		6.9					HPLC–MS/MS	Taihu Lake, China	Sediment	(Xu et al., 2014)	table 2
sulphamethazine	Sulfonamides	n.d.	654		252.7					HPLC–MS/MS	Taihu Lake, China	Surface Water	(Xu et al., 2014)	table 2
sulphamethazine	Sulfonamides	n.d.	99.8		39.8					HPLC–MS/MS	Taihu Lake, China	Sediment	(Xu et al., 2014)	table 2
sulphamethoxazole	Sulfonamides	n.d.	114.7		48.4					HPLC–MS/MS	Taihu Lake, China	Surface Water	(Xu et al., 2014)	table 2
sulphamethoxazole	Sulfonamides	n.d.	49.3		16.1					HPLC–MS/MS	Taihu Lake, China	Sediment	(Xu et al., 2014)	table 2
sulphathiazole	Sulfonamides	n.d.	134.5		45.9	May	2010			HPLC–MS/MS	Taihu Lake, China	Surface Water	(Xu et al., 2014)	table 2
sulphathiazole	Sulfonamides	n.d.	51.7		17.8					HPLC–MS/MS	Taihu Lake, China	Sediment	(Xu et al., 2014)	table 2
sulphisoxazole	Sulfonamides	n.d.	61.4		44.4					HPLC–MS/MS	Taihu Lake, China	Surface Water	(Xu et al., 2014)	table 2
sulphisoxazole	Sulfonamides	n.d.	22.6		11					HPLC–MS/MS	Taihu Lake, China	Sediment	(Xu et al., 2014)	table 2
Tetracycline	Tetracyclines	25000	410000	35000	107400	Oct-Dec	1998	100%	8	LC/ESI-MS	Iowa and Ohio, USA	Lagoon water	(Campagnolo et al., 2002)	Table 2
Tetracycline	Tetracyclines	1000	1000	1000	1000	Oct-Dec	1998	14%	7	LC/ESI-MS	Iowa and Ohio, USA	Stream water	(Campagnolo et al., 2002)	Table 2
Tetracycline	Tetracyclines	n.d.	54.3		4.2	July	2012	15%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
Tetracycline	Tetracyclines	n.d.	21.7		3.5	July	2012	85%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
Tetracycline	Tetracyclines				5				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Tetracycline	Tetracyclines				1.47				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Tetracycline	Tetracyclines	6	95.7	30.4	40			100%	15	UHPLC-MS/MS	Dongjiang River, China	Sediment	(Chen et al., 2018)	Table 1
Tetracycline	Tetracyclines	0	10.8			Wet (Jun)	2015	59%	18	SPE–UPLC–MS/MS	Poyang Lake, China	Freshwater lake	(Ding et al., 2017)	Table 1, pg 141
Tetracycline	Tetracyclines		n.d.	n.d.					14	HPLC	Germany	River water	(Hirsch et al., 1999)	Table 3, pg. 114
Tetracycline	Tetracyclines	12	2093					11%	27	HPLC-MS	Han River, South Korea	River Water	(Kim et al., 2016)	Table 3, pg 350
Tetracycline	Tetracyclines	23	37					8%	37	HPLC-MS	Kyungahn Stream	River Water	(Kim et al., 2016)	Table 3, pg 350
Tetracycline	Tetracyclines		0.11	0.11				1%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
Tetracycline	Tetracyclines		110					1%	84	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Tetracycline	Tetracyclines	n.d.	110							LC-MS/MS	Snake Creek, GA, USA	Surface Water	(Lindsey et al., 2001)	Table 4
Tetracycline	Tetracyclines	n.d.	21.51		2.17	Dec	2015	28%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Tetracycline	Tetracyclines	n.d.	n.d.		n.d.	Aug	2016	0%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Tetracycline	Tetracyclines	n.d.	313				2008	30%	20	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Minh et al., 2009)	Table 5, pg 1058
Tetracycline	Tetracyclines	0	3.82		1.03	Summer	2011	65	20	HPLC-MS	Dalian, China	Seawater	(Na et al., 2013)	Table 2, pg 235
Tetracycline	Tetracyclines	1.66	1.74		1.68	Summer	2011	100	20	HPLC-MS	Dalian, China	Sediment	(Na et al., 2013)	Table 2, pg 235
Tetracycline	Tetracyclines	n.d.	n.d.	n		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Tetracycline	Tetracyclines	n.d.	6.15	n		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Tetracycline	Tetracyclines	n.d.	6.84	n		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Tetracycline	Tetracyclines	n.d.	2.16	0.914		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
Tetracycline	Tetracyclines				3.3				20	HPLC–MS/MS	Austria	Farm soil	Martínez-Carballo et al., 2007)	Table 3
Tetracycline	Tetracyclines	3.59	23.31		10.05	Winter			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Sediment	(Kafaei et al., 2018)	Table S5
Tetracycline	Tetracyclines	3.53	29.36		11.73	Summer			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Sediment	(Kafaei et al., 2018)	Table S5
Tetracycline	Tetracyclines	4	71.15		20.37	Winter			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Seawater	(Kafaei et al., 2018)	Table S5
Tetracycline	Tetracyclines	21.57	52.69		31.27	Summer			8	HPLC–ESI-MS/MS	Persian Gulf, Iran	Seawater	(Kafaei et al., 2018)	Table S5
Tetracycline	Tetracyclines	0	15.8			Autumn	2011	22.2	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Tetracycline	Tetracyclines	0	137.4			Spring	2014	66.7	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Tetracycline	Tetracyclines	0	6			Autumn	2011	28	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Tetracycline	Tetracyclines	0	115.2			Spring	2014	73.7	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Tetracycline	Tetracyclines	n.d.	87.9		43.2					HPLC–MS/MS	Taihu Lake, China	Surface Water	(Xu et al., 2014)	table 2
Tetracycline	Tetracyclines	n.d.	112.2		47.9					HPLC–MS/MS	Taihu Lake, China	Sediment	(Xu et al., 2014)	table 2
Tetracycline	Tetracyclines	n.d.	n.d.	n.d		July	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Tetracycline	Tetracyclines	n.d.	n.d.	n.d		October	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Tetracycline	Tetracyclines	n.d.	n.d.	n.d		January	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Tetracycline	Tetracyclines	n.d.	2.37	n.d		May	2012	71%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
tetracycline	Tetracyclines	n.d.	21.1		2.535	September	2008	36%	14	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430
tetracycline	Tetracyclines	3.97	40.9		22.46	February	2009	92%	13	RRLC-MS/MS	Pearl River, China	Sediment	(Yang et al., 2010)	Table 3, pg 3430
thiamphenicol	Chloramphenicols	n.d.	0.6		0.5	July	2012	85%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	River water	(Chen and Zhou, 2014)	Table 2, pg 609
thiamphenicol	Chloramphenicols	n.d.	1.3		0.4	July	2012	69%	13	UHPLC-MS/MS	Huangpu River, Shanghai,China	Sediment	(Chen and Zhou, 2014)	Table 2, pg 609
thiamphenicol	Chloramphenicols	n.d.	n.d.	n.d.		July	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
thiamphenicol	Chloramphenicols	n.d.	n.d.	n.d.		October	2011	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
thiamphenicol	Chloramphenicols	n.d.	n.d.	n.d.		January	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
thiamphenicol	Chloramphenicols	n.d.	n.d.	n.d.		May	2012	0%	7	UHPLC-MS/MS	Yangtze River Estuary, China	Sediment	(Shi et al., 2014)	table 2, pg 320
thiamphenicol	Chloramphenicols	3.33	86.6	15.2		July	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
thiamphenicol	Chloramphenicols	n.d.	74	17.4		October	2011	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
thiamphenicol	Chloramphenicols	n.d.	110	62.9		January	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
thiamphenicol	Chloramphenicols	6.56	44.2	19.2		May	2012	100%	7	UHPLC-MS/MS	Yangtze River Estuary, China	River Water	(Yan et al., 2013)	table 2, pg 24
Tilmicosin	Macrolides	n.d.	6.67		2.3		2006/7	50%	4	HPLC-MS-MS	River Arno, Italy	River water	(Zuccato et al., 2010)	Table 4, pg 1047
Tilmicosin	Macrolides	n.d.	8.93		2.5		2006/7	25%	4	HPLC-MS-MS	River Po, Italy	River water	(Zuccato et al., 2010)	Table 3, pg 1058
Trimethoprim	Sulfonamides	<10	36	<10	<10			36		LC- MSMS	United Kingdom	River water	(Ashton et al., 2004)	Table 6, pg. 175
Trimethoprim	Sulfonamides	<10	42	<10	12			38		LC- MSMS	United Kingdom	River water	(Ashton et al., 2004)	Table 6, pg. 175
Trimethoprim	Sulfonamides	2500	2500	2500	2500	Oct-Dec	1998	100%	8	LC/ESI-MS	Iowa and Ohio, USA	Lagoon water	(Campagnolo et al., 2002)	Table 2
Trimethoprim	Sulfonamides	60	270	150	160	Oct-Dec	1998	57%	7	LC/ESI-MS	Iowa and Ohio, USA	Stream water	(Campagnolo et al., 2002)	Table 2
Trimethoprim	Sulfonamides				0.73				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Trimethoprim	Sulfonamides				1.08				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Trimethoprim	Sulfonamides		4				2001-2002			HPLC-MS/MS.	Nethe, Germany	River, surface water	(Christian et al., 2003)	Table 3
Trimethoprim	Sulfonamides		8				2001-2002			HPLC-MS/MS.	Rotterbach, Germany	River, surface water	(Christian et al., 2003)	Table 3

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Trimethoprim	Sulfonamides		12				2001-2002			HPLC-MS/MS.	Weser, Germany	River, surface water	(Christian et al., 2003)	Table 3
Trimethoprim	Sulfonamides		3				2001-2002			HPLC-MS/MS.	Wormkebach, Germany	River, surface water	(Christian et al., 2003)	Table 3
Trimethoprim	Sulfonamides				6.6		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Trimethoprim	Sulfonamides				52		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Trimethoprim	Sulfonamides				26		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Trimethoprim	Sulfonamides	nd	6.22					4%	50	LC-MS/MS	Minnesota, USA	Lake water	(Ferrey et al., 2015)	Table 3
Trimethoprim	Sulfonamides		17						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Trimethoprim	Sulfonamides		660						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Trimethoprim	Sulfonamides		4000						2	HPLC	Isakavagu-Nakkavagu river India	River water	(Fick et al., 2009)	Table 3, pg. 2525
Trimethoprim	Sulfonamides		0.2	n.d.					52	HPLC	Germany	River water	(Hirsch et al., 1999)	Table 3, pg. 114
Trimethoprim	Sulfonamides	10	587					22%	27	HPLC-MS	Han River, South Korea	River Water	(Kim et al., 2016)	Table 3, pg 350
Trimethoprim	Sulfonamides	10	27					32%	37	HPLC-MS	Kyungahn Stream	River Water	(Kim et al., 2016)	Table 3, pg 350
trimethoprim	Sulfonamides		0.15	0.71				13%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
Trimethoprim	Sulfonamides		710					13%	104	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Trimethoprim	Sulfonamides	n.d.	216				2008	50%	20	HPLC-MS-MS	Victoria Harbour, Hong Kong	Seawater	(Minh et al., 2009)	Table 5, pg 1058
Trimethoprim	Sulfonamides				0.49				20	HPLC–MS/MS	Austria	Farm soil	Martínez-Carballo et al., 2007)	Table 3
Trimethoprim	Sulfonamides		23		17		2006	36%	11	UPLC-MS/MS	Caudebec, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Trimethoprim	Sulfonamides		31		20		2006	50%	6	UPLC-MS/MS	Honfleur, Siene	River Water	(Tamtam et al., 2008)	Table 2, pg 88
Trimethoprim	Sulfonamides		16		12		2006	70%	10	UPLC-MS/MS	La Bouille, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Trimethoprim	Sulfonamides		45		16		2006	58%	12	UPLC-MS/MS	Poses, Siene River, France	River water	(Tamtam et al., 2008)	Table 2, pg 88
Trimethoprim	Sulfonamides		27		27		2006	20%	5	UPLC-MS/MS	Tancarville, Siene	River water	(Tamtam et al., 2008)	Table 2, pg 88
Trimethoprim	Sulfonamides	0	19			Autumn	2011	88.9	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Trimethoprim	Sulfonamides	0	7.2			Spring	2014	91.7	12	RP-LC	Jianghan Plain, China	Surface Water	(Tong et al., 2014)	Table 2, pg 185
Trimethoprim	Sulfonamides	0	0.2			Autumn	2011	4	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Trimethoprim	Sulfonamides	0	5.2			Spring	2014	68.4	27	RP-LC	Jianghan Plain, China	Groundwater	(Tong et al., 2014)	Table 2, pg 185
Trimethoprim	Sulfonamides	0.08	1.04							HPLC	Vietnam	River water	(Le and Munekage, 2004)	Table 23, pg. 926
Trimethoprim	Sulfonamides	0.08	2.03							HPLC	Vietnam	River water	(Le and Munekage, 2004)	Table 23, pg. 926
Trimethoprim	Sulfonamides	9.02	734.61							HPLC	Vietnam	River sediment	(Le and Munekage, 2004)	Table 23, pg. 926
Trimethoprim	Sulfonamides	n.d.	40.8		12					HPLC–MS/MS	Taihu Lake, China	Surface Water	(Xu et al., 2014)	table 2
Trimethoprim	Sulfonamides	n.d.	39.3		9.3					HPLC–MS/MS	Taihu Lake, China	Sediment	(Xu et al., 2014)	table 2
Trimethoprim	Sulfonamides	n.d.	13600		1133	September	2009	96%	23	HPLC-ESI-MS-MS	Laizhou Bay, China	River Water	(Zhang et al., 2012)	Table 1, pg 210
Trimethoprim	Sulfonamides	n.d.	3.77			october	2010		35	HPLC-ESI-MS-MS	Beibu Gulf	Seawater	(Zheng et al., 2012)	Table 1. pg 28
Trimethroprim	Sulfonamides	n.d.	2.25		0.32	Dec	2015	28%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Trimethroprim	Sulfonamides	n.d.	2.12		0.39	Aug	2016	50%	18	UPLC	Donting Lake, China	Lake water	(Liu et al., 2018)	Table 2
Trimethroprim	Sulfonamides		1.2		0.7	Spring	2010	100		UPLC MS/MS	Mar Menor lagoon, Spain	Sediment	(Moreno-González et al., 2015)	Table 2
Tylosin	Macrolides				1.29				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Tylosin	Macrolides				1.06				3	RRLC-MS/MS	Hailing island, China	Seawater	(Chen et al., 2015)	Sup data
Tylosin	Macrolide	n.d.	4.8	n.d.	0.6			47%	15	UHPLC-MS/MS	Dongjiang River, China	Sediment	(Chen et al., 2018)	Table 1
Tylosin	Macrolide		0.04	0.28				14%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
tylosin	Macrolide		280					14%	115	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
Tylosin	Macrolide	n.d.	1.88	n.d.	0.1			7%	27	HPLC-LC-MS/MS	Baiyangdian Lake, China	Surface Water	(Li et al., 2012)	Table 1
Tylosin	Macrolide	n.d.	n.d.	n.d.	n.d.			0%	45	HPLC-LC-MS/MS	Baiyangdian Lake, China	Sediment	(Li et al., 2012)	Table 1
Tylosin	Macrolide		0.27	0.03	0.07	june/august	2013-2019	88%	290	POCIS	Iowa River, USA	River water	(Washington et al., 2018)	Table 2, pg 366
Tylosin	Macrolide		6.92	0.1	0.87	june/august	2013-2020	52%	290	POCIS	Iowa River, USA	River water	(Washington et al., 2018)	Table 2, pg 367
Tylosin	Macrolide		0.61	0.06	0.1	june/august	2013-2021	57%	290	POCIS	Iowa River, USA	River water	(Washington et al., 2018)	Table 2, pg 368

Antibiotic	Antibiotic class	Min Concentration ng/L	Max Concentration ng/L	Median concentration ng/L	Mean concentration ng/L	Season	Year	Detection Frequency %	n	Detection method	Location	Type of environment	Reference	Page number
Tylosin	Macrolide		22.8	0.08	3.45	june/august	2013-2022	48%	290	POCIS	Iowa River, USA	River water	(Washington et al., 2018)	Table 2, pg 369
Tylosin	Macrolides	n.d.	n.d.		n.d.		2006/7	0%	4	HPLC-MS-MS	River Arno, Italy	River water	(Zuccato et al., 2010)	Table 4, pg 1047
Tylosin	Macrolides	n.d.	n.d.		n.d.		2006/7	0%	4	HPLC-MS-MS	River Po, Italy	River water	(Zuccato et al., 2010)	Table 3, pg 1059
Vancomycin	Glycopeptides	n.d.	7	1.1	1.4			60%	15	UHPLC-MS/MS	Dongjiang River, China	Sediment	(Chen et al., 2018)	Table 1
Vancomycin	Glycopeptides				90		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Vancomycin	Glycopeptides				22		2010/11		6	LC-MS/MS	Charmoise River, France	Sediment	(Dinh et al., 2017)	Table 2, pg 488
Vancomycin	Glycopeptides	0.44	5.17		2.6		2006/7	100%	4	HPLC-MS-MS	River Arno, Italy	River water	(Zuccato et al., 2010)	Table 4, pg 1047
Vancomycin	Glycopeptides	0.59	11.69		4.8		2006/7	100%	4	HPLC-MS-MS	River Po, Italy	River water	(Zuccato et al., 2010)	Table 3, pg 1055
virginamycin	streptogramin		n.d.	n.d.				0%		LC/MS-ESI	USA	Stream water	(Kolpin et al., 2002)	Table 1
virginamycin	streptogramin		n.d.					0%	104	LC/MS-ESI	U.S.	Stream Water	(Kolpin et al., 2002)	Table 1, pg. 1204
virginiamycin	streptogramin	187	187					4%	27	HPLC-MS	Han River, South Korea	River Water	(Kim et al., 2016)	Table 3, pg 350
virginiamycin	streptogramin	n.d.	n.d.					0%	37	HPLC-MS	Kyungahn Stream	River Water	(Kim et al., 2016)	Table 3, pg 350

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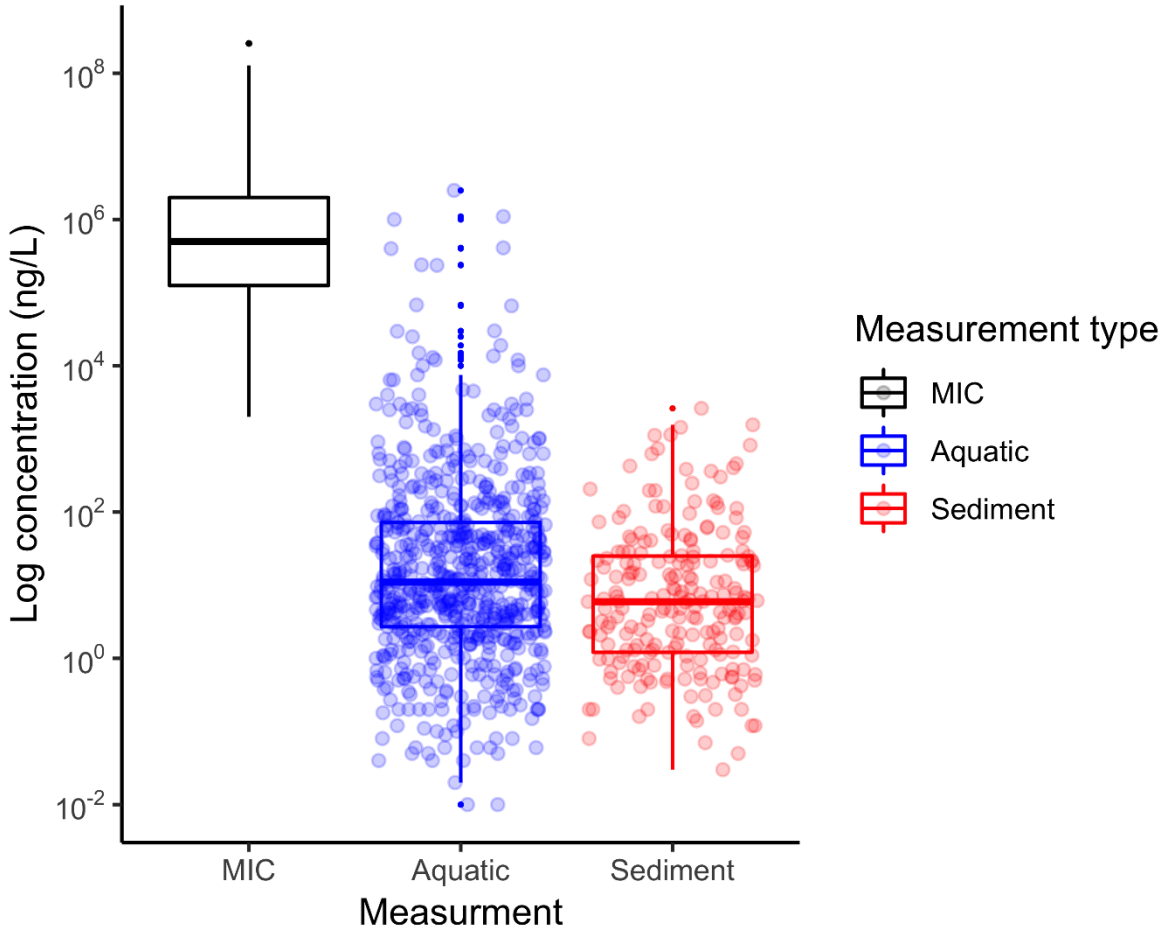
564 Table S2: Standard antibiotic acronyms and classes

Antibiotic	Acronym	Class
Amoxicillin	AMX	B-lactam
Azithromycin	AZM	Macrolides
Cefalexin	CEF	Cefalosporins
Chloramphenicol	CHL	chloramphenicols
Chlortetracycline	CTC	Tetracyclines
Ciprofloxacin	CIP	Fluoroquinolones
Clarithromycin	CLR	Macrolides
Clindamycin	CLI	Fluoroquinolones
Doxycycline	DOX	Tetracyclines
Enoxacin	ENO	Fluoroquinolones
Enrofloxacin	ENR	Fluoroquinolones
Erythromycin	ERY	Macrolides
Erythromycin-H20	ERY-H20	Macrolides
Florfenicol	FFC	chloramphenicols
Flumequine	FLU	Fluoroquinolones
Lincomycin	LIN	Lincosamides
Lomefloxacin	LMX	Fluoroquinolones
Norfloxacin	NOR	Fluoroquinolones
Ofloxacin	OFX	Fluoroquinolones
Oxolinic acid	OXA	Sulfonamides
Oxytetracycline	OTC	Tetracyclines
Penicillin	PEN	B-lactam
Roxithromycin	ROX	Macrolides
Sarafloxacin	SAR	Fluoroquinolones
Sulfadiazine	SDZ	Sulfonamides
Sulfadimethoxine	SDM	Sulfonamides
sulfadimidine	SDI	Sulfonamides
Sulfamerazine	SFMr	Sulfonamides
Sulfamethazine	SMT	Sulfonamides
Sulfamethoxazole	SMX	Sulfonamides
Sulfamethoxypyridazine	SMP	Sulfonamides
sulfapyridine	SP	Sulfonamides
sulfaquinoxaline	SQX	Sulfonamides
Sulfathiazole	SZ	Sulfonamides
Tetracycline	TET	Tetracyclines
Thiamphenicol	TAP	chloramphenicols
Trimethoprim	TMP	Sulfonamides
Tylosin	TYL	Macrolides
Vancomycin	VAN	Glycopeptides

565 Figure S1: Pooled MICs and antibiotic concentrations for sediment and aquatic samples



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Chapter 2: Sub-inhibitory concentrations of kanamycin fix *fusA* mutations in an environmental *Pseudomonas* sp.

Antibiotics can disseminate into the environment and persist at sub-inhibitory concentrations. It is not known what effect such low levels of pollution might have on environmental organisms. Consequently, in this chapter we examined the effect of subinhibitory concentrations of the antibiotic kanamycin on *Pseudomonas protegens,* an environmental bacterium.

Pseudomonas protegens was exposed to 1/10 the MIC of kanamycin via a serial plating experiment. This concentration is representative of concentrations that might be found in the environment, based on the findings outlined in Chapter 1 of this thesis. Exposure to this concentration of kanamycin rapidly fixed a mutation in *fusA,* and this resulted in a four-fold increase in antibiotic resistance.

This finding demonstrates that even low concentrations of antibiotics can drive selection of resistant bacteria. This further suggests that similar selection events are currently occurring in environmental bacteria, contributing to the global problem of antibiotic resistance.

This chapter is the product of a working collaboration between myself, Timothy Ghaly and Michael Gillings. I was predominantly involved in the design of the study, performing the laboratory experiments, interpretation of the data and the drafting of the manuscript. Michael Gillings was involved in the design of the study and contributed to the drafting of the manuscript. Timothy Ghaly was involved in the interpretation of whole genome sequencing data.

Detailed Contributions

	Louise Chow	Michael Gillings	Timothy Ghaly
Design	50%	50%	-
Laboratory work	100%	-	-
Analysis	50%	-	50%
Writing	80%	20%	-

This chapter has been prepared for publication in *FEMS Letters*. The formatting, referencing and word limits adhere to their author guidelines. Figures have been embedded in the text for the purpose of this thesis.

Sub-inhibitory concentrations of kanamycin fix *fusA* mutations in an environmental *Pseudomonas* sp.

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Keywords: antibiotic; pollution; evolution; resistance; aminoglycosides

Abstract

Overuse of antibiotics in medical and agricultural sectors places selection pressure on bacteria, and promotes resistance. Antibiotics can also pollute the environment, where they can persist, and drive evolution of resistance in environmental bacteria. Antibiotic resistant infections are one of the greatest threats to human health, and the selection of resistance in environmental organisms will add to this burden. To explore the dynamics of resistance in environmental compartments, we carried out a serial plating experiment in which an environmental bacterium was exposed to sub-inhibitory concentrations of kanamycin. Several lines showed a rapid increase in kanamycin resistance. Using whole genome sequencing, a single base pair substitution in the *fusA* region was identified, and this mutation fixed after as few as 10 passages. These findings suggest that similar selection events could be occurring in environmental bacteria and will contribute to the growing global problem of antibiotic resistance.

Introduction

Antibiotic usage is central to modern medicine at every level, from prevention to treatment and recovery. Between 2000 and 2015, antibiotic use in health care increased by 65% (Klein *et al.*, 2018). Antibiotics are also widely used in agriculture as a feed supplements and growth promoters, and agricultural use could be four times clinical use (Martin *et al.*, 2015, Robinson *et al.*, 2016). This increasing consumption drives antibiotic resistance via selection for advantageous mutations and for laterally transferred resistance elements (Gillings, 2017).

Emergence of multiple drug resistant strains of bacteria, such as methicillin resistant *Staphylococcus aureus* and *Mycobacterium tuberculosis* poses a significant threat to human health. It has been estimated that by 2050, deaths due to antibiotic resistant pathogens might eclipse cancer as the leading cause of mortality (O'Neill, 2014, WHO, 2014). Antibiotic resistant pathogens have the most impact in lower socio-economic communities where the high cost of second-line antibiotics make them unavailable, and population sizes can be significantly higher (Van Boeckel *et al.*, 2014).

It is clear that clinical use of antibiotics can drive the generation of resistance via selection pressure. However, we also need to examine what happens to antibiotics after their clinical use, because release of antibiotics from waste treatment facilities, and their entry into the environment through agricultural run-off or as pharmaceutical waste (Gothwal & Shashidhar, 2016) has the potential for widespread evolutionary effects . Depending on the antibiotic in question, up to 70-90% of the ingested dose is not metabolized, but is excreted unchanged (Lipsitch *et al.*, 2002, Berge *et al.*, 2005). This means that large quantities of antibiotics make their way into the environment, since current waste treatments do not remove antibiotics efficiently (Sarmah *et al.*, 2006). Antibiotics used in animal husbandry, aquaculture (Cabello, 2006) or fruit spraying (McManus *et al.*, 2002) may enter the

environment directly, or through manuring crops with animal waste containing excreted antibiotics. Antibiotics and their metabolites may also be found in high concentrations downstream from pharmaceutical plants (Dong *et al.*, 2009, Larsson, 2014, Gothwal & Shashidhar, 2016).

When antibiotics are prescribed, the aim is to reach concentrations that eliminate the target pathogen. However, if some cells survive this concentration because they carry genes that confer resistance, these cells and genes can re-populate the microbiome and become a component of waste streams. Therefore, humans and animals should not just be thought of as a source of antibiotics but also as a source of antibiotic resistance genes (Rodriguez-Mozaz *et al.*, 2015, Gillings, 2018).

The antibiotics and antibiotic resistance genes entering the environment by various routes are receiving increasing attention as pollutants, because of their potential to perturb normal microbial communities and to promote antibiotic resistance (Pruden *et al.*, 2013, Zhu *et al.*, 2017, Gillings, 2018). More thought needs to be given to the persistence of antibiotics in the environment at sub-inhibitory concentrations and the effects that this might be having on environmental microbiota. The combination of antibiotics and resistance gene pollution is likely to lead to environmental microorganisms becoming antibiotic resistant, with potential consequences for human and animal health.

Here we examined this possibility by exposing an environmental bacterium to sub-inhibitory concentrations of kanamycin over multiple generations. We followed the appearance of resistance using phenotypic tests and identified the genes involved using whole genome sequencing.

Methods

Bacterial isolates

Pseudomonas protegens strain PF-5 (formerly *Pseudomonas fluorescens* PF-5) was selected for this study (GenBank: CP000076.1. (Paulsen *et al.*, 2005). *Ps. protegens* PF-5 is a common gram-negative soil bacterium studied for its biocontrol properties (Loper *et al.*, 2012).

Ps. protegens PF-5 was obtained from Professor Ian Paulsen, Macquarie University. Bacteria were maintained on LB Agar plates (0.01% tryptone, 0.005% yeast extract, 0.005% sodium chloride, 0.015% Agar) at 25°C. Single colonies were re-suspended in 50% v/v glycerol and M9 salts (Miller, 1972) and placed at -80°C for long term storage.

Serial Plating Experiments

A serial plating experiment was performed to determine the effect of sub-clinical concentration of antibiotics on subsequent generations of bacteria. The methods followed published methods and are briefly outlined here (Chow *et al.*, 2015).

The antibiotic kanamycin was used for this study. It is an aminoglycoside antibiotic, listed on the World Health Organization's list of essential medicines. It is considered a second line treatment and is usually reserved for treating severe bacterial infections and tuberculosis (WHO, 2017). It targets the 30S ribosomal subunit, inhibiting protein synthesis. The Minimum Inhibitory Concentration (MIC) of kanamycin for *Ps. protegens* PF-5 was determined to be 8 mg/L using a standard MIC measurement test (Wiegand *et al.*, 2008).

For serial plating experiments, a single overnight colony was used to inoculate two sets of triplicate plates; control LB agar plates and LB agar plates containing 0.8mg/L kanamycin sulfate, 1/10 of the MIC for *Ps. protegens* PF-5 (Chow *et al.*, 2015). Plates were

incubated at 25°C for 48 hr, referred to here, for convenience, as one passage. Following each 48 hr incubation period, a randomly selected well-separated colony was used to continue the serial plating. Every five passages, three well isolated colonies were selected from each plate for DNA extraction, PCR analysis and long term storage. The first of these three colonies was also used to continue the serial plating. DNA extractions for PCR analysis were carried out using a bead-beating method and were stored at -20°C (Yeates & Gillings, 1998, Gillings, 2014).

After 40 passages, the MICs were measured again using a standard MIC measurement test to determine whether there had been any changes in resistance to kanamycin (Wiegand *et al.*, 2008). The MIC (determined as OD_{600} less than 0.05 indicating no growth) for three replicates were averaged and a two-sample t-test was used to determine if there was significant difference between the control lines and the lines exposed to kanamycin. Isolates from the 40^{th} passage were also prepared for whole-genome sequencing.

DNA Extraction

DNA extractions for PCR analysis were carried out using a bead-beating method and were stored at -20°C (Yeates & Gillings, 1998, Gillings, 2014).

DNA for whole-genome sequencing was required to be higher quality and concentration and was therefore extracted using a standard CTAB phenol:chloroform:isoamyl alcohol protocol (Sambrook & Russell, 2001). In brief, cells were lysed in a CTAB/$NaCl_2$ solution. DNA was purified using phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with isopropanol. The sample was further purified with an RNase treatment before precipitation and resuspension in TE buffer.

***fusA* screening**

Primers were designed to amplify the fusA gene (fusA1: 5'AAGGGGAGACTGAATCAGCC, fusA2: 5"GTTATCCGTGCTGAAGTGCC) using PCR. This gene region was amplified from all stored passages of the three kanamycin exposure lines and the control lines.

DNA sequencing and Analysis

Illumina sequencing was performed by Macrogen (South Korea). Sequence data were analysed with breseq (Deatherage, 2014). (Genbank accession number CP032352, CP032353)

PCR products were DNA sequenced using Sanger protocols by Macrogen (South Korea). Sequencing results were analysed using Geneius R9 Version 9.1.6. (Genbank accession number MH813471)

Results

After 40 passages of *Ps. protegens* PF5 in the presence of 1/10 the MIC for kanamycin, all three experimental lines exhibited increased resistance to kanamycin, while the control lines remained unchanged (Figure 1). All three kanamycin lines displayed significantly higher MICs for kanamycin when compared to the control lines (two sample t-test; Line 1 $p=0.01245$; Line 2 $p=0.00066$; Line 3 $p=0.02184$). Lines 2 and 3 exhibited a fourfold increase in resistance and Line 1 had doubled its resistance to kanamycin.

Whole genome sequencing of the generation 40 passages identified a novel single base pair mutation in the translational elongation factor G (fusA) gene in both Lines 2 and 3. The mutation was a transition, from a guanine to an adenine, resulting in an amino acid

substitution in the FusA protein. Mutations in this gene have been previously reported to confer kanamycin resistance (Mogre et al., 2014, Ibacache-Quiroga et al., 2018).

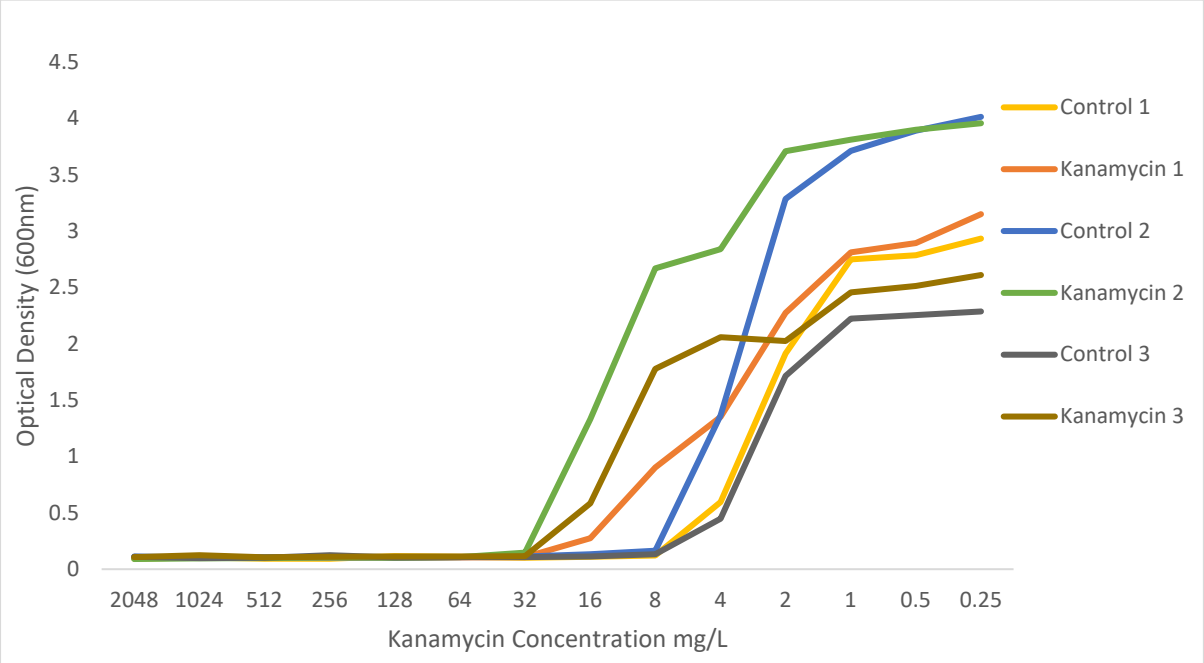

Figure 1. Minimum inhibitory concentration of kanamycin for control and kanamycin exposed lines of *ps. protegens PF5* at passage 40. Kanamycin lines 2 and 3 show a four-fold increase in kanamycin resistance, kanamycin line 1 has doubled in resistance. All three control lines remain unchanged, following 40 passages in a serial plating experiment.

To determine the passage at which these mutations occurred, we used allele-specific PCR with primers that terminated on the mutated nucleotide. Nested PCR for *fusA* was used to amplify this gene from all passages. The *fusA* mutation fixed in lines 2 and 3 between passages 5 and 10 and was consistently present from passage 10 onwards. The identity of the mutation was confirmed via sequencing the PCR products.

A number of other mutations were observed in each of the experimental and control lines (Table S1). These mutations were independent, and never occurred in more than one line, as might be expected from a mutation accumulation experiment such as the one performed here. This also establishes that there was no cross-contamination between any experimental and control lines across the entire experiment. None of the mutations observed has previously been associated with a kanamycin resistance phenotype.

Discussion

Here we showed that exposure to a sub-inhibitory concentration of kanamycin fixed a mutation in the translational elongation factor G gene (*fusA*) in two independent lineages of *Ps. protegens*. This mutation fixed after only five to ten passages, and did so in a mutation accumulation experiment where the opportunities for selection were significantly reduced, as outlined below.

The design of the experiment involved serial plating of single bacterial colonies, each chosen at random from the well separated colonies on a streak plate. Consequently, each *fusA* mutation had to arise during the growth of a single colony, which then had to be chosen for plating at the next passage. From the mixture of genotypes streak-plated at this passage, a colony arising from a cell that exhibited the mutation had to then be chosen to continue the passaging process. Even though there was a great deal of stochasticity inherent in this process, the observation that two lines independently fixed the same *fusA* mutation implies that the true rate of *fusA* mutation in this system is considerably higher.

Mutations in *fusA* have been associated with resistance to kanamycin and other aminoglycosides in *E. coli* (Mogre *et al.*, 2014, Ibacache-Quiroga *et al.*, 2018). Mutations to *fusA* confer resistance to kanamycin because *fusA* interacts with the 30S ribosomal subunit, which is the target site for aminoglycosides such as kanamycin (Pulk & Cate, 2013, Garneau-Tsodikova & Labby, 2016). Fixation of *fusA*-driven kanamycin resistance occurred independently in two lines of an environmental species of *Pseudomonas*. This makes it reasonable to assume similar mutations in *fusA* could be occurring in environmental bacteria exposed to sub-inhibitory levels of aminoglycosides.

Often, antibiotic resistance is a stepwise process, where point mutation confers a degree of resistance, while a second or third mutation provides increasingly higher levels of

resistance (Ibacache-Quiroga *et al.*, 2018). Here we observed a single mutation in the FusA gene. A second mutation in the FusA gene has been documented to provide a higher level of resistance, and has been observed when bacteria were exposed to ½ the MIC (Mogre *et al.*, 2014). It has also been determined that a mutation to *fusA* does not necessarily have a negative effect on growth rate. Even in the absence of kanamycin, *fusA* mutants are not outcompeted by the wild type, suggesting this is a low-cost mutation to maintain (Mogre *et al.*, 2014).

There is little information available on the typical environmental levels of kanamycin contamination. It is known that kanamycin is poorly absorbed, and 40-80% of the therapeutic dose is excreted unchanged. It is also stable, and soluble in water, suggesting it could persist in the environment for extended periods of time (Kunin, 2006). The half-life of kanamycin is 14-28 days depending on soil type, with traces (4.8-22.9%) of antibiotic still present after 63 days (Sun *et al.*, 2013). Kanamycin B has been detected in seawater at concentrations ranging from 0.1-1.5 ug/L (Tahrani *et al.*, 2016). Considering dilution in the ocean, it is possible that at some point in the waste stream feeding this seawater that the concentration of kanamycin was 1/10 the MIC for a range of environmental bacteria. In this case, we would predict that similar *fusA* mutations are likely to be fixed in a wide range of environmental bacteria. High level resistance to aminoglycosides, including kanamycin, has been documented in bacteria isolated from environmental compartments, suggesting that the mutation processes and selection we have documented here have already occurred in environmental bacteria exposed to aminoglycoside pollution (Rice *et al.*, 1995).

The efficacy of kanamycin needs to be preserved because it is effective against multi-drug resistant tuberculosis (MDR-TB). Globally, TB is one of the top 10 causes of death and with first line antibiotic treatments failing to treat MDR-TB, we need second line treatments such as kanamycin and streptomycin (WHO, 2017). Here we have demonstrated how rapidly

219 an environmental bacterium can generate resistance to kanamycin. Such environmental
220 bacteria have the potential to become opportunistic pathogens or to donate their resistance
221 genes via lateral gene transfer.

222 Aminoglycosides, including kanamycin, continue to be used prophylactically in
223 animal husbandry (Kemper, 2008) and to spray fruit (Dibner & Richards, 2005). There is a
224 clear pathway for this use to result in significant environmental contamination, and the
225 generation of resistant bacteria. These antibiotics should be restricted from widespread use in
226 agriculture to preserve our second line of defense antibiotics.

227

228 **Conflict of interest**

229 The authors declare that the research was conducted in the absence of any commercial or
230 financial relationships that could be construed as a potential conflict of interest.

231 **Funding**

232 Work was funded by The Faculty of Science and Engineering Macquarie University and the
233 Australian Government Research Training Program (RTP).

234 **Acknowledgements**

235 The authors would like to acknowledge the members of the EMMA lab, Marita Holley and
236 Sasha Tetu for their invaluable contributions to this research.

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Supplementary data

Table 1. All mutations detected in *Pseudomonas protegens* PF-5 in control lines and kanamycin lines at passage 40 following serial plating experiment. Whole genome Illumina sequencing was used and data were analysed with breseq. “x” denotes a base pair substitution. “Position” indicates position of mutation in *Pseudomonas protegens* strain PF-5 (GenBank: CP000076.1).

Position	Gene	Description	Pf5 Control 2	Pf5 Control 3	Pf5 Kan 1	Pf5 Kan 2	Pf5 Kan 3
3,808,580	PFL_3300 / PFL_3301	putative membrane-bound lytic murein transglycosylase/ABC transporter, periplasmic substrate-binding protein					x
4,110,893	gacA	response regulator GacA	x				
4,110,932	gacA	response regulator GacA					x
5,163,067	gacS	sensor protein GacS		x			
5,163,295	gacS	sensor protein GacS			x		
5,379,340	PFL_6255	conserved hypothetical protein					x
6,364,018	fusA	translation elongation factor G				x	x

Chapter 3: Sub-inhibitory exposure to ciprofloxacin selects for de novo *gyrA* mutations in an environmental species of *Pseudomonas*

In this chapter we examine the effect of low levels of the antibiotic ciprofloxacin on *Pseudomonas protegens,* an environmental bacterium. The bacterium was serially plated on agar containing 1/10 the MIC of ciprofloxacin for this strain. The data collected in Chapter 1 suggest that the concentration used in these experiments is representative of concentrations that might be found in the environment. Repeated plating on subinhibitory concentrations of ciprofloxacin rapidly fixed *gyrA* mutations in the passaged lines. This mutation resulted in an eight-fold increase in the MIC for ciprofloxacin.

These observations suggest that selection can be driven by low concentrations of antibiotics and that similar selection events could be occurring at a high frequency in environmental bacteria. Consequently, ciprofloxacin pollution could disrupt normal microbial ecology and contribute to the global problem of antibiotic resistance.

This chapter is the product of a working collaboration between myself, Timothy Ghaly and Michael Gillings. I was predominantly involved in the design of the study, the laboratory experiments, interpretation of the data and the drafting of the manuscript. Michael Gillings was involved in the design of the study, and contributed to drafting and editing the manuscript. Timothy Ghaly was involved in the analysis of whole genome sequencing data.

Detailed Contributions

	Louise Chow	Michael Gillings	Timothy Ghaly
Design	50%	50%	-
Laboratory work	100%	-	-
Analysis	50%	-	50%
writing	80%	20%	-

This chapter has been prepared for publication in *Frontiers in Microbiology*. The formatting, referencing and word limits adhere to their author guidelines. Figures have been embedded in the text for the purpose of this thesis.

Sub-inhibitory exposure to ciprofloxacin selects for *de novo gyrA* mutations in an environmental species of *Pseudomonas*

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Keywords: antibiotic; pollution; SOS response; evolution; resistance

Abstract

Antibiotics and their metabolites can enter the environment via diverse routes, and persist at low, but biologically relevant concentrations. Even low levels of antibiotics can trigger the SOS response in bacteria, which upregulates mutation rates. This increases the chance of a beneficial mutation occurring, such as one conferring resistance to antibiotics, and then becoming fixed within bacterial populations. Here, we conducted a serial plating experiment using the environmental bacterium, *Pseudomonas protegens* Pf-5, which was exposed to 1/10 the MIC of ciprofloxacin over 40 single colony passages. We detected a single base pair mutation in the gyrase A gene, causing an eight-fold increase in resistance to ciprofloxacin. We established that the mutation occurred between passage 15 and 20 and was fixed from generation 20 onwards. We predict that similar mutations will occur in other environmental bacteria when exposed to low levels of ciprofloxacin and will contribute to the growing global problem of antibiotic resistance.

Introduction

The use of antibiotics in medicine and agriculture has precipitated a crisis in medical treatment. The widespread emergence of multi-drug resistant bacterial infections poses a significant threat to human health. In spite of attempts to minimize antibiotic consumption, usage increased by 65% from 2000 to 2015 (Klein et al., 2018).This worrying trend is fueling increasing emergence and spread of antibiotic resistant strains of bacteria, and raises concerns that we could return to a post-antibiotic era (Alanis, 2005), and it has been estimated that by 2050 antimicrobial resistant infections will account for 10 million deaths annually (O'Neill, 2014; WHO, 2014). There will be a significant impacts on global economy, possibly equivalent to the Global Financial Crisis of 2008, and could see an extra 28 million people fall into poverty (Adeyi, 2017).

Antibiotics are widely used for treatment of infections, but many procedures are reliant on prophylactic use of antibiotics that would be too risky to perform without antibiotics. Medicine is not the only sector that benefits from antibiotic usage. Agriculture is heavily reliant on antibiotics, with approximately 80% of antibiotic consumption being in this sector (Food and Administration, 2014). This use is particularly in mass animal husbandry (Dibner and Richards, 2005), aquaculture (Cabello, 2006) and fruit spraying (McManus et al., 2002).

In all these uses, there is a low absorption rate. This often results in 70-90% of the antibiotic dose being excreted unchanged (Lipsitch et al., 2002; Berge et al., 2005). Secondary metabolites of many antibiotics are also still active antimicrobials (Homem and Santos, 2011). For human waste, antibiotics pass into sewage waste treatment facilities where, due to their small molecular size and relative stability, they survive treatment. They are then released into the environment along with waste water effluent or sewage sludge. In

agriculture, antibiotics have a direct route to the environment, through disposal of animal waste and manuring of crops from animals treated with antibiotics (Haller et al., 2002). In aquaculture, antibiotics are directly introduced into the environment as antibiotic-supplemented fish feed (Cabello, 2006). Antibiotics also enter the environment via pharmaceutical run-off (Larsson, 2014), and landfill leachate (Chung et al., 2018) where they can reach concentrations higher than clinical levels.

Antibiotics entering the environment should be thought of as pollutants, due to their ability to disrupt normal microbial communities (Pruden et al., 2006). Furthermore, in the gut of humans and animals, some bacteria will be able to persist during antibiotic therapy due to possession of resistance determinants. These bacteria will be shed in feces, and consequently, humans and animals are sources of both antibiotics and of bacteria that carry genes conferring resistance (Gillings, 2018). Antibiotic pollution sees a gradient of antibiotic radiating from areas of human population, and along this gradient different concentrations of antibiotics will have significant biological effects (Andersson and Hughes, 2012). Sub-inhibitory concentrations of antibiotics induce the bacterial SOS response, triggered by DNA damage. The SOS response upregulates expression of error-prone DNA polymerase, and increases rates of transposition and recombination. These effects increases the likelihood of mutations, including those conferring resistance (Baharoglu and Mazel, 2014).

Antibiotics are increasingly being reported in water and soil samples. This has prompted a call for better understanding of the effects of sub-inhibitory concentrations of antibiotics in the environment (Harris et al., 2013). Here we examined the effect of sub-inhibitory concentrations of the antibiotic ciprofloxacin on an environmental *Pseudomonas* strain and show that this exposure can rapidly generate resistance.

Materials and Methods

Bacterial isolates

Pseudomonas protegens strain PF-5 (formerly *Pseudomonas fluorescens* PF-5) was selected for this study (GenBank: CP000076.1). *Pseudomonas protegens* PF-5 is a common gram-negative soil bacterium studied for its biocontrol properties (Loper et al., 2012).

Pseudomonas protegens PF-5 was obtained from Professor Ian Paulsen, Macquarie University. Bacteria were maintained on LB Agar plates (0.01% tryptone, 0.005% yeast extract, 0.005% sodium chloride, 0.015% Agar) at 25°C. Single colonies were re-suspended in equal parts 50% glycerol and M9 salts and held at -80°C for long term storage.

Serial Plating Experiments

A serial plating experiment was performed to determine the effect of sub-clinical concentration of antibiotics on subsequent generations of bacteria. The methods followed published methods and are only briefly outlined here (Chow et al., 2015).

The antibiotic ciprofloxacin was selected for this study. It is currently listed in the World Health Organisation Essential Medicines List (WHO, 2017). Ciprofloxacin is a broad spectrum fluoroquinolone used to treat both Gram-positive and Gram-negative bacterial infections. It inhibits DNA gyrase, which in turn prevents DNA replication (LeBel, 1988). The Minimum Inhibitory Concentration (MIC) of ciprofloxacin for *Pseudomonas protegens* PF-5 was determined using a standard MIC measurement test (Wiegand et al., 2008).

For serial plating experiments, a single overnight colony was used to inoculate two sets of triplicate plates; control LB agar plates and LB agar plates containing 0.025 mg/L ciprofloxacin, which was determined to be 1/10 of the MIC for *Ps. protegens* PF-5 (Chow et

al., 2015). Plates were incubated at 25°C for 48 hr, referred to here, for convenience, as one passage. After incubation, a single, well-separated colony from each plate was used to continue the serial plating. Every five passages, three well-isolated colonies were selected from each plate for DNA extraction, PCR analysis and long term storage. The first of these three colonies was also used to continue the serial plating. Glycerol stocks were prepared in equal parts 50% glycerol and M9 salts and held at -80°C for long term storage.

DNA Extractions

DNA extractions were carried out using a bead-beating method (Yeates and Gillings, 1998; Gillings, 2014). DNA fingerprints generated by BOX-PCR were used for rapid detection of mutations and DNA rearrangements, and to control for possible contamination. BOX-PCR protocols followed published methods (Sup. Data Table 1) (Gillings and Holley, 1997).

After 40 passages, the MICs of all lines were re-tested (Wiegand et al., 2008), and the samples were prepared for whole-genome sequencing. DNA was extracted using a standard CTAB phenol:chloroform:isoamyl alcohol protocol (Sambrook and Russell, 2001). In brief, cells were lysed in a CTAB/$NaCl_2$ solution. DNA was purified using phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with isopropanol. The sample was further purified with an RNase treatment before precipitation and resuspension in TE buffer.

***gyrA* screening**

In one of the experimental lines, a single base substitution in the DNA gyrase (*gyrA*) gene was detected. A simple, rapid PCR method was developed to both confirm this mutation and determine during which passage this mutation occurred. A PCR primer set comprised of an allele-specific primer nested within a forward and reverse primer was used (Sup. Data Table

1). This produces two bands in samples where the *gyrA* mutation is present. All stored passages of all experimental and control lines were screened using this method.

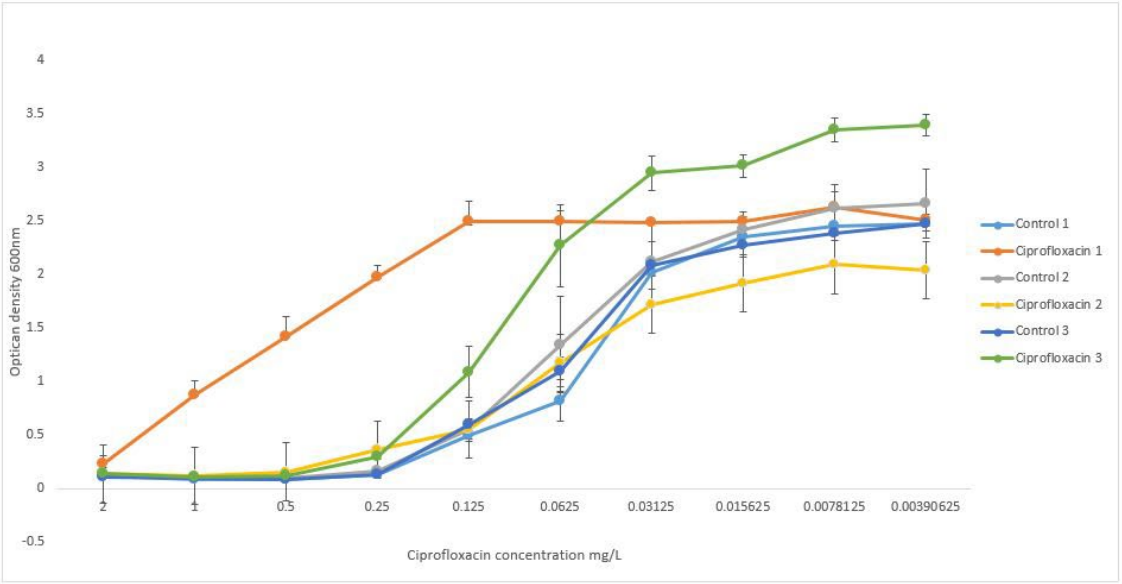
DNA Sequencing and Analysis

Whole genome Pacific BioSciences (Pacbio) sequencing and Illumina sequencing were performed by Macrogen (South Korea). Sequence data were assembled with Canu 1.7, aligned using Mauve Version 2.4.0 and analysed using Geneius R9 Version 9.1.6. (Sup. Data, Table 2) (Accession number MH813470).

In order to confirm the single base substitution detected in the allele specific PCR, a second PCR was carried out only using the forward and reverse primers (Sup. Data Table 1) and these PCR products were sequenced using Sanger sequencing (Macrogen, South Korea). Sequencing results were analysed using Geneius R9 Version 9.1.6. (Accession number CP032358)

Results and Discussion

After 40 passages, the MIC of all lines was determined. One line of *Ps. protegens* PF-5 that had been exposed to 1/10 MIC ciprofloxacin exhibited an eight-fold increase in resistance to ciprofloxacin (Figure 1).



147

148 Figure 1. Minimum inhibitory concentration of ciprofloxacin against three lines of
149 Pseudomonas protegens Pf5 Generation 40. Ciprofloxacin line 1 (yellow) shows an eight-fold
150 increase to ciprofloxacin while lines 2 and 3 and all three control lines remain unchanged.

151

152 Whole genome sequencing of all lines at passage 40, identified a single base mutation
153 in the gyrase A gene (gyrA) of the line with elevated MIC. The single base pair substitution
154 was at nucleotide position 259, codon 87, from G to A. This changes the *gyrA* codon 87 from
155 aspartic acid to asparagine. Allele specific PCR allowed us to determine that this mutation
156 occurred between passage 15 and 20 and was present from generation 20 onwards, as seen as
157 a double banded PCR product (Figure 2). The mutation was confirmed through Sanger
158 sequencing. The MIC was determined for the passages before and after generation 20, it was
159 found that the same line increased in resistance between generations 15 and 20, further
160 confirming that the increase in resistance is likely due to the mutation in gyrA. There was
161 generally a higher rate of point mutations in lines exposed to ciprofloxacin compared to the
162 control lines (Sup. Data. Table 2).

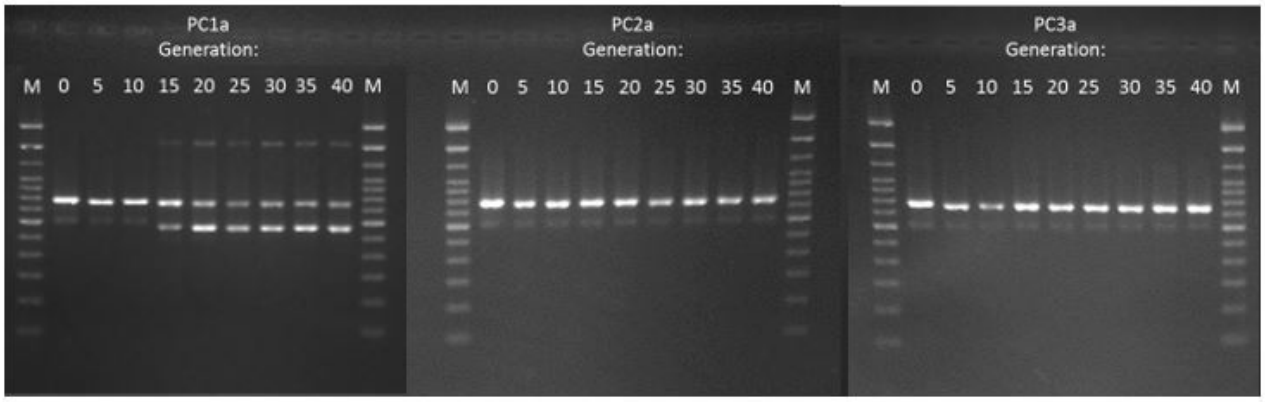


Figure 2. Electrophoresis gel of *Ps. protegens* pf5 Generation 40 lines 1, 2 and 3 (PC1a, PC2a, PC3a respectively). A double banded product is present in line 1 indicating the presence of a mutation, a single banded product is present in lines 2 and 3 indicating no mutation. M = 100bp ladder.

Ciprofloxacin belongs to the class of antibiotics called fluoroquinolones. The effect of fluoroquinolones on *Escherichia coli* has been well documented. Several mutations have been observed in *gyrA* in *E. coli* and are most commonly found in the Quinolone-Resistance Determining Region (QRDR). At least seven mutations in *gyrA* codons have been found to confer resistance to fluoroquinolones in *E.coli* (Weigel et al., 1998; Ruiz, 2003). One of these mutations is identical to the mutation found here in *Ps. protegens.*

Mutations conferring resistance are often a stepwise process, where a single point mutation will confer a degree of resistance while a second will confer a higher degree of resistance and so on. (Ruiz, 2003; Su et al., 2013). In *E. coli*, the presence of a single mutation, as we have seen here, will to some degree confer resistance. The mutation at codon 87 is the second most documented mutation in quinolone resistant *E.coli.* The addition of a second mutation at codon 83 confers a significantly higher level of resistance to quinolones (Ruiz, 2003). It is possible that here we have documented the first step in a multi-step progress to significant antibacterial resistance.

The mutation we documented here was fixed under sub-inhibitory concentrations of ciprofloxacin and no overt selection. By using agar plates instead of liquid cultures, we

184 removed competition between cells. Single, well-isolated colonies were chosen from the
185 plate. Any mutations must have occurred in the original cell, or during the growth of the
186 single colony, and the subsequent streaking of that colony must have resulted in further well-
187 isolated single mutant colonies that were chosen at the next passage. Consequently, fixation
188 of any mutant in a passaged lineage is not certain, and relies on some element of chance.
189 These factors show that similar mutations probably occurred much more often than we
190 observed.

191 These findings are significant, because they show that there is a high likelihood of
192 similar mutations occurring in environmental *Pseudomonas* strain. The *de novo* mutation
193 occurred, and fixed, surprisingly quickly (after only 15 passages), and did so under exposure
194 to environmentally relevant concentrations of ciprofloxacin.

195 A gradient of antibiotic concentration radiates from areas of dense human population.
196 The concentrations along this gradient will vary, and while it is unlikely they will be found at
197 clinically relevant levels, they could be found at the kinds of sub-inhibitory concentrations
198 used in our experiments. Sub-inhibitory concentrations of antibiotics increase mutation rates
199 via the SOS response (Andersson and Hughes, 2012; Chow et al., 2015). The SOS response
200 is a general response to DNA damage, such as the damage inflicted by antibiotics, and its
201 relationship with upregulation of mutation is well documented. In this experiment a number
202 of point mutations were detected in both the experimental and control lines, as might be
203 expected from a mutation accumulation experiment such as the one performed here. The
204 variation in mutations between lines establishes that there was no cross-contamination
205 between both the experimental lines and the control lines across the entire experiment. Of
206 note, several mutations were detected in the sensor histidine kinase RpeA and sensor protein
207 GacA and GacS regions (Sup. Data Table 2). These regions are not well understood but are

thought to be involved with quorum sensing (Kay et al., 2006), and so it makes sense that we would see them in a plating experiment.

There are approximately 40 genes involved in the SOS response, several of which are translesion DNA polymerases which allow replication machinery to bypass damaged regions of DNA. This maintains chromosomal integrity but also significantly increases the likelihood of base substitutions (Baharoglu and Mazel, 2014) (Mesak et al., 2008) (Cirz et al., 2006). Fluoroquinolones such as ciprofloxacin upregulate the SOS response as they affect DNA replication. Prolonged exposure to fluoroquinolones, even at low concentrations, can upregulate mutation rates 2-9 fold (Long et al., 2016).

Fluoroquinolones are a widely used class of antibiotics and ciprofloxacin is one of the most commonly used (Acar and Goldstein, 1997). These antibiotics are chemically stable, and relatively resistant to hydrolysis and high temperatures. They are sensitive to UV light although binding to cations such as iron and zinc provides some resistance UV degradation. Metabolites of fluoroquinolones can still be active antimicrobials. For example, the main metabolite of the widely used veterinary fluoroquinolone, enrofloxacin, is ciprofloxacin (Sukul and Spiteller, 2007).

Fluoroquinolones readily bind to sediment, however, the level of biological activity after sorption to sediments is affected by the type of sediment and the strength of sorption. Sorption of fluoroquinolones into soil reduces bioavailability and concentration in solution but sorption also protects fluoroquinolones from photo degradation and biodegradation (Girardi et al., 2011). A sewage simulation found that 65% of fluoroquinolones was absorbed via sorption and 35% was found in the effluent (Kümmerer et al., 2000). While sorption of Fluoroquinolones to soil reduces the risk of surface and ground water contamination, it increases the exposure of soil dwelling microorganisms to fluoroquinolones (Alder et al.,

2004). High concentrations of fluoroquinolones have been found in sludge from wastewater recycling plants. This sludge is often applied as fertilizer and in topsoil where this sewage sludge has been applied ciprofloxacin concentrations were found to be between 1.4-2.4 mg/kg. Furthermore, wastewater from a hospital in Switzerland contained 17.2-29.4ug/L of ciprofloxacin (Alder et al., 2004). It is estimated that 80-90% of ciprofloxacin is absorbed into sludge and that after waste water treatment, sludge contains approximately 3mg/kg of ciprofloxacin (Girardi et al., 2011). Human and animal waste is used as manure, in the US approximately 4 million dry tones of manure were applied to crops in the year 2004. Estimating at a concentration of 3mg/kg of ciprofloxacin, this would amount to 12,000 kilograms of ciprofloxacin applied to crops in 2004 in the US alone. These concentrations easily encompass 1/10 the MIC of a range of bacterial species (The European Committee on Antimicrobial Susceptibility Testing), suggesting that the results seen here could be an indication of what is occurring in the environment.

The problem of antibiotic pollution needs to be addressed at several levels. Antibiotics should be prevented from entering the environment: pharmaceutical waste should not be allowed to run into rivers; human waste should not be used to manure crops; and animal waste should be treated before being used as manure. Antibiotic needs to be monitored and controlled globally in both human medicine and agriculture in order to control the growing problem of antibiotic resistant infections.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

256 **Funding**

257 Work was funded by The Faculty of Science and Engineering Macquarie University and the

258 Australian Government Research Training Program (RTP).

259

260 **Acknowledgements**

261 The authors would like to acknowledge the members of the EMMA lab, Marita Holley and

262 Sasha Tetu for their invaluable contributions to this research.

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Supplementary Data

Table 1. Thermal cycling primers, mastermix and programs.

PCR	Primers	Mastermix per reaction	Thermal Cycle
BOX	BOXA1R: 5'CTACGGCAAGGCGACGCTGACG	22.5 µL PCR water, 25 µL GoTaq® white (Promega), 0.5 µL 1 mg/ml RNAse, 1µL 50µM BOXA1R primer	94°C 3 mins 94°C 30s 52°C 30s 68°C 8 mins x 35 68°C 15mins 4°C hold
gyrA allele specific screening	gyrA 1 (F): GCTGAAACAGTCCTACCTCG gyrA 2 (allele specific): GGTGATACCGCGGTGTACA gyrA 3 (R): CGCCTTGTTCAGTTGGTAAGG	22.5 µL PCR water, 25 µL GoTaq® white (Promega), 0.5 µL 1 mg/ml RNAse, 0.5µL 50µM gyrA 1, 0.5µL 50µM gyrA 2, 0.5µL 50µM gyrA 3	94°C 3 min 94°C 30 sec 68°C 30 sec 72°C 2 min x35 72°C 5 min 4°C hold
gyrA allele specific for sanger sequencing	gyrA 1 (F): GCTGAAACAGTCCTACCTCG gyrA 3 (R): CGCCTTGTTCAGTTGGTAAGG	22.5 µL PCR water, 25 µL GoTaq® white (Promega), 0.5 µL 1 mg/ml RNAse, 0.5µL 50µM gyrA 1, 0.5µL 50µM gyrA 3	94°C 3 min 94°C 30 sec 68°C 30 sec 72°C 2 min x35 72°C 5 min 4°C hold

Supplementary data

Table 2. All mutations detected in *Pseudomonas protegens* PF-5 in control lines and ciprofloxacin lines at passage 40 following serial plating experiment. Whole genome Illumina sequencing was used and data were analysed with breseq. "x" denotes a base pair substitution. "Position" indicates position of mutation in *Pseudomonas protegens* strain PF-5 (GenBank: CP000076.1).

Position	Gene	Description	Pf5 Control 2	Pf5 Control 3	Pf5 Cipro 1	Pf5 Cipro 2	Pf5 Cipro 3
122,631	PFL_0118 / rrsA	CoA-transferase, family III/16S ribosomal RNA				x	
627,715	PFL_0537 / thiC	type I secretion outer membrane protein, TolC family/thiamine biosynthesis protein ThiC			x		
786,942	PFL_0675	response regulator/GGDEF domain protein				x	
3,773,825	rpeA	sensor histidine kinase RpeA			x		
3,774,007	rpeA	sensor histidine kinase RpeA					x
3,774,031	rpeA	sensor histidine kinase RpeA				x	
4,110,893	gacA	response regulator GacA	x				
4,443,715	PFL_3842	conserved hypothetical protein			x		
5,017,593	gyrA	DNA gyrase, A subunit			x		
5,162,326	gacS	sensor protein GacS			x		
5,163,067	gacS	sensor protein GacS		x			
5,616,936	gltJ	glutamate/aspartate ABC transporter, permease protein GltJ					x
6,137,387	PFL_5367	conserved hypothetical protein					x

Chapter 4: Effect of sub-inhibitory concentrations of antibiotics on *Acinetobacter baumannii*

In this chapter, I examined the response of bacteria to subinhibitory levels of antibiotics and the potential role of the SOS response. *Acinetobacter baumannii* was exposed to low levels of ciprofloxacin in a serial plating experiment. Since the SOS response is mediated by RecA, we compared wild type and RecA knockout mutants of *Acinetobacter baumannii*, to determine the role of the SOS system in response to subclinical levels of the antibiotic ciprofloxacin. We found no increase in antibiotic resistance after 40 passages. Ordinarily, the SOS response upregulates error-prone DNA polymerases and other genetic systems that increase the rate at which variation is generated, and this in turn increases the likelihood that antibiotic resistance mutations appear. However, *Acinetobacter baumannii* is missing some of the regulatory elements necessary to induce the SOS response, suggesting that *Acinetobacter baumannii* might not respond to DNA damage in the same way as other bacterial species. This highlights the need for more research into how *Acinetobacter baumannii* responds to DNA damage, since this species is an increasingly important nosocomial bacterial infection in which multi-drug resistant strains are becoming common.

This chapter is the product of a working collaboration between myself and Michael Gillings. I was predominantly involved in the design of the study, the collection of data, analysis of the data, and drafting of the manuscript. Michael Gillings contributed to the design of the study, analysis of data and preparation of the final manuscript.

Detailed Contributions

	Louise Chow	Michael Gillings
Design	70%	30%
Laboratory work	100%	-
Analysis	80%	20%
Writing	80%	10%

Effect of sub-inhibitory concentrations of antibiotics on *Acinetobacter baumannii*

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Keywords: antibiotic resistance; SOS response, *recA*, ciprofloxacin

Abstract

Antibiotics are disseminated into aquatic environments via human waste streams, agricultural run-off and pharmaceutical effluent, where they can persist at low, but biologically relevant, concentrations. Antibiotic pollution establishes a selection gradient for resistance and may also raise the frequency of events that can generate resistance: point mutations; recombination; and lateral gene transfer. This study examined the response of *Acinetobacter baumannii* to sub-inhibitory levels of antibiotics, and the role of the SOS system in this response. SOS is a general response to DNA damage, and is mediated by RecA. Through a serial plating experiment, we examined the response to *Acinetobacter baumannii* to low levels of ciprofloxacin. By using a *recA* knockout of *Acinetobacter baumannii*, we examined the potential role of the SOS response when exposed to subclinical levels of the antibiotic ciprofloxacin. We found no increase in antibiotic resistance after 40 passages. *Acinetobacter baumannii* has no SOS box, which is required to induce the SOS response and promote elevated mutation rates. This suggests that *Acinetobacter baumannii* does not respond to DNA damage in the same way as other bacterial species.

Introduction

Antibiotic resistance has been identified as one of the greatest threats to human health for the 21st century. It is estimated that by 2050, antibiotic resistant infections will account for 10 million deaths annually, compared to the current 700,000 (O'Neill, 2014, WHO, 2014). Overuse and misuse of antibiotics in the medical and agricultural sectors have provided selection for resistant bacteria, and it is estimated that 70% of pathogens now exhibit resistance to at least one, if not more, antibiotics (Berdy, 2012).

Antibiotics are widely used in medicine to treat infections, however many surgical procedures are also reliant on the prophylactic use of antibiotics. Antibiotics are extensively used in animal production to prevent disease and infection, and as a growth promoter (Hilbert & Smulders, 2004, Bednorz *et al.*, 2013). It has been estimated that up to 70% of antibiotics produced are used in agriculture (Lipsitch *et al.*, 2002, Berge *et al.*, 2005). Antibiotics are also used in aquaculture (Cabello, 2006) and fruit spraying (McManus *et al.*, 2002).

A relatively small amount of the antibiotics consumed by humans and animals are metabolised, leading to 30-90% of antibiotics being excreted, unchanged, into waste treatment facilities, which are unable to remove them. Antibiotics can also directly enter the environment through agriculture (Lipsitch *et al.*, 2002, Berge *et al.*, 2005, Sarmah *et al.*, 2006), through effluent from pharmaceutical factories (Dong *et al.*, 2009, Li *et al.*, 2010) and landfill leachate (Barnes *et al.*, 2004). Antibiotics entering the environment via these various pathways should be classified as pollutants, however there is little regulation on their release.

There has been increasing attention given to the possible impacts of antibiotic pollution in the environment, as it is becoming clear that antibiotics are able to persist in the environment, where they continue to interrupt normal microbial processes (Andersson & Hughes, 2012, Bengtsson-Palme & Larsson, 2016). Sub-inhibitory levels of antibiotics are known to trigger the SOS response, a general response to DNA damage (Mesak *et al.*, 2008,

Andersson & Hughes, 2012). The SOS response upregulates expression of error-prone DNA polymerase, and increases rates of transposition and recombination. These effects increase the likelihood of mutations, including those conferring resistance (Baharoglu and Mazel, 2014). The SOS response is mediated by the repressor LexA and the inducer RecA, and allows bacteria to quickly adapt to stressful environments (Michel, 2005). This is further enhanced by the ability of bacteria to transfer genetic elements via horizontal gene transfer, meaning that a beneficial mutation that arises in a single bacterial cell can rapidly disseminate throughout bacterial populations.

Here we examined the potential role of the SOS response in *Acinetobacter baumannii* in response to low levels of antibiotics. To examine the role of RecA in response to DNA damage, a *recA* knockout was used. The *recA* knockout and the wild type strains were exposed to low levels of antibiotics in a serial plating experiment.

Materials and Methods

Bacterial isolates

Acinetobacter baumannii AB5075 was used for this study. *Acintobacter baumannii* AB5075 is a highly virulent strain of *Acinetobacter baumannii* that is commonly used as a model strain when investigating the effect of antibiotic treatment (Jacobs et al., 2014). *A. baumannii* is an opportunistic pathogen that is of interest as it is increasingly being identified as a nosocomial infection (Peleg *et al.*, 2008). Two strains were used in this study, a wild type and a *recA* knockout. *A. baumannii* was obtained from the Manoil Lab *Acinetobacter baumannii* Mutant Library (Gallagher *et al.*, 2015). Bacteria were maintained on LB Agar plates (0.01% tryptone, 0.005% yeast extract, 0.005% sodium chloride, 0.015% agar) at 25°C.

Antibiotic treatment

Ciprofloxacin was used for this study; it is a second generation fluoroquinolone used to treat a broad spectrum of infections. It inhibits DNA gyrase, which in turn prevents DNA replication (LeBel, 1988). The minimum inhibitory concentration (MIC) of ciprofloxacin for both strains was determined using a standard MIC measurement test (Wiegand *et al.*, 2008). and was 256 mg/L for both strains of *Acinetobacter baumannii*.

DNA extraction

DNA was extracted from bacterial cultures using a bead-beating method (Yeates & Gillings, 1998, Gillings, 2014). Briefly, a single, well isolated colony from an overnight culture was resuspended in a lysing matrix tube with CLS-TC buffer (MP Biomedicals). Cells were physically lysed by treatment in a FastPrep FP120 (BIO 101 Savant) machine for 30s at 5.5m/s before being centrifuged in an Eppendorf 5417C, for 5 minutes at 14,000x g. Protein precipitation, binding and washing of DNA, and subsequent elution in TE buffer were as previously described (Yeates & Gillings, 1998, Gillings, 2014). Purified DNA was stored at -20°C.

Repetitive Element PCR

DNA fingerprints were generated using repetitive extragenic palindromic (REP) and BOX PCR (Reboli *et al.*, 1994, Vila *et al.*, 1996) One µL of DNA was mixed with 9µL of Genereleaser ™ (Bioventures Inc.) in a 0.5mL PCR strip tube, and heated on high for 7 min in a 650 W microwave oven with a microwave sink. Tubes were then held at 80°C for 5 min in an Eppendorf Master Cycle Epigradient S PCR machine, before 40µL of PCR master mix was mixed into each tube. The PCR master mix per reaction was as follows: 13.5 µL PCR water, 25 µL GoTaq® white (Promega), 0.5 µL 1 mg/ml RNAse, and 0.5µL 50µM REP

forward primer (5'IIIGCGCCGICATCAGGC) and 0.5µL 50µM REP reverse primer (5'ACGTCTTATCAGATTCAC) or, 1µL 50µM BOXA1R primer (5'CTACGGCAAGGCGACGCTGACG). Negative controls containing Genereleaser™ only and water only were included in each PCR. A REP-PCR cycle (94°C 3min, 35 cycles (94°C 1min, 40°C 1min, 68°C 8min), 68°C 16min) or BOX-PCR cycle (94°C 3min, 39 cycles (94°C 30secs, 52°C 30secs, 68°C 8min), 68°C 15min) was then performed. Primers were synthesised by Sigma-Aldrich Inc.

Agarose Electrophoresis

PCR products were separated on 2% agarose gels poured in Tris-Borate-EDTA (TBE) buffer. A 100 base pair ladder (Invitrogen) was included on each gel. Samples were loaded with one quarter volume of bromophenol blue loading dye (0.45 M Tris-borate, 0.01 EDTA, 40% sucrose, 0.25% bromophenol blue). Gels were run in Tris-Borate-EDTA (TBE) at 110 volts for 50 to 80 min and were stained with GelRed™ (Biotium). Gel images were captured using a Gel logic 2200 PRO camera and Carestream MI computer software.

Serial Plating Experiments

A single colony of each strain was used to inoculate a series of eight plates of both control LB agar plates containing no antibiotic and LB plates containing 1/10 the MIC for ciprofloxacin. Plates were incubated at 25°C for 48 hr, referred to here, for convenience, as one passage.

After incubation for 48hr, a single well-separated colony from each plate was used to continue the serial plating and was also streaked onto a LB plate for DNA extractions and generation of glycerol stocks (Chow *et al.*, 2015). Repetitive Element PCRs were carried out

to monitor changes in DNA banding patterns and to monitor for possible contamination of the cultures.

Results

Detectable genome changes

Every five passages, BOX and REP-PCR were carried out to detect genome changes, as these methods are known to be highly effective in discriminating between strains of *A. baumanii* (Vila et al., 1996) . The basis of the REP-PCRs is explained in Reboli et al (1994) (Reboli *et al.*, 1994) but, in brief, relies on amplification of conserved regions based on repetitive extragenic palindromic (REP) elements. The basis of BOX-PCRs is explained in Gillings & Holley (1997) (Gillings & Holley, 1997), but, in brief, relies on amplification of regions between two random, but reproducible priming sites. Consequently, amplicons are sensitive to mutations in the priming sites, and to indels across the amplified regions.

REP-PCR assays did show minor changes to banding patterns, but these occurred across all treatments and strains (Figure 1). In other words, there was no evidence for elevated rates of either mutation or genome rearrangement in ciprofloxacin treated lines. If the minor changes in banding patterns do represent mutational changes, these were not associated with either the ciprofloxacin treatment or the *recA* status on the strains. Minor banding changes could be due to stochasticity in the PCR amplifications. BOX-PCR assays also exhibited minor changes to banding patterns. Wild-type lines and *recA* knockout lines both exhibited these minor changes, regardless of their exposure to ciprofloxacin (Figure 2).

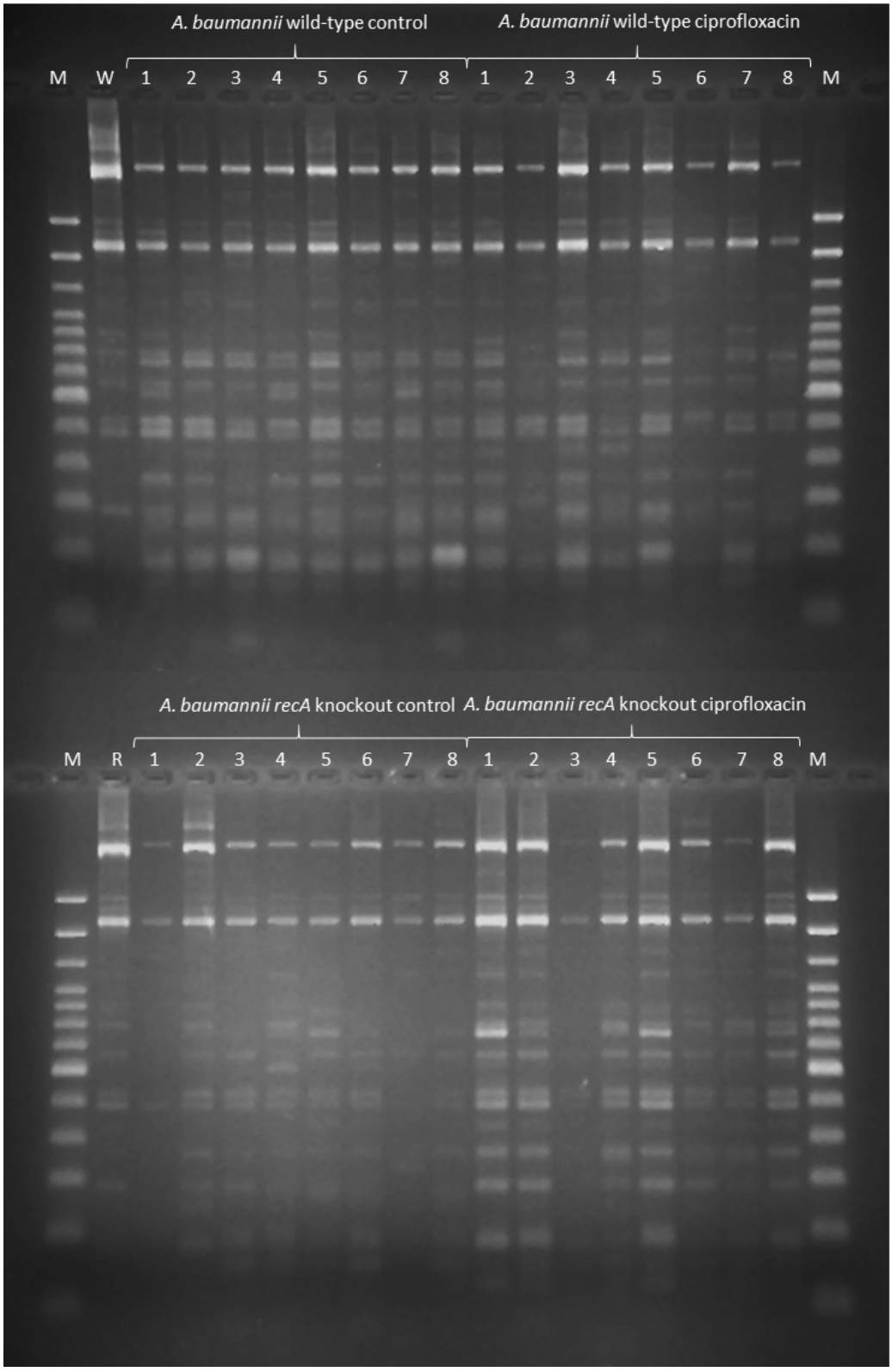

Figure 1 Figure 1: REP-PCR of passage 40 *A. baumannii* lines. Lanes "M" are 100bp ladder molecular weight markers. Lanes "W" are the stock wild-type strain and Lanes "R" are the original *recA* knockout strain. The top panel is generation 40 *A.baumannii* wild-type strain, control and ciprofloxacin exposed lines. The second panel is generation 40 *A.baumannii recA* knockout strain, control and ciprofloxacin exposed lines.

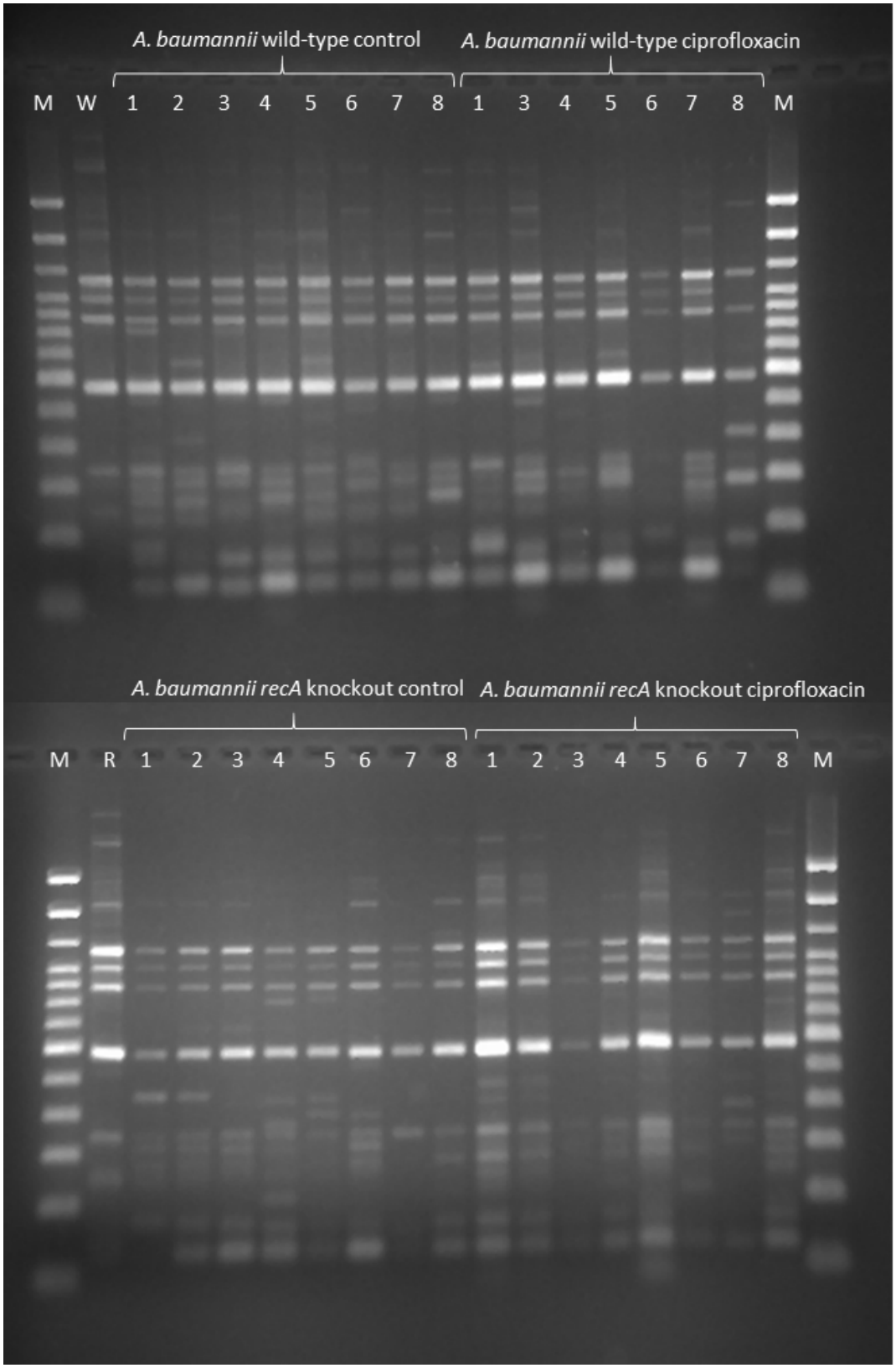

Figure 2: BOX-PCR of passage 40 *A.baumannii* lines. Lanes "M" are 100bp ladder molecular weight markers. Lanes "W" are the stock wild-type strain and Lanes "R" are the original *recA* knockout strain. The top panel is generation 40 *A.baumannii* wild-type strain, control and ciprofloxacin exposed lines. The second panel is generation 40 *A.baumannii recA* knockout strain, control and ciprofloxacin exposed lines.

To determine if any of the minor genotypic changes were associated with a change in resistance status, the MIC of each passaged line was determined to detect any significant differences in MIC from the original stock culture. All lines remained at the original MIC of 256mg/L ciprofloxacin, regardless of treatment or *recA* genotype (Figure 3).

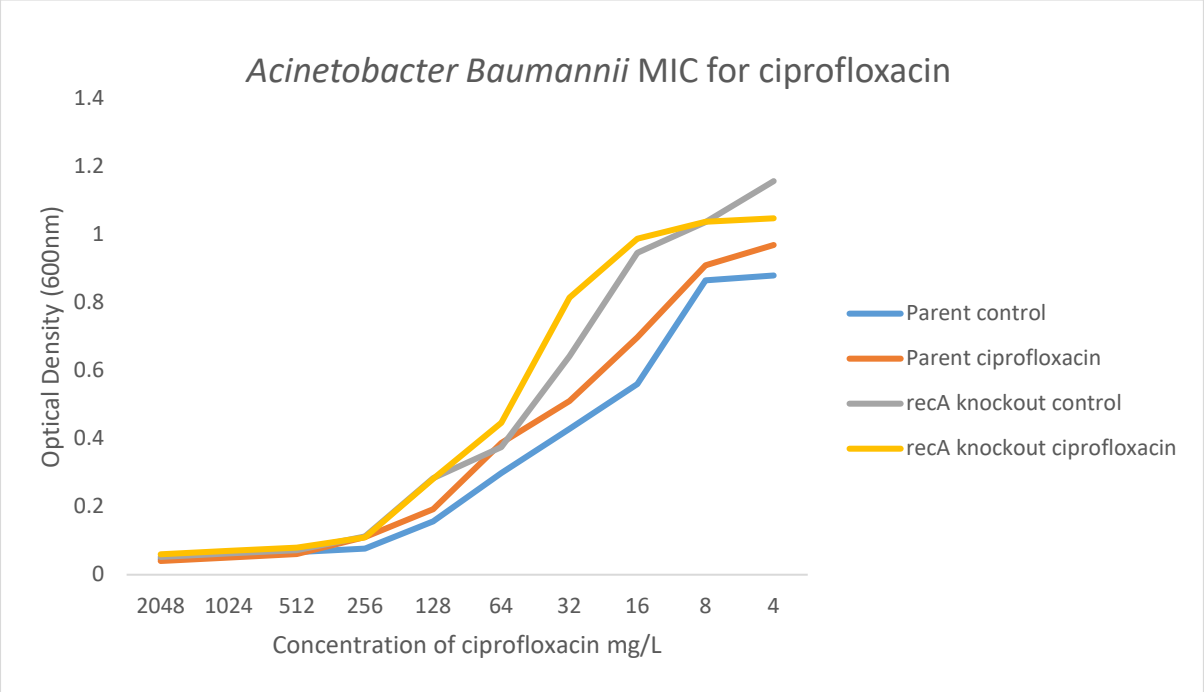

Figure 3: The MIC of ciprofloxacin for *A.baumannii* at passage 40. The four lines are the average of 8 replicates.

Discussion

To examine the potential role of the SOS response in *Acinetobacter baumannii,* we exposed a *recA* knockout and wild type strain to low levels of antibiotics in a serial plating experiment. As RecA is the inducer of the SOS response, the *Acinetobacter baumannii recA* knockout would not be able to induce the SOS response. REP and BOX PCRs were used to track genome changes and MIC testing was used to determine changes in resistance to antibiotics.

All lines remained at their original MIC of 256mg/L of ciprofloxacin, indicating that after 40 passages at 1/10 the MIC there was no increase in resistance. This finding is different

187 from studies on other bacterial species, where significant increases in MIC were observed
188 after as few as five passages on sub-inhibitory ciprofloxacin (Chow *et al.*, 2015).

189 DNA fingerprinting techniques were used to monitor potential changes to the
190 bacterial genome. These techniques examine a sub-sample of genomic regions, generating
191 amplicons that are sensitive to point mutations in primer binding sites and to indels within the
192 amplified region (Gillings and Holley 1997). REP PCR and BOX PCR both detected minor
193 changes to banding patterns in the passaged lines, however these were not confined to the
194 lines exposed to ciprofloxacin, and both wild type and the *recA* knockout control lines also
195 had changes in banding patterns. This suggests that the banding pattern changes were
196 unrelated to either *recA* status or ciprofloxacin exposure. Determining whether these banding
197 changes were due to genetic drift caused by repeated bottlenecks, or arise from the
198 stochastics of the PCR process are beyond the current study.

199 *A. baumannii* may not respond to DNA damage in the same way that most bacteria
200 do. Many bacteria respond to DNA damage by triggering the SOS response. The SOS
201 response is controlled by the LexA repressor and the RecA inducer. Under normal conditions,
202 LexA prevents SOS genes from being expressed by binding to the SOS box, located in the
203 promoter region of the SOS genes (Michel, 2005). Following DNA damage, the protein RecA
204 is recruited onto single stranded DNA where it stimulates cleavage of the LexA repressor,
205 inactivating it and therefore allowing the expression of approximately 40 SOS genes. SOS
206 genes are mainly involved in DNA repair, and include highly error prone polymerases, which
207 increases mutation rates. (Laureti *et al.*, 2013, Baharoglu & Mazel, 2014). However, in *A.*
208 *baumannii*, it seems that the SOS response operates independently from the LexA repressor
209 and the RecA inducer, probably using a non-lexA repressor, as seen in *Acinetobacter*
210 *calcoaceticus* (Rauch *et al.*, 1996). The fact that *Acinetobacter baumannii* can induce RecA
211 via another pathway might explain why both strains exhibited similar results in our

experiments, as the *recA* gene is not required. It seems that RecA is needed for bacterial repair, not due to its normal role as an inducer in the SOS response, but through a recombinational repair pathway (Aranda *et al.*, 2011). A similar role of RecA is seen in *Neisseria gonorrhoeae* where RecA is involved in protection from oxidative DNA damage (Stohl & Seifert, 2006).

Ciprofloxacin damages DNA and is well known to trigger induction of the SOS response (Cirz *et al.*, 2006, López *et al.*, 2007). In studies that use *recA* knockouts or a non-cleavable LexA repressor, the SOS response cannot be triggered, and increased resistance to antibiotics cannot arise as a consequence of SOS driven error-prone DNA replication (Michel, 2005). The fact that no lines of *A. baumannii* exhibited increased resistance suggests that the SOS response is not triggered by antibiotic induced DNA damage in *A. baumannii.* No *Acinetobacter spp.* have been documented to have an SOS box. This suggests that the response of *Acinetobacter* to DNA damage could involve systems other than the SOS response (Aranda *et al.*, 2013). Consequently, *Acinetobacter baumannii* may not respond to low levels of antibiotics as other bacterial species do, where it is well documented that sub-clinical concentrations trigger the SOS response and increase mutation rates (Mesak *et al.*, 2008, Andersson & Hughes, 2014, Chow *et al.*, 2015).

These findings highlight that there is more to be discovered about *Acinetobacter baumannii*'s response to DNA damage. *Acinetobacter baumannii* is increasingly being recorded as a nosocomial pathogen, and there are many reports of multi-drug resistance, including strains that are resistant to all known antibiotics (Peleg *et al.*, 2008). It clearly has the ability to acquire resistance via mutation and horizontal gene transfer. More research is needed into *Acinetobacter baumannii* to determine how it repairs damaged DNA, its general stress response, and how it generates high levels of resistance to antibiotics.

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Chapter 5: Minimum inhibitory concentrations of antibiotics in biofilms

Bacteria in biofilms display significantly more resistance to antibiotics compared to their planktonic counterparts, and this makes treating infections caused by biofilm forming bacteria more difficult. Despite this, there is no standardised methodology to quantify this increase in resistance, making it difficult to compare and replicate results, and to devise treatment regimens for biofilms. In this chapter we examined the increased resistance to antibiotics that biofilms display.

We developed a novel method for measuring the minimal inhibitory concentration (MIC) of antibiotics in biofilms. Using a standard tube biofilm experimental set up, we incrementally increased antibiotic concentration. Optical density readings were used to measure cell dispersal from the biofilm, and were used to indicate the biofilm MIC. We document increased resistance of 8 to 512 times the corresponding planktonic MIC, depending on the antibiotic tested. This protocol is a useful tool for predicting and tracking resistance phenomena in biofilms.

The publication in this chapter the product of a working collaboration between myself, Timothy Ghaly and Michael Gillings. I was predominantly involved in the design, analysis, and interpretation of the data and the drafting of the manuscript. Michael Gillings contributed to the drafting of the manuscript. Timothy Ghaly contributed to the laboratory experiments.

Detailed contributions

	Louise Chow	Michael Gillings	Timothy Ghaly
Design	80%	10%	10%
Laboratory experiments	70%	-	30%
Analysis	90%	-	10%
Writing	80%	20%	-

This chapter has been prepared for publication in *Letters in Applied Microbi*ology. The formatting, referencing and word limits adhere to their author guidelines. Figures have been embedded in the text for the purpose of this thesis.

Minimum inhibitory concentrations of antibiotics in biofilms

Running title: Antibiotics in biofilms

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Keywords: aquatic biofilms, antibiotic resistance, *Pseudomonas protegens*, *Escherichia coli, MIC*

Significance and Impact of Study

Bacteria in biofilms display higher resistance to antibiotics than their planktonic counterparts which makes treating infections caused from biolfilm forming bacteria more difficult. Despite this, we lack a standardised methodology to quantify this increase in resistance, making it difficult to compare and replicate results. Here we adapt protocols used for liquid and solid media to develop a novel method for measuring the minimal inhibitory concentration (MIC) of antibiotics in biofilms. This study provides a rapid an inexpensive method by which to measure the MIC of antibiotics in biofilms, which can be used to further investigate how antibiotics affect biofilms.

Abstract

Biofilms are found in almost all environments, and play a significant role in medicine, with some 65% of bacterial infections being caused by biofilm associated bacteria. Biofilms provide a structured environment where bacteria can thrive, because they provide protection against environmental stressors. Bacteria in biofilms display significantly more resistance to antibiotics compared to their planktonic counterparts and this makes treating infections caused by biofilms more difficult. Despite this, we lack a standardised methodology to quantify this increase in resistance, making it difficult to compare and replicate results. Here we adapt protocols used for liquid and solid media to develop a novel method for measuring the minimal inhibitory concentration (MIC) of antibiotics in biofilms. We document increased resistance of 8 to 512 times the corresponding planktonic MIC. Here, we present a simple and inexpensive method for testing the antibiotic susceptibility of bacteria in biofilms. This protocol may be a useful tool for predicting and tracking resistance phenomena in biofilms.

Keywords: aquatic biofilms, antibiotic resistance, *Pseudomonas protegens*, *Escherichia coli, MIC*

Introduction

Biofilms are complex matrices of bacteria and polymeric material (Donlan, 2002). They can form on a wide range of substrates, including environmental surfaces and living or dead tissue in animals or humans. Bacterial biofilms can comprise a single species but are often multi-species assemblages (Costerton et al., 1995, Balcázar et al., 2015).

Research into biofilms has been gaining attention due to their importance in human medicine, with an estimated 65% of bacterial infections being caused by bacteria residing in biofilms (Ito et al., 2009, Lewis, 2008). Infections caused by biofilms are difficult to treat and often recur after treatment. Medical implants such as catheters, pacemakers, heart valves and endotracheal tubes are prone to chronic infection caused by biofilms (Hall-Stoodley et al., 2004, Donlan, 2001). Urinary tract infections are the most common type of nosocomial infection, and are commonly caused by biofilm formation in catheters (Hatt and Rather, 2008). Chronic lung infections in cystic fibrosis patients are also examples of biofilm-mediated infection (Bjarnsholt et al., 2009). To add to this concern, biofilms grow slowly and may not produce detectable symptoms until the biofilm is well established (Costerton et al., 1999).

The fact that biofilms are found in virtually all environments indicates there are significant advantages for bacteria residing in a biofilm. Biofilms provide a structured environment in which bacteria can thrive in otherwise hostile environments, because they provide protection against environmental stressors. Bacteria can be 10 to 1000 fold more resistant to antibiotics when in a biofilm than when in a planktonic state (Anderson and O'Toole, 2008, Penesyan et al., 2015, Mah and O'toole, 2001, Costerton et al., 1995, Ito et al., 2009). The mechanisms for increased resistance are diverse, with factors such as decreased antibiotic diffusion and antibiotic degradation being just two possible mechanisms (Stewart and Costerton, 2001, Penesyan et al., 2015), and these mechanisms probably differ between

different types of biofilms and between species. However, planktonic cells derived from biofilms do not maintain increased resistance (Lewis, 2008). This indicates that the increased resistance seen in biofilms is not due to mutations or transfer of resistance genes, but rather to the structure and properties of the biofilm (Stewart and Costerton, 2001).

Control of biofilms is a priority for medical and industrial applications. However, the dynamics of bacterial growth and the assessment of antibiotic resistance is usually tested with planktonic cells. There is a lack of methodology for testing these parameters in biofilms, making it difficult to standardise and replicate results. Understanding the role of biofilms in the rise of antibiotic resistance requires methods for assessing the way different antibiotics penetrate, degrade and affect biofilms (Hall-Stoodley et al., 2004). In particular, the Minimum Inhibitory Concentration (MIC) of antibiotics will vary significantly between biofilm and planktonic cells. Here we describe a novel protocol for determining the MIC of antibiotics using a standard tube biofilm (Peterson et al., 2011, Schaefer et al., 2001).

Results and discussion

Control lines, which had no antibiotic added, exhibited fairly consistent optical density throughout the experiment (Fig. 1 and 2). This demonstrates that once the biofilms were established, a constant number of cells were being shed into the flow-through medium. For the experimental treatments, antibiotics were initially added to the flow through medium at the measured MIC for planktonic cells. As expected, this concentration had no effect on the cell density for either test species, using any of the antibiotics. Consequently, we could conclude that the MIC for planktonic cells had little or no effect on the biofilm cells.

As antibiotic concentrations were incrementally doubled, cell density did begin to decline, although this decline appeared at different concentrations for the different antibiotics tested. When optical density fell to zero, this was estimated as the biofilm MIC. For *Ps.*

protegens, the biofilm MIC was 8 times and 512 times the planktonic MIC for ciprofloxacin and trimethoprim, respectively (Fig 1). Initial declines in cell density began earlier in the concentration gradient, at 4 and 128 times the MIC for ciprofloxacin and tetracycline, respectively.

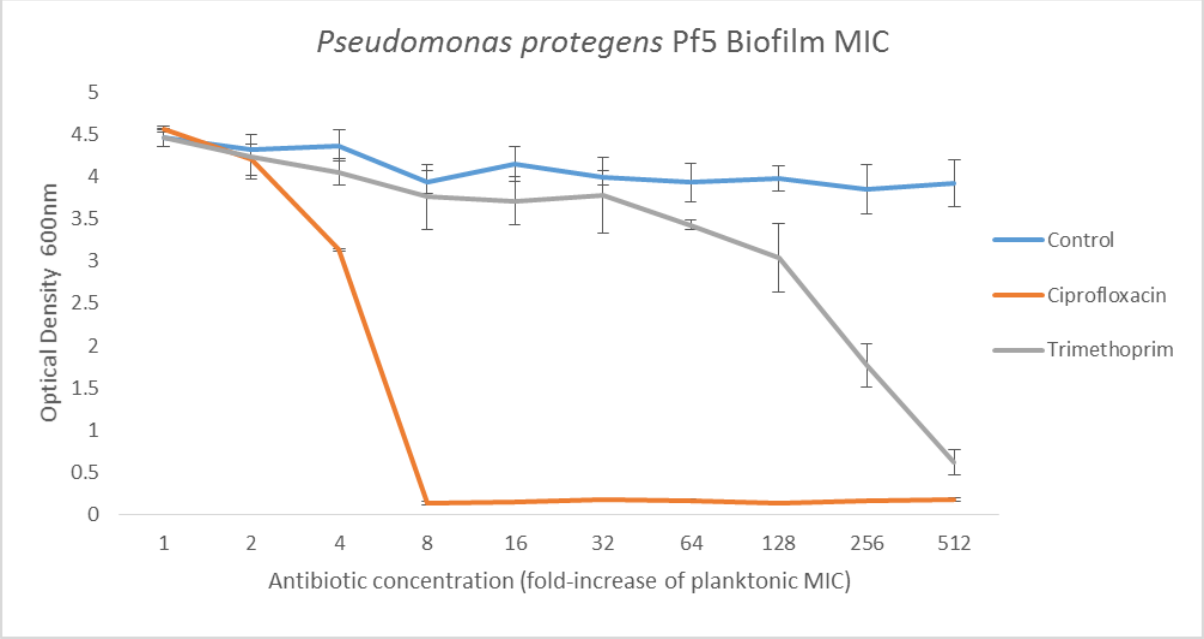

Fig. 1. Optical density readings (± 1 S.E) of flow through cells shed from *Pseudomonas protegens* biofilms exposed to increasing concentrations of ciprofloxacin and trimethoprim. When optical density fell to zero, this was estimated as the minimum inhibitory concentration (MIC) as it indicated the biofilm was no longer shedding cells. The biofilm MIC was 8 and 512 times the planktonic MIC for ciprofloxacin and tetracycline, respectively (n = 3 replicate lines). The control lines remained constant throughout the experiment.

When looking at *E. coli,* declines in optical density began as soon as antibiotic was added to the biofilm. This trend continued in a more or less linear fashion with increasing concentrations of antibiotic, until optical density approached zero at 256 times the planktonic MIC. At greater concentrations, the antibiotic interfered with optical density measurements.

The biofilm MIC for *E. coli* was consequently estimated as approximately 256 times the planktonic MIC for all three antibiotics (Fig 2).

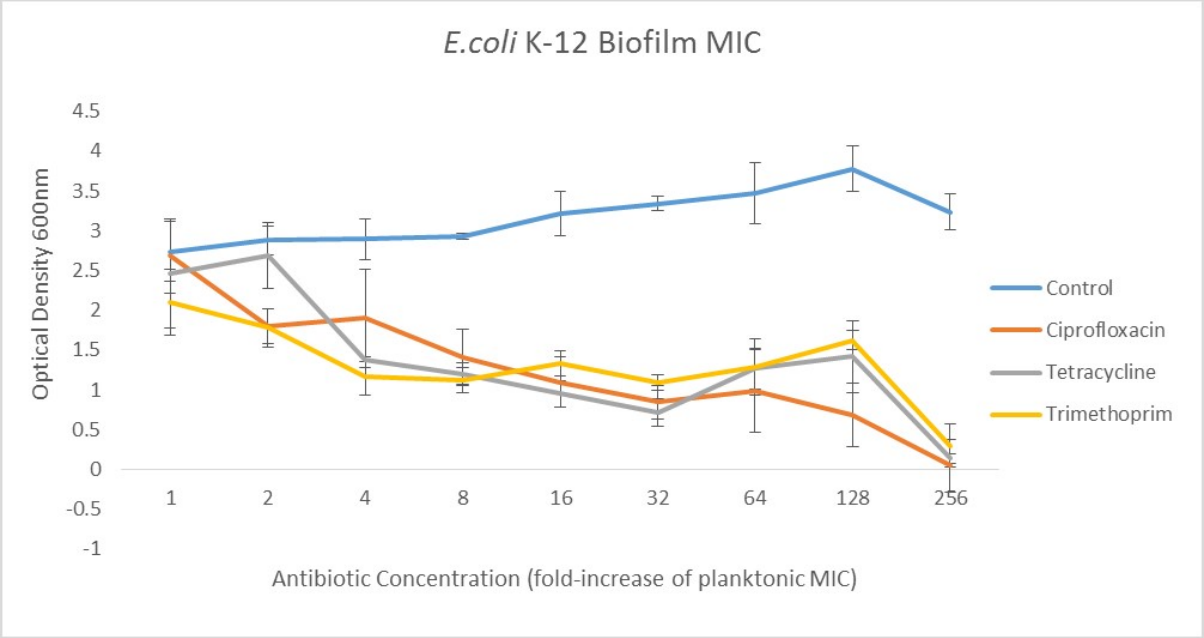

Fig. 2. Optical density readings (± 1 S.E) of flow through cells shed from *Escherichia coli* K12 biofilms exposed to increasing concentrations of ciprofloxacin, trimethoprim and tetracycline. When optical density fell to zero, this was estimated as the biofilm minimum inhibitory concentration (MIC) as it indicated the biofilm was no longer shedding cells. The biofilm MIC was 256 times the planktonic MIC for all three antibiotic treatments (n = 3 replicate lines). The control lines remained constant throughout the experiment.

All cells collected from the flow through were re-tested for antibiotic resistance. In every case, the liberated cells exhibited the same MIC as the original tests on planktonic cells, demonstrating that resistant strains had not been selected during the experiment.

Here we developed a simple, reproducible method for testing bacterial biofilms for sensitivity to antibiotics. We show that bacterial biofilms exhibit an increase in resistance to antibiotics, confirming that biofilm MICs are significantly higher than planktonic MICs. The estimated MIC values fall within the predicted range of a 10 to 1000 fold increase in resistance

in bacterial biofilms (Costerton et al., 1995, Mah and O'toole, 2001). These increases in MIC were not due to mutations accumulated during the experiment, since liberated planktonic cells were susceptible to the original MIC.

This method could be very useful for rapidly assessing large numbers of species/antibiotic combinations, and can do done rapidly and inexpensively. Such tests might be particularly important, because it appears that different antibiotics might exhibit differential increases in MIC against single bacterial species, and that different species might exhibit different response profiles in biofilms. Tube biofilms are also likely to be a better model of naturally occurring aquatic biofilms than the more commonly used static microtiter plate assays.

The mechanisms behind increased resistance in biofilms are likely to be multifactorial (Penesyan et al., 2015). Antibiotics might not be able to penetrate the biofilm or diffuse slowly once they enter the biofilm. Antibiotic penetration and diffusion varies between types of antibiotics and between biofilms formed by different bacterial species. Reduced availability of nutrients within the biofilm puts some bacterial cells into a semi-dormant state where they are less susceptible to antibiotics (Costerton et al., 1999). Degradation of antibiotics by some members of a biofilm community could benefit all cells in the biofilm.

Consequently, the resistance of biofilm cells arises from the physical and functional attributes of the biofilms themselves, and not from genetic changes in the cells. The planktonic cells that are shed from biofilms do not retain increased resistance. Cells that disperse from biofilms as a result of stress, damage to the biofilm or physical sloughing of biofilm sections maintain many biofilm characteristics, such as antibiotic resistance (Donlan, 2002).

It is clear that biofilms display significantly higher resistance to antibiotics than their planktonic counterparts. This creates problems for medical treatment and for maintenance of

medical or industrial equipment. Understanding the properties of biofilms requires a simple and tractable laboratory model for biofilm growth. This system we have described here is one such model but is limited as it only describes in vitro effects.

Materials and Methods:

Bacterial isolates

Pseudomonas protegens PF-5 (formerly *Pseudomonas fluorescens* PF-5) and *Escherichia coli* K12 were selected for this study. *Ps. protegens* is a common Gram-negative soil bacterium studied for its biocontrol properties (Loper et al., 2012). Isolates were obtained from Professor Ian Paulsen, Macquarie University. *E. coli* is a common Gram-negative bacterium important in clinical settings, particularly due to their role in urinary tract infections. *Escherichia coli* K-12 was obtained from the Keio collection (Baba et al., 2006).

Bacteria were maintained on LB Agar plates (0.01% tryptone, 0.005% yeast extract, 0.005% sodium chloride, 0.015% Agar) at 37°C. Single colonies were re-suspended in equal parts 50% glycerol and M9 salts and held at -80°C for long term storage.

Antibiotics

The antibiotics ciprofloxacin, tetracycline and trimethoprim were used for this study. Ciprofloxacin is currently listed in the World Health Organization Essential Medicines List (WHO, 2017). It is a broad-spectrum fluoroquinolone used to treat both Gram-positive and Gram-negative bacterial infections. It inhibits DNA gyrase, which in turn prevents DNA replication (LeBel, 1988). Tetracycline is a broad spectrum antibiotic that is used to treat both Gram-positive and Gram-negative bacterial infections. It prevents proteins synthesis by preventing attachment of aminoacyl-tRNA to the ribosomal acceptor site (Chopra and Roberts,

2001). Trimethoprim inhibits synthesis of DNA by inhibiting the reduction of dihydrofolic acid to tetrahydrofolic acid (Gleckman et al., 1981). Trimethoprim is currently listed in the World Health Organization Essential Medicines List (WHO, 2017).

Biofilm formation

The biofilm set up followed a standard Tube Biofilm protocol (Peterson et al., 2011). Sterile Luria-Bertani medium (0.01% tryptone, 0.005% yeast extract, 0.005% sodium chloride, 0.015% Agar) was pumped through sterile Tygon Tubing using a IPC High Precision Multichannel Dispenser (ISMATEC) at a flow rate of 50 µL per minute. The tubing was inoculated with a 1:100 dilution of an overnight culture of a single colony of *E. coli* or *Ps. protegens* and biofilm growth established over three days (72hrs) at 37°C. At this time point the biofilm was fully established, and planktonic cells were being shed into the medium

MIC determination

To determine a starting concentration for addition of antibiotics into the biofilms, the MIC of planktonic cells was determined. The MICs for ciprofloxacin and trimethoprim was determined for *Ps. protegens*. The MICs of ciprofloxacin, trimethoprim and tetracycline were determined for *E. coli*. MICs were determined using a standard MIC measurement test (Wiegand et al., 2008). Tetracycline was not used for *Ps. protegens* as it displayed intrinsic resistance to this antibiotic.

Antibiotics were added to the sterile medium being pumped into the tubing starting at a concentration of 1 x the planktonic MIC. The pump was left to run for 90 minutes before flow through was collected and the optical density was read on a Pherastar FS spectrometer at 600 nm. Optical density readings were used as an indicator of cell dispersal from the biofilm, indicating the growth or inhibition of the biofilm. Optical density readings were blanked against LB medium with the corresponding concentration of antibiotic for each sample.

Antibiotic concentrations were doubled every 90 minutes and flow-through collected. Three replicate lines for each treatment and three control lines with no antibiotics were carried through the experiment.

To determine whether any increase in resistance was due to mutation or the properties of the biofilm matrix itself, all samples were inoculated into sterile LB broth and incubated overnight at 37°C. The MICs were measured for the re-cultured planktonic cells. of ciprofloxacin and trimethoprim for *Ps. protegens* PF-5 and ciprofloxacin, trimethoprim and tetracycline for *E. coli* was determined on the planktonic cells, again using a standard MIC test (Wiegand et al., 2008).

Acknowledgements

The authors would like to acknowledge the members of the EMMA lab, Anahit Penesyan, Marita Holley and Sasha Tetu for their invaluable contributions to this research.

Funding

Work was funded by The Faculty of Science and Engineering Macquarie University and the Australian Government Research Training Program (RTP).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 6: The effect of sub-inhibitory antibiotic concentrations on biofilms

In this chapter we examine the effect of low levels of the antibiotic ciprofloxacin on *E.coli* growing in biofilms. Using a standard tube biofilm set up, we subjected the biofilm to a low concentration of antibiotics to examine the phenotypic and genetic consequences of exposure. Wild type and *recA* knockout mutants were used to examine the role of the SOS response in the observed responses.

Using whole genome sequencing, we identified two consistent single base pair mutations. The first mutation was observed in *treB* and was only seen in the wild type, suggesting that it was driven by a response to DNA damage mediated by the SOS system. The second mutation, in *yebT*, was observed in both the wild type and the *recA* knockouts, suggesting that this appearance of this mutation operated independently from the SOS response.

This chapter is the product of a working collaboration between myself, Timothy Ghaly and Michael Gillings. I was predominantly involved in the design of the study, the laboratory experiments, interpretation of the data and the drafting of the manuscript. Michael Gillings was involved in the design of the study and contributed to the drafting of the manuscript. Timothy Ghaly was involved in the laboratory experiments, analysis and interpretation of the DNA sequencing data.

Detailed Contribution

	Louise Chow	Michael Gillings	Timothy Ghaly
Design	50%	40%	10%
Laboratory work	50%	-	50%
Analysis	50%	-	50%
Writing	80%	20%	-

This chapter has been prepared for publication in *NPJ Biofilms and Microbiomes*. The formatting, referencing and word limits adhere to their author guidelines. Figures have been embedded in the text for the purpose of this thesis.

Sub-inhibitory concentrations of antibiotics: Effects on biofilms

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Keywords: sub-inhibitory; aquatic; antibiotic resistance; pollution; pharmaceutical

Abstract

Antibiotics disseminate into the environment via waste effluent and agricultural run-off. Here they can persist at low concentrations, and should be thought of as pollutants due to their continued ability to disrupt microbial ecosystems. In particular, low levels of antibiotics are known to trigger the SOS response, which is a general bacterial response to DNA damage. As the majority of bacteria are found in biofilms, it is important to investigate what effect environmentally relevant concentrations of antibiotics might have on bacteria in this state. Biofilms are complex matrices of bacteria, polysaccharides and proteins that display significantly higher antibiotic resistance than bacteria in a planktonic state. To replicate what might be happening in the environment, we used an experimental biofilm set up, and subjected *E. coli* biofilms to low concentrations of antibiotics. Biofilms formed from wild type and a *recA* knockout mutant were compared, to determine the role of SOS in response to low levels of antibiotics. Using whole genome sequencing, we identified two relevant mutations, one in *treB* (trehalose-specific enzyme II^{Tre}) which was seen in all three independent lines of the wild type bacteria, suggesting that it was a response to DNA damage mediated by the SOS response. The second mutation, in *yebT*, a mammalian cell entry (MCE) domain protein, was seen in both the wild type and the *recA* knockouts, suggesting that this mutation operated independently from the SOS response.

Introduction

Antibiotic resistant infections are one of the main threats to human health in the 21st century, with estimates that they will account for 10 million deaths annually by 2050 [1]. Antibiotics are widely used in both human medicine and agriculture, with many medical and farming practices being reliant on antibiotics to treat and prevent infections. With growing concerns over the impact of antibiotic resistant infections, many countries have employed monitoring strategies and treatment protocols. However, current stewardship measures are not effective, and there has been a 65% increase in antibiotic usage between 2000 and 2015 [2].

Antibiotics are poorly metabolized, with 70-90% of antibiotics that are consumed being excreted unchanged [3,4]. Since current waste treatments do not remove antibiotics efficiently [5], they can enter the environment and persist for significant lengths of time. Likewise, in agriculture, the majority of antibiotic is excreted and enters the environment directly, or through manuring of land with animal waste. Antibiotics can also enter the environment via pharmaceutical run-off [6], fruit spraying [7], and landfill leachate [8]. Antibiotics entering the environment via these different routes should be thought of as pollutants, due to their ability to persist in the environment and disrupt normal microbial functions.

Overuse of antibiotics in both the agricultural and medical fields has placed selective pressure on bacteria, giving advantages to strains that have acquired mechanisms to avoid the damaging effects of antibiotics. When antibiotics are used the aim is to reach a treatment concentration at or above the minimum inhibitory concentration (MIC). However, when antibiotic pollution enters the environment there is a gradient of antibiotic concentration radiating from areas of human population. This means that antibiotics will be found at concentrations below the MIC. It is becoming increasingly evident that these low

concentrations of antibiotics increase mutation rates via the SOS response [9,10]. The SOS response is a general bacterial response to DNA damage, such as the damage inflicted by antibiotics, and its relationship with upregulation of mutation is well documented [11,12]. There are approximately 40 genes involved in the SOS response, several of which are error prone DNA polymerases that allow replication machinery to bypass damaged regions of DNA. This maintains chromosomal integrity, but also significantly increases the likelihood of base substitutions [11,13 14]. The SOS response is mediated by two main proteins, a repressor, LexA and an inducer, RecA [12].

In vitro studies normally examine the mechanisms by which bacteria respond to low levels of antibiotics by using bacteria in the planktonic state. These studies, while important and useful, do not examine the biofilm state, in which most bacteria are normally found. Approximately 99% of bacteria exist in biofilms, both in the environment and within living organisms [15]. Biofilms are complex matrices of bacteria, polysaccharides and proteins that adhere to surfaces [16]. They can consist of one species but are more often comprised of several species of bacteria, leading to stable interactions between bacterial cells that can increase fitness [17,18].

Bacteria in biofilms are more resistant to antibiotics than their planktonic counterparts, and this makes treating infections caused by biofilms more difficult [19]. An increase of 10 to 1000 fold in resistance is common [20]. The mechanisms of this increased resistance are not well documented. Factors such as decreased antibiotic diffusion and metabolic activity are just two possible mechanisms [21]. Planktonic cells that are shed as a result of normal growth and development of the biofilm are unlikely to retain increased resistance, and it is unknown what triggers shedding of cells in this manner [16]. However, cells that disperse from the biofilm as a result of stress (such as lack of nutrients or antibiotics),

damage to the biofilm, or cells that are physically sloughed off the biofilm, can maintain many biofilm characteristics, such as antibiotic resistance [16].

The susceptibility and effect of antibiotics on biofilms cannot be studied via standard MIC and antibiotic exposure tests as these report the effect of antibiotics on bacteria in the planktonic state [22]. In order to effectively study the effect of antibiotics on biofilms studies must attempt to reproduce *in vivo* conditions. Here we used a tube biofilm set up, and once the biofilm was well established it was exposed to a low level of antibiotics, similar to the concentration we would expect to see in the environment. This was done to examine how biofilms would react to environmental levels of antibiotic pollution.

Methods

Bacterial Isolates

Escherichia coli was used for this experiment. *E.coli* is a common Gram negative bacterium found in both the environment and within warm-blooded organisms. A wild type and isogenic *E.coli recA* knockout strain were compared to examine the potential role of the SOS response in the generation of mutations. *E.coli* K-12 BW25113 wild type and *recA* knockout strains were obtained from the Keio collection [23].

Bacteria were maintained on Luria-Bertani Agar plates (0.01% tryptone, 0.005% yeast extract, 0.005% sodium chloride, 0.015% Agar) at 37°C. Single colonies were re-suspended in equal parts 50% glycerol and M9 salts and held at -80°C for long term storage.

Antibiotic treatment

Ciprofloxacin was selected for use in this study. Ciprofloxacin is a broad-spectrum fluoroquinolone used to treat both Gram-positive and Gram-negative bacterial infections. It inhibits DNA gyrase, which in turn prevents DNA replication[24]. Ciprofloxacin is well documented to induce the SOS response. The planktonic minimum inhibitory concentration of ciprofloxacin for the *E. coli* wild type and *recA* knockout strains was determined to be 0.03125 mg/L using a standard MIC protocol [25].

Biofilm experiment

The biofilm set up followed a standard Tube Biofilm set up [26], consisting of sterile Luria-Bertani medium (0.01% tryptone, 0.005% yeast extract, 0.005% sodium chloride, 0.015% agar), pumped through sterile Tygon Tubing using a IPC High Precision Multichannel Dispenser (ISMATEC) at a flow rate of 50uL per minute. The tubing was inoculated with either *E. coli* wild type or *recA* knockout strains using an overnight culture diluted to an OD600 of 0.5 in LB. The biofilm was established over 72hrs at 37°C at a flow rate of 50uL per minute. After biofilm establishment, ciprofloxacin was added to the sterile media at 1/10 the planktonic MIC. A control line without antibiotics for both the *E.coli* wild type and *recA* knockout strains was also carried through the experiment. Three replicates were used for each of the four experimental lines. The biofilm was left to run a further 72hrs at 37°C. The run-off was collected and spread onto LB Agar plates at a dilution of 10^{-6}. The plates were incubated overnight at 37°C. Ten single colonies were randomly picked from each plate and re-suspended in LB broth and incubated overnight at 37°C. DNA extractions and glycerol stocks (equal parts 50% glycerol and M9 salts) were prepared from the overnight growth.

DNA Extraction

DNA was extracted using a standard CTAB phenol:chloroform:isoamyl alcohol protocol [27]. In brief, 0.5mL of overnight cultures were used, cells were lysed in a CTAB/$NaCl_2$ solution.

DNA was purified using phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with isopropanol. The sample was further purified with an RNase treatment before precipitation and resuspension in TE buffer.

DNA sequencing and Analysis

Illumina HiSeq4000, 100bp paired-end sequencing was performed at the Macrogen sequencing facility (Seoul, South Korea). 68 samples were sequenced in 3 lanes (24 sample multiplex). Mutations were detected by mapping the short read data to the *E. coli* K12 BW25113 reference genome (CP009273) using breseq (Deatherage, 2014).

Results

Point mutations were detected in both the wild type and *recA* knockout experimental lines with a mean of 1.3 and 1.47 mutations per sample respectively (Genbank accession number PRJNA507271) (Table S1). There were slightly more mutations in the experimental *recA* knockout lines but this was not significant (Students T-test, n=30, p= 0.1933). Point mutations were also detected in both the wild type and *recA* knockout control lines with a mean of 1 and 1.67 mutations per sample respectively (Genbank accession number PRJNA507271) (Table S1). Once again there were slightly more mutations in the control *recA* knockout lines but this was not significant (Students T-test, n=3, p= 0.3015). For both experimental and control lines, and both wild type and *recA* knockouts, most mutations were only seen in a single isolate, however, there were two point mutations that were found independently in several isolates. Such events are candidates for mutations that provide a selective advantage under the experimental conditions. Independent mutations were observed in *treB*, a gene encoding the trehalose-specific enzyme II^{Tre}, involved in trehalose transport, and a mutation in *yebT*, which encodes a mammalian cell entry (MCE) domain protein.

A mutation in *treB* was recovered in 26 of the 30 wild-type colonies recovered from the ciprofloxacin treatment, but from none of the *recA* knockout colonies, nor from any of the colonies recovered from the no antibiotic controls. The mutation was seen in all three replicate biofilms. The mutation was a G to an A, in the 113th codon, changing the amino acid from valine to glutamic acid. Valine is the normal amino acid present in the *E. coli* culture stocks used for this experiment and is the amino acid encoded in the *E. coli* reference genome (CP009273). Because all experimental and control lines were initiated from stock cultures with valine encoded at this position, the mutation we observed has arisen and fixed in multiple, independent events in all replicates of the ciprofloxacin-treated wild type biofilms. This mutation to glutamic acid in TreB was also found in 10 other bacterial sequences deposited in Genbank (Species and Genbank accession numbers *Escherichia coli* CFT073 (AE014075.1), *Vibrio vulnificus* CMCP6 (AE016795.3), *Salmonella enterica* (AE014613.1), *Shigella flexneri* 2457T (AE014073.1), AA163993.1, *Shigella flexneri* Shi06HN006 (CP004057.1), *Clostridium sporogenes* (CP009225.1), *Erwinia carotovora* (BX950851.1), *Yersinia pestis* (AL590842.1), and *Klebsiella pneumoniae* (FO834906.1)). This indicates that this is a common mutation that might confer a selective advantage.

A single base pair mutation in the YebT (also known as MAM7) MCE domain protein was observed in 19/30 of the *recA* knockout colonies and 9/30 wild-type colonies that were exposed to ciprofloxacin. The mutation was seen in all three replicates of both wild type and *recA* knockout lines. The same mutation was observed in a single colony of the wild-type control lines that were not exposed to ciprofloxacin. The mutation was a T to a C in the 814th codon, changing tyrosine to histidine. Tyrosine is the normal amino acid present in the *E. coli* culture stocks used for this experiment and is the amino acid encoded in the *E. coli* reference genome (CP009273). Because all experimental and control lines were initiated from stock cultures with tyrosine encoded at this position, the mutation we observed arose and was fixed

independently in multiple treatments during the course of the experiment. The amino acid residue (tyrosine) in YebT is highly conserved in other bacterial species (eg Species and Genbank accession numbers, *Citrobacter werkmanii* (NZ_BBMW00000000), *Enterobacter cloacae* UW5 (CP002886.1), *Erwinia sp.* Ejp617 (CP002124.1), *Escherichia coli* str. K-12 substr. MG1655 (U00096.3), *Klebsiella pneumoniae* (LK022720.1), *Pantoea ananatis* (CP001875.2), *Salmonella enterica* serovar typhimurium (CM001062.1), *Serratia plymuthica* (CP006250.1), and *Yersinia pestis* CO92 (AL590842.1)). This indicates that this is normally a conserved region of the protein, and the mutation observed here could confer some selective advantage under the conditions of the experiment.

Discussion

To examine the effect of low levels of antibiotics on biofilms, we exposed an established *E. coli* biofilm to a sub-inhibitory concentration of the antibiotic ciprofloxacin. This was done to examine how biofilms might respond to environmentally relevant levels of antibiotic pollution. Through whole genome sequencing we were able to identify several point mutations in *E. coli* when exposed to 1/10 the MIC of ciprofloxacin. By using both a wild type strain and a *recA* knockout strain of *E. coli* we aimed to identify the whether the SOS response was activated in response to these low levels of ciprofloxacin, as RecA is the inducer of the SOS response.

The *recA* knockout lines had marginally more mutations compared to the wild type (mean of 1.47 and 1.3 mutations per sample respectively), however this was not significant ($p= 0.1933$)(Table S1). These findings run counter to the expectation that environmental levels of antibiotics would trigger the SOS response which would in turn up-regulate mutation rate in the wild type lines[13,28]. The *recA* knockout strain of *E. coli* should not have

been able to trigger the SOS response, and we might expect fewer mutations in this line when compared to the wildtype lines. Of course the rate of *de novo* mutation is not the same as the number of mutations that subsequently fix via selection and can thus be detected, so our genome survey was affected by both positive and negative selection. It is also possible that 1/10 the MIC was not a high enough concentration to trigger the SOS response in biofilms due to an inherently decreased antibiotic susceptibility facilitated by the biofilm. As such, 1/10 the planktonic cell MIC may represent a concentration as low as 1/10,000 the biofilm MIC[21]. This might explain why there were not significantly more mutations in the wild-type lines compared to the *recA* knockout lines.

Even with these caveats, our experiments did detect mutations that are candidates for events that could have been driven by SOS mechanisms and subsequent selection events. A single base pair mutation in *treB* was seen in 26 of the 30 isolates from all three replicates of wild type strains exposed to ciprofloxacin. This mutation was not detected in any of the *recA* knockout lines exposed to ciprofloxacin, or in any of the unexposed wild type or *recA* control lines. Because this mutation was observed in all three replicates of the wild-type lines, the mutation must have arisen independently, and been selected to high frequency, at least three times. It is important to note that these events occurred over a very short time frame of 72 hours exposure.

Since this mutation only occurred in wild-type samples exposed to ciprofloxacin, and not in similarly exposed *recA* mutant lines, it is possibly a consequence of the SOS response mediated by RecA. TreB is a transmembrane transporter involved in the transport of trehalose, which helps in protection against environmental stress[29], and ciprofloxacin is documented to cause oxidative stress [30,31]. Furthermore, in *E. coli*, down-regulation of *treB* has been documented in response to sub-MIC levels of Quinoxalines[32], and clinical levels of ampicillin and ofloxacin also repress expression of *treB*[33]. So it seems that following

environmental stress, such as exposure to antibiotics, *treB* may be downregulated or repressed, possibly to reduce the entry of antibiotics, as TreB is a transmembrane transporter [32]. It is possible that a change in the amino acid residue from valine to glutamic acid could alter the function or down-regulate/repress *treB*, reducing the entry of antibiotics. This is potentially why this particular amino acid substitution is also found in a range of other bacterial strains as it may provide protection against the harmful effect of antibiotics. The relationship between TreB and RecA is unknown, but it is possible that RecA has role in the generation of this mutation since it was not seen in the *recA* knockout lines.

Other mutations were observed to occur and fix independently in multiple instances, although these do not appear to be mediated by SOS phenomena. A single base pair mutation in the gene *yebT* was seen in 19/30 of the *recA* knockout isolates and 9/30 wild-type isolates recovered from ciprofloxacin exposed biofilms. This mutation was much less frequent in control lines, occurring in just one isolate from the wild-type control. The mutation occurred in all three replicates of both wild-type and the *recA* knockout lines exposed to ciprofloxacin. This indicates that the mutation arose independently and rose to high frequency at least three times in each of these lines across a 72 hour exposure to ciprofloxacin. The fact that this mutation was common in both the wild-type and the *recA* knockout, but occurred in only one of the control lines suggests that this mutation was driven to high frequency by the ciprofloxacin treatment itself. A change in amino acid residue from tyrosine to histidine could alter the function of YebT. In *E.coli*, YebT helps maintain outer membrane lipid asymmetry, facilitates the transport of lipids, and plays a role in adhesion [34-36]. Factors such as outer membrane structure, transport and adhesion are important in biofilm formation, so alteration of the function of YebT might affect these processes [22].

These findings highlight the need for more research into the mechanisms of biofilm formation and functioning. In particular, the effects of antibiotics on biofilms and the bacteria

residing in them are likely to be significantly different from their effects on planktonic cells. Most research has thus far been conducted on the planktonic state, and this does not reflect the condition of the majority of environmental bacteria. It is thought that bacteria in biofilms display an increase in resistance of 10 to 1000 times that of the planktonic state [19,37] [20,38,39], and the potential for novel evolutionary responses in biofilms, such as those observed here, has been little explored.

Research into the complexities of biofilms is increasing as their importance in medicine and the generation of antibiotic resistance is recognized. More needs to be known about the mechanisms by which biofilms exhibit increased resistance to antibiotics. This will make treating and managing biofilms easier. We also need to know what effect environmental exposure to antibiotics is having on the acquisition and spread of mutations in biofilms, such as those conferring resistance.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

Work was funded by The Faculty of Science and Engineering Macquarie University and the Australian Government Research Training Program (RTP).

Acknowledgements

The authors would like to acknowledge the members of the EMMA lab, Anahit Penesyan, Marita Holley and Sasha Tetu for their invaluable contributions to this research.

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Tables:

Table S1: Table of mutations detected by mapping the short read data to the *E. coli* K12 BW25113 reference genome (CP009273) using breseq (Deatherage, 2014). The row labeled "Mutation" indicates the base pair substitution. The row labeled "Position" indicates the position in the reference genome CP009273. "x" in the rows labeled with sample names indicates a base pair substitution.

Mutation	C→T	C→T	T→C	A→G	T→G	T→C	T→A	T→C	IS5 (+) +4 bp	IS5 (+) +4 bp	G→A	Δ1 bp	G→A	T→C	A→C	G→A	C→T	T→G	A→T
Positon	1,191,676	1,191,688	1,191,701	1,191,703	1,257,757	1,264,647	1,454,056	1,914,206	1,972,818	1,972,967	2,109,395	2,168,808	2,400,041	2,719,426	2,744,787	2,817,147	4,179,376	4,350,315	4,455,660
Gene	icd →	icd →	icd →	icd →	ispE ←	ldrA ←	paaH →	yebT →	Intergenic	intergenic	wcaL ←	gatZ ←	lrhA ←	Intergenic	nadK →	Intergenic	rpoC →	cadC ←	treB ←
E. coli Parent original isolate																			
E. coli Parent Control 1a													X						
E. coli Parent Control 2a																			
E. coli Parent Control 3a								X		X									
E. coli Parent Control 1a																			X
E. coli Parent Control 1b																			X
E. coli Parent Control 1c						X													
E. coli Parent Control 1d																			X
E. coli Parent Control 1e																			X
E. coli Parent Control 1f																			X
E. coli Parent Control 1g																			X
E. coli Parent Control 1h																			X
E. coli Parent Control 1i																			X
E. coli Parent Control 1j																		X	
E. coli Parent Control 2a																			X
E. coli Parent Control 2b																			
E. coli Parent Control 2c																			
E. coli Parent Control 2d												X							X
E. coli Parent Control 2e																			X
E. coli Parent Control 2f																			X
E. coli Parent Control 2g																			X
E. coli Parent Control 2h																			X
E. coli Parent Control 2i																			X

Mutation	C→T	C→T	T→C	A→G	T→G	T→C	T→A	T→C	IS5 (+) +4 bp	IS5 (+) +4 bp	G→A	Δ1 bp	G→A	T→C	A→C	G→A	C→T	T→G	A→T
Positon	1,191,676	1,191,688	1,191,701	1,191,703	1,257,757	1,264,647	1,454,056	1,914,206	1,972,818	1,972,967	2,109,395	2,168,808	2,400,041	2,719,426	2,744,787	2,817,147	4,179,376	4,350,315	4,455,660
Gene	icd →	icd →	icd →	icd →	ispE ←	ldrA ←	paaH →	yebT →	Intergenic	intergenic	wcaL ←	gatZ ←	lrhA ←	Intergenic	nadK →	Intergenic	rpoC →	cadC ←	treB ←
E. coli Parent Control 2j																			X
E. coli Parent Control 3a								X											X
E. coli Parent Control 3b								X											X
E. coli Parent Control 3c								X											X
E. coli Parent Control 3d								X											X
E. coli Parent Control 3e								X											X
E. coli Parent Control 3f								X											X
E. coli Parent Control 3g								X											X
E. coli Parent Control 3h								X											X
E. coli Parent Control 3i								X											X
E. coli Parent Control 3j								X											X
E. coli RecA-original isolate																			
E. coli RecA- Control 1a														X					
E. coli RecA- Control 2a														X					
E. coli RecA- Control 3a					X									X		X			
E. coli RecA- Control 1a								X											
E. coli RecA- Control 1b								X											
E. coli RecA- Control 1c														X					
E. coli RecA- Control 1d								X											
E. coli RecA- Control 1e														X					
E. coli RecA- Control 1f								X											
E. coli RecA- Control 1g								X											
E. coli RecA- Control 1h								X											
E. coli RecA- Control 1i								X											
E. coli RecA- Control 1j														X					

Mutation	C→T	C→T	T→C	A→G	T→G	T→C	T→A	T→C	IS5 (+) +4 bp	IS5 (+) +4 bp	G→A	Δ1 bp	G→A	T→C	A→C	G→A	C→T	T→G	A→T
Positon	1,191,676	1,191,688	1,191,701	1,191,703	1,257,757	1,264,647	1,454,056	1,914,206	1,972,818	1,972,967	2,109,395	2,168,808	2,400,041	2,719,426	2,744,787	2,817,147	4,179,376	4,350,315	4,455,660
Gene	icd →	icd →	icd →	icd →	ispE ←	ldrA ←	paaH →	yebT →	Intergenic	intergenic	wcaL ←	gatZ ←	lrhA ←	Intergenic	nadK →	Intergenic	rpoC →	cadC ←	treB ←
E. coli RecA-Control 2a								X											
E. coli RecA-Control 2b							X	X											
E. coli RecA-Control 2c														X					
E. coli RecA-Control 2d								X											
E. coli RecA-Control 2e								X											
E. coli RecA-Control 2f														X					
E. coli RecA-Control 2g								X	X								X		
E. coli RecA-Control 2h	X	X	X	X			X												
E. coli RecA-Control 2i														X		X			
E. coli RecA-Control 2j								X	X										
E. coli RecA-Control 3a								X		X									
E. coli RecA-Control 3b														X		X			
E. coli RecA-Control 3c														X					
E. coli RecA-Control 3d								X							X				
E. coli RecA-Control 3e								X											
E. coli RecA-Control 3f											X			X					
E. coli RecA-Control 3g								X											
E. coli RecA-Control 3h								X		X									
E. coli RecA-Control 3i														X					
E. coli RecA-Control 3j								X											

General Conclusions and Discussion

Antibiotic resistance is one of the most significant threats to human health. It has been predicted that by 2050 antibiotic resistant infections will account for 10 million deaths annually (O'Neill 2014). This crisis has been driven by the ability of bacteria to rapidly evolve resistance mechanisms. Such resistance mechanisms become fixed in bacterial populations when they are exposed to the selection pressures of antibiotics, especially when these agents are over used, or used incorrectly. Approximately 70% of nosocomial infections are now resistant to at least one type of antibiotic (Zhang, Kinkelaar et al. 2011), and the breadth of this resistance is expected to increase as bacteria acquire resistance genes that confer increasingly higher levels of resistance to multiple classes of antimicrobials.

Antibiotics are constantly being released into the environment via human waste streams and agricultural run-off (Lipsitch, Singer et al. 2002, Berge, Atwill et al. 2005, Sarmah, Meyer et al. 2006). They can persist in the environment for significant periods of time, where they disrupt normal microbial processes, and should therefore be thought of as pollutants (Kümmerer 2009, Monteiro and Boxall 2010). Added to this problem are the resistance genes themselves. With constant selection pressure, the numbers of cells containing resistance genes has increased significantly over the last 70 years (Knapp, Dolfing et al. 2009). These bacterial cells are also released into the environment, with the consequence that resistance genes have also become significant pollutants (Zhu, Johnson et al. 2013, Zhu, Zhao et al. 2017). The simultaneous release of selective agents, and the genes that confer resistance to those agents, creates ideal conditions for selection of new combinations of resistance determinants in an ever-growing diversity of bacterial species. These circumstances are likely to accelerate the resistance crisis.

Research into antibiotic pollution is a rapidly growing area, as we become increasingly aware of their potential effects on bacterial evolution in environmental compartments. However, the concentrations of antibiotics found in the environment are largely unknown, as are the effects that these concentrations may have on environmental bacteria. These two questions are the subject of the research reported in this thesis.

Occurrence of antibiotics in the environment

There are significant knowledge gaps in understanding the concentrations of antibiotics in the environment, and what effect these concentrations might have on selection for acquisition of antibiotic resistance. To examine this problem, I investigated the concentrations of diverse antibiotics that have been reported from a range of environments. I surveyed the available literature to establish the range of environmental concentrations for all classes of clinically and agriculturally relevant antibiotics (Chapter 1). These data were tabulated to determine whether these concentrations could be biologically significant. Ranges were compared to the MICs available for bacterial species in the EUCAST (www.eucast.org) database. There was significant overlap between measured environmental concentrations of antibiotics and the range of antibiotic concentrations that could impose selection. This determination was made on the basis of the minimum selective concentration, which is usually defined as 0.25 to 0.004 of the MIC (Gullberg, Cao et al. 2011).

The fact that environmental concentrations of antibiotics often fall into the range where they are likely to be influencing microbial ecology, means they are likely to be driving the selection of antibiotic resistant bacteria. These findings provide a useful benchmark for future regulation over the environmental levels of antibiotics. They also confirm the idea that

antibiotics are present in the environment at concentrations where they are likely to be increasing the rate of selection events and contributing to the problem of antibiotic resistance.

Effect of environmental concentrations of antibiotics

Having determined the range of concentrations of antibiotics known to be present in the environment, I designed experiments to determine whether these concentrations could have biological consequences. I needed to determine how environmentally relevant concentrations of antibiotics might affect the generation of resistance, and in particular the involvement of systems that up-regulate mechanisms which generate genetic diversity. These responses could include increased rates of mutation, recombination, transposition and lateral gene transfer. I also needed to distinguish how these different effects might play out for both planktonic cells and for cells in biofilms.

I designed experimental evolution assays to determine how quickly resistance arises in the presence of sub-inhibitory concentrations of antibiotics (Chapter 2, 3 & 4). I exposed bacteria to low, but environmentally relevant concentrations of antibiotics in a serial plating experiment over 40 passages. Exposed bacterial lines rapidly acquired increased resistance to the experimental antibiotics, as measured by significant increases in their MIC. Because the lines were pure cultures, the resistance could not have arisen by lateral gene transfer, and consequently must have arisen by mutation in the bacterial genomes. Whole genome sequencing was used to investigate this possibility.

Consistent point mutations in genes relevant to resistance were found in independent lines, and for both antibiotics tested. In lines exposed to ciprofloxacin, a consistent point mutation was found in *gyrA* (Chapter 3). This mutation was at the same amino acid residue as previously reported to confer resistance to quinolone antibiotics in clinical pathogens,

although in our experiments we used an environmental organism not associated with human or animal disease. This finding strongly suggests that the pollution of environmental compartments with antibiotics is of a significant magnitude to impose selection pressure, and to fix antibiotic resistance mutations in novel organisms.

Similar results occurred in the experimental evolution of lines exposed to sub-inhibitory concentrations of kanamycin. Point mutations were detected in *fusA*, this again being a gene previously implicated in resistance to kanamycin (Chapter 2). The mutation affected residues known to be associated with resistance in clinical isolates. Consequently the kinds of results obtained during exposure to ciprofloxacin were replicated in experimental evolution experiments using kanamycin. This confirms the idea that antibiotic pollution is capable of generating resistant strains of environmental bacteria. Given the diversity of antibiotics known to pollute waterways, the generation of soil and aquatic bacteria that carry multiple resistance determinants seems inevitable.

I also examined the effect of low concentrations of antibiotics on bacterial biofilms. Bacteria in biofilms are more resistant to antibiotics than their planktonic counterparts. An increase of 10 to 1000 fold in resistance is commonly reported (Mah and O'Toole 2001). This makes treating infections caused by biofilms more difficult (Anderson and O'Toole 2008). The susceptibility and effect of antibiotics on biofilms cannot be studied via standard tests, as these generally examine effect of antibiotics on bacteria in the planktonic state (Donlan 2001).

To effectively study the effect of antibiotics on biofilms, I first had to modify the protocols normally used to determine MICs in liquid culture and on agar plates (Chapter 5). This resulted in a novel method, using biofilms grown in plastic tubing, to effectively determine the MIC of antibiotics on cells growing in biofilms. Once this method was

developed, I then used it to perform experimental evolution on bacterial biofilms exposed to environmentally relevant concentrations of antibiotics (Chapter 6). Again, whole genome sequencing was used on cells shed from the biofilm to identify point mutations. Consistent mutations were detected in *treB* and *yebT.* While these genes are not known to be directly involved in antibiotic resistance, they are respectively associated with stress responses, or with adhesion and regulation of membrane lipids. Again, these mutations occurred exclusively, or overwhelmingly, in the antibiotic exposed treatments, confirming the idea that pollution with these agents can drive the fixation of novel mutations under environmental conditions.

Mutations that confer resistance often occur in a stepwise process, where a single point mutation confers a degree of resistance while a second confers a higher degree of resistance and so on. (Ruiz 2003, Su, Khatun et al. 2013). It is useful to be able to track single gene mutations associated with antibiotic resistance, as the spread of the resistant strain of bacteria can then be tracked and monitored. This is important, as it gives us information about the routes and modes of transmission which can then be used as an indicator of what we might expect in future outbreaks. Similarly, other genomic events could help disseminate resistance, such as acquisition of resistance genes onto mobile DNA elements. Various classes of integrons have become important means of dissemination after their acquisition into transposons and plasmids (Gillings 2014).

The experimental evolution protocols we described here might also have use in prediction of novel phenotypes relevant to controlling bacterial growth. The identification of point mutations that accumulate during antimicrobial exposure is now experimentally feasible given the rapidly declining costs of genome sequencing (Gillings, Paulsen et al. 2017). Such experiments might provide examples of genes that could be important to antibiotic resistance

in the future, and give us an idea of what kind of phenotypes might arise in environmental organisms.

The general conclusion from this work is that concentrations of the antibiotics found as pollutants in environmental compartments are sufficiently high to have significant effects on environmental bacteria. These concentrations cause *de novo* mutations that confer increased resistance. While it is not yet clear if antibiotic resistance in environmental microorganisms will have whole ecosystem effects, it does seem likely that this phenomenon will affect microbial ecology and cell-cell interactions. Acquisition of resistance is essentially stochastic, relying on rare events at a single point in time, coincident with relevant selection pressures that then allow newly resistant lineages to compete, and increase in abundance. Widespread pollution with antibiotics enhances the rates at which key mutational events are likely to occur, while simultaneously providing the selection regime to promote survival of newly resistant cells. The potential is clear for environmental organisms to acquire resistance genes, which could then be disseminated globally through lateral gene transfer, or to become significant pathogens in their own right. Antibiotic pollution joins overuse and misuse as a significant threat to human health and the preservation of the efficacy of antibiotics.

The future of antibiotic pollution and antibiotic resistance

The findings of this thesis supports the need for antibiotic pollution to be regulated. There are various routes by which antibiotics enter the environment, and these routes need to be assessed and monitored. While individual countries are beginning to tackle this problem, single, isolated interventions are likely to have little impact. Antibiotic stewardship and pollution control must be a global effort to combat the problem of resistance and to preserve

the efficacy of antibiotics. There are a number of actions that will help to preserve antibiotic efficacy by regulating antibiotic pollution, and modifying our use of antibiotics.

Antibiotic pollution

Antibiotics enter the environment via a number of routes, including human waste streams, agricultural run-off and pharmaceutical effluent. Identifying the relative importance and magnitude of each of these sources could be a first step in ameliorating antibiotic pollution.

Pharmaceutical production plants often release pharmaceutical waste into adjacent water bodies. Antibiotic released from pharmaceutical plants have been detected at concentrations in the mg/L range, sometimes up to 1000 times the treatment concentration (Larsson, de Pedro et al. 2007). This concentration far exceeds the concentrations likely to select for resistance that are documented in this thesis. There needs to be greater transparency in the manufacturing process, and in the mechanisms for disposal of pharmaceutical waste. Both consumers and governments can make informed choices, and put pressure on pharmaceutical manufacturers to treat run-off.

Of all antibiotics manufactured globally, approximately 70-80% are used in agriculture (Rushton and Stärk). This use is mainly in mass animal husbandry (Dibner and Richards 2005), aquaculture (Cabello 2006), fruit spraying (McManus, Stockwell et al. 2002) and manuring of crops with animal waste (Chee-Sanford, Mackie et al. 2009, Heuer, Schmitt et al. 2011). Antibiotics can directly enter the environment through these routes.

The majority of the antibiotics consumed in human medicine are excreted unchanged, making their way into waste water treatment plants. These plants are then polluted with antibiotics, and also with genes that confer resistance. They therefore become a hotspot for the generation and acquisition of antibiotic resistance (Schlüter, Szczepanowski et al. 2007, Gillings and Stokes 2012, Rizzo, Manaia et al. 2013). Multi-drug resistant bacteria are often

found in the sludge and sediment arising from waste water treatment plants (Reinthaler, Posch et al. 2003, Kim and Aga 2007, Rizzo, Manaia et al. 2013). Since the sludge from these plants can be used for land application, resistant bacteria are spread to agricultural soil.

Current practices in waste water treatment plants do not remove or degrade antibiotics. There are some options for treatment of human waste that would degrade the antibiotics so that the sludge can be used. For example, reverse osmosis membranes (Li, Zhang et al. 2018) or photo degradation through turning of sludge (Sturini, Speltini et al. 2012) can result in significant antibiotic removal or degradation.

Currently there are no regulations or environmental limits on antibiotic pollution, in contrast to many other pollutants such as chlorine, oil and grease, heavy metals, sulfates or nitrogen. All of these pollutants can be monitored, have reference standards, and if necessary, treatment protocols (Carraro, Bonetta et al. 2016). Antibiotics need to be recognized as pollutants and to have similar regulatory status. There should be global guidelines for the reference standards and treatment protocols for antibiotic pollution in human waste streams, agricultural run-off and pharmaceutical effluent. Once antibiotics have regulatory status, it would be easier to enforce treatment of waste, and more research into effective removal methods would follow.

Modified use and practice in human medicine

Even with the knowledge of the significant problem of antibiotic resistance, usage of antibiotics in human medicine has increased 65% from 2000 to 2015 (O'Neill 2014). Modification of antibiotic use in human medicine and better stewardship can preserve the efficacy of antibiotics. Improved and faster tests to determine the need and type of antibiotic can reduce the unnecessary prescription of antibiotics, particularly broad-spectrum antibiotics. Improved identification of resistant strains of bacterial would also allow us to

monitor and track resistant strains (Ellington, Kistler et al. 2006, Böckelmann, Dörries et al. 2009, Poirel, Walsh et al. 2011).

Practices that reduce antibiotic consumption, such as vaccines or hygiene systems, particularly in low-income countries, can be highly effective. For example, when clean water and basic sanitation are available, diarrheal diseases decrease, (Nandi, Megiddo et al. 2017). Effective use of vaccines can reduce future antibiotic needs (Lee, Reveles et al. 2014). New antimicrobials will be ineffective in solving the resistance problem in the long term if these novel drugs are used in the same way that existing antibiotics have been used.

Modified use and practice in agriculture

Banning or limiting the usage and type of antibiotics in agriculture needs to be globally enforced. Antibiotics considered critically important (WHO 2017) should be conserved for human treatment and banned from use in agriculture. This the most direct way to control the release of antibiotics into the environment. In Denmark (Aarestrup, Seyfarth et al. 2001) and the USA (Sapkota, Hulet et al. 2011) the use of feed supplemented with antibiotics was banned and led to a reduction of antibiotic resistance seen in faecal bacteria. The implications of this are two fold, animal production can continue to be viable without antibiotic use, and it is possible to reverse the occurrence of antibiotic resistance among farmed animals. However, while there might be a decline in antibiotic resistance following the removal of antibiotics, the environment will still continue to be polluted with low concentrations of antibiotics and, as discussed in this thesis, these concentrations will continue to select for resistance. Furthermore, because of the low fitness cost associated with antibiotic resistance generated this way, we can expect to see resistance determinants to persist in bacteria for significant lengths of time (Andersson and Hughes 2010, Johnsen, Townsend et al. 2011). This again

highlights the need for a unified, long term, global responses, as a single, isolated responses, such as those in Denmark and the USA, are not enough to combat the problem.

When antibiotics enter the environment, they can persist at biologically relevant concentrations for significant periods of time. Exposure to these levels of antibiotics can lead to upregulation of mutation and DNA transfer driven by the SOS response (Mesak, Miao et al. 2008, Jorgensen, Wassermann et al. 2013, Andersson and Hughes 2014, Chow, Waldron et al. 2015). These processes increase the likelihood of bacteria acquiring antibiotic resistance via mutation or lateral gene transfer, and consequently poses a significant threat to human health. We acknowledge that antibiotic usage is ingrained into every step of modern medicine (Classen, Evans et al. 1992, Zaman, Hussain et al. 2017), and that mass food production might not be possible without prophylactic usage of antibiotics (Dibner and Richards 2005, Cabello 2006). However, there needs to be a shift in antibiotic monitoring, usage and control at every level. The true extent of antibiotic use must be known in order to form workable solutions. Current antibiotic usage is unsustainable, and set the conditions for loss of human life, decreased livestock production and significant economic costs.

The fact that antibiotics are losing their effectiveness after decades of misuse cannot be ignored. Common infectious diseases like tuberculosis (Velayati, Masjedi et al. 2009), pneumonia (Ho, Que et al. 1999), sexually transmitted infections (Ohnishi, Golparian et al. 2011, Unemo and Nicholas 2012) and diarrheal infections (Nakaya, Yasuhara et al. 2003) are becoming untreatable due to the rise of drug resistant strains. The spread of antibiotic resistance is a global phenomenon. Although resistance genes may arise in one location they can rapidly spread to all parts of the globe. Addressing the problem of antibiotic resistance requires a rapid, and unified global response to preserve antibiotic effectiveness.

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Appendices

PLOS ONE

RESEARCH ARTICLE

Evolution of class 1 integrons: Mobilization and dispersal via food-borne bacteria

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OPEN ACCESS

Citation: Ghaly TM, Chow L, Asher AJ, Waldron LS, Gillings MR (2017) Evolution of class 1 integrons: Mobilization and dispersal via food-borne bacteria. PLoS ONE 12(6): e0179169. https://doi.org/10.1371/journal.pone.0179169

Editor: Finbarr Hayes, University of Manchester, UNITED KINGDOM

Received: February 12, 2017

Accepted: May 24, 2017

Published: June 6, 2017

Data Availability Statement: DNA sequences described in this study were deposited in GenBank as accession numbers KY126369-KY126372 and NFUM00000000. All other relevant data are within the paper and its Supporting Information files.

Funding: This work was funded by an Australian Research Council Discovery Grant (grant number: DP130103839). The funders had no role in study design, data collection and analysis, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abstract

Class 1 integrons have played a major role in the global dissemination of antibiotic resistance. Reconstructing the history of class 1 integrons might help us control further spread of antibiotic resistance by understanding how human activities influence microbial evolution. Here we describe a class 1 integron that represents an intermediate stage in the evolutionary history of clinical integrons. It was embedded in a series of nested transposons, carried on an IncP plasmid resident in *Enterobacter*, isolated from the surface of baby spinach leaves. Based on the structure of this integron, we present a modified hypothesis for integron assembly, where the ancestral clinical class 1 integron was captured from a betaproteobacterial chromosome to form a Tn*402*-like transposon. This transposon then inserted into a plasmid-borne Tn*21*-like ancestor while in an environmental setting, possibly a bacterium resident in the phyllosphere. We suggest that the *qacE* gene cassette, conferring resistance to biocides, together with the mercury resistance operon carried by Tn*21*, provided a selective advantage when this bacterium made its way into the human commensal flora via food. The integron characterized here was located in Tn*6007*, which along with Tn*6008*, forms part of the larger Tn*6006* transposon, itself inserted into another transposable element to form the Tn*21*-like transposon, Tn*6005*. This element has previously been described from the human microbiota, but with a promoter mutation that upregulates integron cassette expression. This element we describe here is from an environmental bacterium, and supports the hypothesis that the ancestral class 1 integron migrated into anthropogenic settings via foodstuffs. Selection pressures brought about by early antimicrobial agents, including mercury, arsenic and disinfectants, promoted its initial fixation, the acquisition of promoter mutations, and subsequent dissemination into various species and pathogens.

Introduction

Class 1 integrons are genetic elements that play a major role in the global dissemination of antibiotic resistance because they can capture gene cassettes from a vast pool of resistance genes [1], and are resident on diverse mobile elements [2]. All class 1 integrons possess *intI1*, a gene that encodes a site-specific recombinase (IntI1), responsible for the insertion and excision of exogenous gene cassettes at the integron-associated recombination site (*attI1*). The gene

cassettes within the integron array are transcribed from a promoter (Pc) located within the coding sequence of *intI1*. This gene capture system allows generation of genomic complexity and acquisition of adaptive phenotypes [3], including resistance to nearly all known classes of antibiotics [4].

The class 1 integrons that are widely distributed in pathogens from clinical settings (hereafter referred to as 'clinical' class 1 integrons) are part of a more diverse group of class 1 integrons found on the chromosomes of environmental bacteria [5]. Clinical class 1 integrons are found embedded in transposons and conjugative plasmids, allowing their rapid dissemination via lateral gene transfer. As a consequence, class 1 integrons have spread to nearly all species of Gram-negative pathogens [6]. Since the clinical class 1 integron has played such a major role in the global spread of multi-drug resistance, it is important to reconstruct its evolutionary history so we can better mitigate antibiotic resistance, and gain insights into how human activities influence bacterial evolution.

It is likely that a single environmental class 1 integron gave rise to the ancestor of clinical class 1 integrons, since these all share a highly conserved *intI1* sequence [7]. In turn, this implies that a single event resulted in the movement of one variant of the class 1 integron into the human microbiota. The descendants of this initial event have given rise to a pool of genetic elements that have successfully spread into diverse bacterial species, and are now universally present in the commensal bacteria of humans and their domesticated animals [6, 8, 9]. Once this ancestral integron made its way into the human commensal or pathogenic flora, it was exposed to various selection pressures, eventually leading to the acquisition of more than 130 different resistance genes [4, 10].

The current model for the evolution of clinical integrons, however, does not answer how the pre-clinical form made its way into the human microbiota, what bacterial host facilitated this transition, or present a clear order of the complex rearrangements that lead to its clinical form, particularly, its association with the mercury resistance Tn*501*-like transposon [11]. The most likely route for movement of class 1 integrons from natural environments into the human microbiota is via water or food-borne bacteria. In particular, bacteria that occur on foodstuffs that are eaten raw, or lightly cooked, are likely candidates.

To investigate this possibility, we examined various foods for their carriage of integron-bearing bacteria, so that we can better understand the evolutionary history of the clinical class 1 integron. Here, we report: screening of baby spinach leaves from retail outlets for class 1 integrons; characterization of these integrons, their gene cassettes, and the mobile elements they reside upon; and how this information helps to inform our understanding of the events leading to the fixation of clinical class 1 integrons in the human microbiota.

Materials and methods

Isolation of single colonies and DNA extraction

Commercial baby spinach leaves were obtained from retail outlets in Sydney, NSW, Australia. Bacteria were isolated from leaves using a stomacher (BagMixer 400W, Interscience, St. Nom, France). Mixed cultures were screened for *intI1* by PCR, and positive cultures were plated out to obtain single colonies, which were re-screened for *intI1* [12]. Genomic DNA was extracted from *intI1*-positive single colonies using bead-beating [13]. DNA yield was assessed using agarose gel electrophoresis.

PCR amplification and analysis

The class 1 integron integrase gene was targeted using primers HS464 and HS463a [14]. Cassette arrays from pre-clinical class 1 integrons were targeted for amplification using primers

MRG284 and MRG285 [15], which amplify the region between *intI1* and immediately after the most distal *attC* site in the cassette array. Clinical class 1 cassette arrays were targeted with the primers HS458 and HS459 [14], which amplify the region between *intI1* and the 3' conserved segment (3'-CS) [16]. To identify integron-positive isolates to species, PCRs targeted 16S rDNA with primers f27 and r1492 [17], the RNA polymerase beta subunit gene (*rpoB*) using primers RpoB-F and RpoB-R, and the 60 kDa heat shock protein gene (*hsp60*) with primers Hsp60-F and Hsp60-R [18]. PCRs targeting the plasmid partitioning gene, *parA*, were used to detect the integron-bearing plasmid, which was characterized via whole genome sequencing of *Enterobacter cloacae* isolate MN73R (described below). Primers par-1 and par-2 were designed to amplify the complete gene (S1 Table).

All PCRs were performed in 50 μL reactions containing 100 ng DNA, 25 μL GoTaq White (Promega, Madison, WI, USA), 0.5 μL of 1 mg/mL RNase and 0.5 μL of each primer (50μM). PCRs were carried out using an Eppendorf Mastercycler Epigradient S thermocycler with the appropriate thermal cycling program. PCR efficiency was assessed using 1–2% agarose gels.

Restriction digests (*Hinf* I) were performed on the MRG284/285 amplicons. All reactions were performed according to the manufacturer's instructions (Promega) and were left to incubate overnight at 37˚C. Analysis of digests was performed on 2% agarose gels, cast and run in TBE buffer [19], and post-stained with GelRed (Biotium, Fremont CA USA).

DNA sequencing and analysis

PCR products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) as per the manufacturer's instructions. Sanger dideoxy sequencing was carried out using the amplification primers at the Macrogen sequencing facility (Seoul, South Korea). Sequence alignments were made using Geneious v 9.0.4 software. Sequences were interrogated by searching against nucleotide databases using blastn algorithms (http://www.ncbi.nlm.nih.gov/BLAST/).

Whole-genome sequencing was carried out for one *intI1*-positive *E. cloacae* isolate (MN73R). Genomic DNA extracted from MN73R was sequenced on an Illumina HiSeq platform at the Garvan Institute of Medical Research (Sydney, Australia). Initial scaffolding was assembled using the A5 pipeline [20, 21], and ordered in Mauve v. 20150226 software against the *E. cloacae* subsp. *cloacae* ATCC 13047 reference genome (accession number NC_0144121). DNA sequences described in this study were deposited in GenBank as accession numbers KY126369-KY126372 and NFUM00000000.

Results

Class 1 integron screening

Washings from fresh spinach leaves were used to establish mixed cultures that were screened for *intI1* using PCR. Positive cultures were then plated to recover individual colonies. Seventy individual colonies recovered from a single collection sample were PCR positive for *intI1*. The cassette arrays of all 70 *intI1*-positive isolates were successfully amplified using primers MRG284/285 (1285 bp amplicon), but could not be amplified using HS458/459, indicating all isolates carried a pre-clinical form of the class 1 integron, prior to the formation of the 3'-CS [22]. The restriction patterns of *Hinf* I digested MRG284/285 amplicons were identical, suggesting the isolates all carried the same cassette array.

Species identification

On the basis of 16S rDNA restriction digests and DNA sequencing of a subset of the isolates (n = 50), all integron-positive isolates were identified as *E. cloacae*. After further DNA

sequencing of *rpoB* and *hsp60* amplicons, these isolates were subsequently identified as *E. cloacae* subsp. *cloacae*. To further analyse the present *E. cloacae* strain, the two largest assembled contigs (994 626 bp and 835 901 bp, respectively) were used in a BLAST search of *E. cloacae* genotypes. The top BLAST alignment for both contigs matched with an endophytic *E. cloacae* strain isolated from a pepper plant (accession number CP003737) [23].

Integron characterization

DNA sequencing of *intI1* and cassette arrays revealed an integron carrying two gene cassettes, neither of which carried a typical cassette-encoded antibiotic resistance gene (Fig 1). The integron did not contain the 3' conserved segment (*qacEΔ1* / *sul1*) usually found in clinical class 1 integrons, suggesting this was a pre-clinical integron. All isolates had an identical integron-integrase sequence, which displayed 99.96% sequence identity (1 bp difference) with a class 1 integron (accession number EU591509) found in *E. cloacae* isolated from human feces [24]. The first cassette contained a gene designated as *MN039*, whose predicted gene product is an NADPH-dependent flavin mononucleotide (FMN) reductase, an enzyme family responsible for the reduction of quinones. The second cassette contained *qacE2*, whose product is an efflux pump that can confer resistance to biocides such as antiseptics and disinfectants [25].

Sequence comparison between the present integron and that previously characterised by Labbate et al [24], showed a single base pair difference in the promoter region, Pc (Fig 2). Four predominant Pc variants occur in class 1 integrons, based on the sequence of their -35 and -10 hexamer motifs. The integron described here had the PcW (weak) promoter, thought to be the ancestral promoter type [26, 27]. The integron described by Labbate et al [24] contained the PcH1 (hybrid 1) promoter (Fig 2).

Genomic landscape of the integron

To understand the evolutionary history of the integron and its potential for lateral transfer, we used the genome sequence to reconstruct the genetic landscape around the integron. The integron was located within a Tn*402*-like transposon, designated Tn*6007* (Fig 1) [24]. The transposons Tn*6007* and Tn*6008* possess homologous pairs of inverted repeats (IRi/IRt), and together

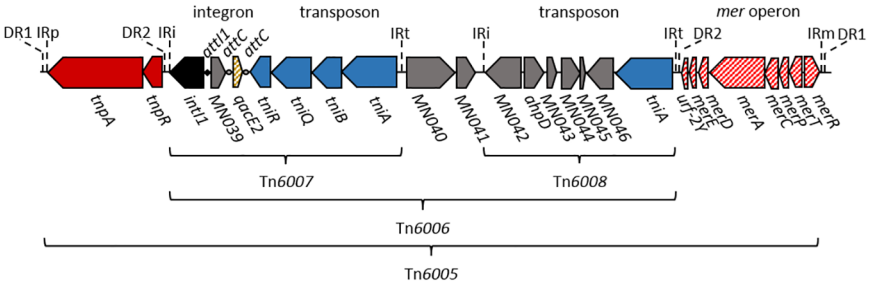

Fig 1. Genomic landscape of the class 1 integron reported in the present study. From left to right, the components are as follows: the direct repeat (DR1) formed by the insertion of Tn*6005*; the inverted repeat for Tn*6005* (IRp); Tn*21*-like transposition genes *tnpA* and *tnpR*; the direct repeat (DR2) formed by the insertion of Tn*6006*; the inverted repeat for Tn*6006*/*6007* (IRi); a class 1 integron with *intI1* and integron-associated recombination site *attI1*, carrying two gene cassettes, *MN039* and *qacE2*, each with a cassette-associated recombination site *attC*; Tn*402*-like transposition genes *tniR*, *tniQ*, *tniB*, *tniA*; the inverted repeat for Tn*6007* (IRt); genes *MN040* and *MN041*; the inverted repeat for Tn*6008* (IRi); the *Tn6008* transposon, carrying genes *MN042*, *ahpD*, *MN043*, *MN044*, *MN045*, *MN046*, and *tniA*; the inverted repeat for Tn*6006*/*6008* (IRt); the direct repeat (DR2) formed by the insertion of Tn*6006*; Tn*21*-like *mer* operon consisting of *urf-2Y*, *merE*, *merD*, *merA*, *merC*, *merP*, *merT* and *merR*; the inverted repeat for Tn*6005* (IRm); and the direct repeat (DR1) formed by the insertion of Tn*6005*. This whole element is embedded in an IncP plasmid whose sequence is lodged as accession number KY126370.

https://doi.org/10.1371/journal.pone.0179169.g001

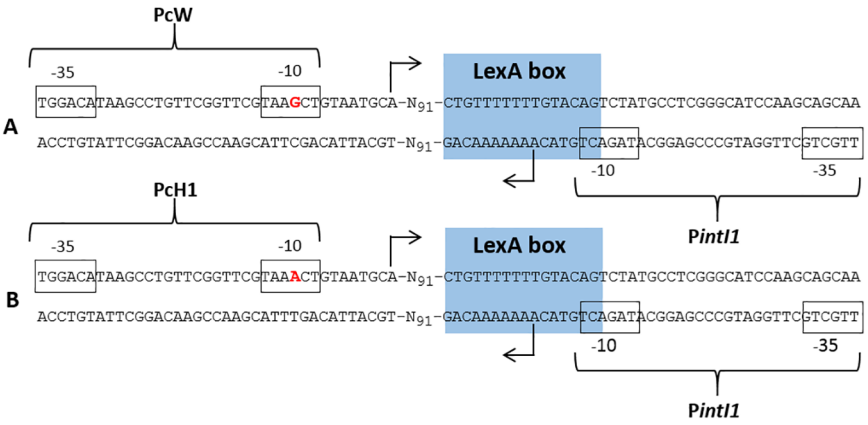

Fig 2. Detailed structure of the class 1 integron promoter regions. The -35 and -10 motifs for the Pc and P*intI1* promoters are boxed; a point mutation in the -10 motif, which distinguishes the PcW and PcH1 promoters, is highlighted in red; the transcription initiation sites are indicated by arrows; the LexA box is shaded in blue (expression of *intI1* is regulated by the SOS response). (A) Promoter region within the class 1 integron described in the present study, containing the PcW promoter variant, which is also present in a number of chromosomal class 1 integrons (accession numbers EU316185 and EU327987-EU327991). This strongly suggests PcW is the ancestral promoter in these integrons; (B) Promoter region within the otherwise identical class 1 integron characterized from the human fecal flora by Labbate et al. (23), which contains the PcH1 promoter.

https://doi.org/10.1371/journal.pone.0179169.g002

form Tn*6006* [24]. The complete Tn*6006* module appears to have transposed as a single unit. This can be inferred by the flanking direct repeats (DR2), which indicate a duplication at the insertion site. Here, Tn*6006* was inserted into the *res* site of a Tn*501*-like transposon, which also carried the *mer* operon. The Tn*501*-like transposon, together with the Tn*6006* insert, formed Tn*6005* (Fig 1) [24]. The Tn*6005* compound transposon was itself located within a 114 969 bp plasmid, designated pOP-I. The plasmid, whose complete sequence is lodged under accession number KY126370, carried a type IV conjugation system. The plasmid also carried the DNA-binding protein gene *kfrA*, which binds to the replication region of IncP-1 plasmids [28]. We therefore designate pOP-I as part of the IncP family of broad-host range plasmids. PCR screening and Sanger sequence analysis of *parA* confirmed the presence of pOP-I in all the *intI1*-positive isolates tested in this study.

Discussion

Class 1 integrons have played a major role in spreading multidrug-resistance in bacteria [29]. However, the clinical class 1 integrons responsible for this dissemination are just one sequence type in a larger family of class 1 integrons that are broadly distributed in environmental bacteria, and which exhibit considerable sequence diversity [5, 11]. Almost without exception, the DNA sequence of the integrase gene (*intI1*) of all clinical class 1 integrons is identical [7]. Given the extensive sequence diversity in this family of elements, the sequence identity amongst clinical class 1 integrons is strong evidence that they have a single, recent common ancestor. Understanding the origins of clinical class 1 integrons and the reasons for their spectacular success will help us understand the dynamics of antibiotic resistance, and the influence that humans have had on the bacterial resistome [30].

Here, we identified a class 1 integron that is a strong candidate for the type of element that might be the common ancestor of the clinical class 1 integrons. The integron was located on the conjugative IncP family plasmid, pOP-I, resident in *E. cloacae* subsp. *cloacae* isolated from the phyllosphere of baby spinach leaves. Various characteristics of this class 1 integron and its

genetic landscape agree with the properties expected of the immediate precursor to the clinical class 1 integron, as outlined below.

The integron described here was inserted into a Tn*402*-like transposon, and was linked to another transposon, Tn*6008*, to form the composite transposon Tn*6006* (Fig 1) [24]. The complete Tn*6006* module has then inserted at the *res* site of a Tn*501*-like transposon (Tn*21* ancestor), carrying a *mer* operon (Fig 1) to generate transposon Tn*6005*, previously described by Labbate et al. [24]. Here, this entire construct was recovered from an environmental bacterium, and consequently appears to predate the infiltration of this kind of element into clinical environments.

All integrons of clinical importance (classes 1, 2 and 3) have inserted into transposable elements, thus markedly increasing their potential for mobility [10, 31]. The immediate ancestor of the clinical class 1 integron is a good example, and is thought to have arisen when a chromosomal integron from an environmental betaproteobacterium inserted into a transposon. This conclusion is based on the discovery of chromosomal integron-integrase genes (*intI1*) with sequence identity to those now seen in clinical integrons [11]. This ancestor was initially mobilized into a *res* hunting transposon of the Tn*402* type [32], and consequently we would expect this first molecular event to result in a clinical *intI1* sequence attached to a complete set of Tn*402* transposition genes, as seen here. Further, chromosomal integrons carry gene cassettes unrelated to the antibiotic resistance cassettes found in contemporary clinical integrons, and this should also be the case in the class 1 ancestor. The integron we describe here was linked to complete Tn*402* transposition machinery, lacked the 3'-CS indicative of clinical forms [22], and carried two non-antibiotic resistance cassettes (Fig 1), as predicted for the model clinical ancestor.

The first of these cassettes (*MN039*), encoded a predicted NADPH-dependent FMN reductase. Enzymes in this family can provide resistance to toxic substances, such as arsenic [33] and other oxidative stressors [34]. The second cassette carried a gene encoding an efflux pump, QacE2. The *qacE2* gene product is an efflux pump for cationic compounds, thus also providing some resistance to disinfectants and antiseptics [25]. Cassettes encoding Qac efflux pumps are common in environmental class 1 integrons, and have been predicted to be part of the earliest forms of clinical class 1 integrons [15], in keeping with expectations for a clinical ancestor.

These observations present an insight into the evolutionary history of clinical class 1 integrons. Transposon Tn*21* and its various derivatives have had a central involvement in the global dissemination of antibiotic resistance determinants. It was previously thought that the ancestral class 1 integron inserted into the ancestral *mer* transposon after partial deletion events of the *qacE* cassette and *tni* module, using transposition machinery supplied in *trans* [35]. Here we suggest an alternate order of assembly, whereby the pre-clinical integron within the *cis*-acting Tn*402* inserted into the Tn*21* ancestor prior to any deletion events. This would also suggest that the formation of Tn*21* occurred in an environmental setting. Under this interpretation, the integron we describe here represents an intermediate stage in the complex rearrangements that preceded the dissemination of clinical class 1 integrons into the human microbiota.

The occurrence of the present integron in an *E. cloacae* strain isolated from baby spinach leaves, provides a plausible route for transmission of environmental integrons into the human microbiota. *E. cloacae* is often found associated with plants, and can be readily isolated from spinach leaves [36], also being found as an endophyte in spinach xylem vessels [37]. The consumption of uncooked spinach leaves therefore provides a plausible mechanism for transfer of *E. cloacae* carrying the pre-clinical class 1 integron into the human gut. Bagged, ready to eat spinach has been shown to be a vehicle for transmission of Gram negative bacteria into the

human microbiota [38], and *E. cloacae* itself is an inhabitant of the digestive tract, where it is a commensal organism [39].

This integron is known to have made the transition into the human gut because the same element has previously been described from human fecal flora [24]. Although it is possible that this is a result of human contamination of agricultural plants, we suggest two reasons why this is unlikely. First, *rpoB* sequence analysis shows the present *E. cloacae* isolate to be representative of environmental strains, isolated from endophytic and soil environments (e.g. accession numbers CP003737, JN627207, LC049166). Furthermore, the two largest assembled contigs (994 626 bp and 835 901 bp, respectively) of the sequenced strain, both exhibited a top BLAST alignment (99% identity) with an enodphytic *E.cloacae* strain isolated from a pepper plant (accession number CP003737) [23]. These sequence alignments suggest that the present isolate is an environmental strain associated with plants, and is unlikely to be a human contaminant.

The second, a single nucleotide variation in the cassette promoter region (Pc), distinguishes the two integrons (Fig 2). There are four main Pc variants defined on the basis of their -35 and -10 hexamer motifs, each with different transcriptional strengths: PcW; PcH1; PcH2; and PcS [26]. There is a 30-fold increase in strength from the weakest promoter, PcW, to the strongest promoter, PcS [40]. The integron in the present study had the PcW variant, which is thought to be the ancestral form, while that characterized by Labbate et al [24] contained PcH1. This change in promoter sequence does not affect the IntI1 amino acid sequence (due to a silent base substitution; Fig 2), and thus does not change IntI1 excision activity [41]. However, PcH1 is associated with a 4.5-fold higher level of cassette expression [40]. Thus, conversion of PcW to PcH1 enhances gene cassette transcription without restricting the capacity for cassette reorganization. Such a mutation is believed to have occurred early in the history of clinical integrons [40]. In support of this, the PcH1 variant is commonly found among clinical forms of class 1 integrons (e.g. accession numbers KX169264, KP099552, LC169585, LC169566, and CP014662), suggesting that this point mutation occurred in anthropogenic settings. Therefore, we propose that the present integron, containing the PcW variant, was the precursor to the integron containing the PcH1 variant, and that this promoter variant was subsequently selected to provide enhanced cassette expression in the environment of the human gut. Hence, the present integron, as well as its bacterial host, appear to have environmental origins, and their occurrence on spinach leaves is unlikely to be a consequence of human contamination.

While we will never know the precise series of events that generated the clinical class 1 integron, we can now reconstruct a plausible scenario based on the properties of the integron we describe here. One variant of the diverse betaproteobacterial chromosomal class 1 integrons (Fig 3A), carrying a *qac* gene cassette, was captured by a Tn*5090*-like transposon, to form Tn*402* (Fig 3B). This hybrid element targeted the *res* site of a plasmid bearing the mercury-resistance Tn*501*-like transposon, to generate the compound transposon Tn*21* (Fig 3C) [35]. The location of this complex mosaic element on a broad host range IncP plasmid would then have allowed its conjugative transfer into a diversity of bacterial hosts.

The subsequent history of the clinical class 1 integron involves molecular diversification and dissemination into diverse hosts. Introduction of the first true antibiotics in the 1930s, the sulfonamides [42], would have selected for acquisition of the resistance gene *sul1*, at the same time deleting part of the *qacE* gene cassette to generate the 3' conserved segment [22], which is now a feature of many clinical class 1 integrons (Fig 3D). Various deletions also occurred in the *tni* transposition module (Fig 3E). These events, plus the collective acquisition of over 130 antibiotic resistance gene cassettes [4] have generated the diverse structures of contemporary clinical class 1 integrons (Fig 3E).

In summary, we have demonstrated the environmental occurrence of a class 1 integron that resides within a complex set of transposons and is capable of being mobilized by a broad host

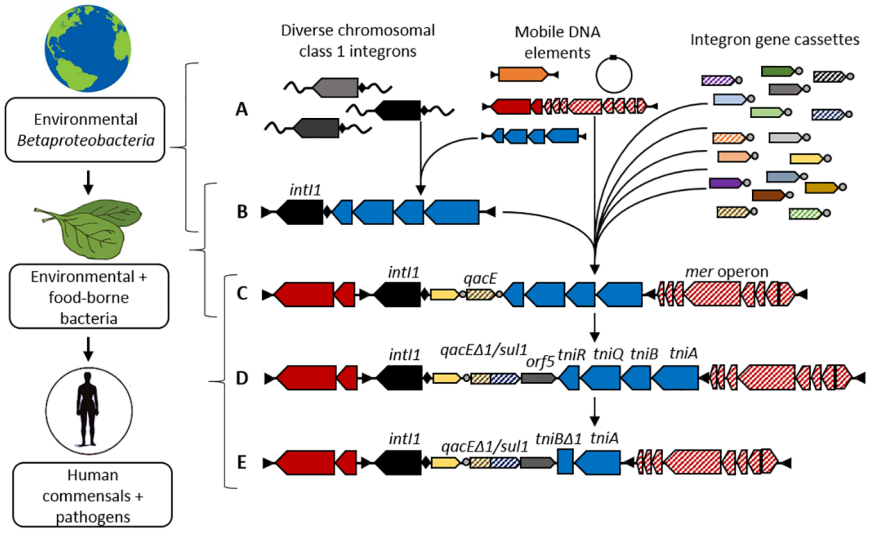

Fig 3. A model for the origin of clinical class 1 integrons. (A) Diverse chromosomal class 1 integrons that are present in environmental *Betaproteobacteria* can interact with mobile DNA elements such as transposons and plasmids in the environment. Integrons have access to a vast pool of integron gene cassettes including the *qacE* cassette that encodes a membrane efflux pump; (B) A single chromosomal integron is captured by a Tn*5090*-like transposon, to generate Tn*402*; (C) Now mobilized, the integron is free to move between a range of bacterial species. In particular, the Tn*402* transposon inserts into the mercury resistance Tn*501*-like transposon, to generate Tn*21*. Residence of this complex DNA element on a broad host range plasmid allows the integron to make its way into the human commensal flora via food-borne bacteria; (D) Once resident within the human microbiota, the integron is fixed by selection, driven by mercury and disinfectants, and after introduction of sulfonamide antibiotics, captures the *sul1* and *orf5* gene cassettes to delete part of the original *qacE* cassette; (E) Partial deletions of the *tni* module, and the collective acquisition of diverse resistance cassettes, lead to the diversity of clinical class 1 integrons that have since disseminated around the globe.

https://doi.org/10.1371/journal.pone.0179169.g003

range IncP plasmid. This DNA element was found in a bacterial species that can colonize both plants and the human gut, and it was recovered from a plant food that can be consumed raw, thus providing a highly plausible route into the human microbiota. Once resident in the microbiota, the possession of genes known to confer resistance to arsenic, mercury and disinfectants supply both the integron and its bacterial host with a means of preferential survival, since all these agents of selection were in use well before the antibiotic era.

From a single molecular rearrangement that happened in a single cell perhaps as recently as 100 years ago, this one sequence variant of the class 1 integron has vastly increased in abundance and distribution. Its spectacular success has been driven by population expansion of humans and their domestic animals, overlaid by the intense selection driven by antimicrobial agents.

Supporting information

S1 Table. PCR primers and annealing temperatures used in this study.
(DOCX)

Acknowledgments

We thank Brittany Gilchrist, Kiana Elliot, Larissa Tromph and Gustavo Tavares for laboratory assistance. We also thank Mary Nolan and Sasha Tetu for comments on earlier drafts of the manuscript. The authors declare no conflict of interest.

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 05 August 2015
doi: 10.3389/fmicb.2015.00803

Potential impacts of aquatic pollutants: sub-clinical antibiotic concentrations induce genome changes and promote antibiotic resistance

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Antibiotics are disseminated into aquatic environments via human waste streams and agricultural run-off. Here they can persist at low, but biologically relevant, concentrations. Antibiotic pollution establishes a selection gradient for resistance and may also raise the frequency of events that generate resistance: point mutations; recombination; and lateral gene transfer. This study examined the response of bacteria to sub-inhibitory levels of antibiotics. *Pseudomonas aeruginosa* and *Pseudomonas protegens* were exposed kanamycin, tetracycline or ciprofloxacin at 1/10 the minimal inhibitory concentration (MIC) in a serial streaking experiment over 40 passages. Significant changes in rep-PCR fingerprints were noted in both species when exposed to sub-inhibitory antibiotic concentrations. These changes were observed in as few as five passages, despite the fact that the protocols used sample less than 0.3% of the genome, in turn suggesting much more widespread alterations to sequence and genome architecture. Experimental lines also displayed variant colony morphologies. The final MICs were significantly higher in some experimental lineages of *P. protegens,* suggesting that 1/10 the MIC induces *de-novo* mutation events that generate resistance phenotypes. The implications of these results are clear: exposure of the environmental microbiome to antibiotic pollution will induce similar changes, including generating newly resistant species that may be of significant concern for human health.

Keywords: antibiotic resistance, microbiome, antibiotic pollution, SOS response, evolution

OPEN ACCESS

Edited by:
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Reviewed by:
Julian Davies, University of British Columbia, Canada
Jose L. Martinez, Centro Nacional de Biotecnología, Spain

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Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *27 May 2015*
Accepted: *22 July 2015*
Published: *05 August 2015*

Citation:
Chow L, Waldron L and Gillings MR (2015) Potential impacts of aquatic pollutants: sub-clinical antibiotic concentrations induce genome changes and promote antibiotic resistance. Front. Microbiol. 6:803. doi: 10.3389/fmicb.2015.00803

Introduction

Antibiotic resistance has been identified as one of the greatest threats to human health for the twenty-first century by the World Health Organisation (WHO, 2014). Overuse and misuse of antibiotics in the medical and agricultural sectors have contributed to the problem, and it is estimated that 70% of pathogens now exhibit resistance to at least one or more antibiotics (Berdy, 2012). In most cases the risk of death is doubled if the individual is infected with a resistant strain of bacteria. In the United States in 2013, there were 23,000 confirmed deaths due to Antibiotic resistance (US CDC) and Europe reports 25,000 deaths per year (2007, ECDC).

The primary human use of antibiotics is medicinal, where they are used to treat a range of bacterial infections. However, misuse and overuse of antibiotics are contributing to the

development of antibacterial resistance. Incorrect prescription of antibiotics, unnecessarily high dosages and over-use all promote resistance (Campoccia et al., 2010; Andersson and Hughes, 2012; Hvistendahl, 2012; Witte, 2013). Antibiotics are also extensively used in agriculture and aquaculture to prevent disease and as a growth promoter (Hilbert and Smulders, 2004; Bednorz et al., 2013). It has been estimated that 50–70% of antibiotics produced in the United States of America are used in agriculture (Lipsitch et al., 2002; Berge et al., 2005).

A relatively small amount of the antibiotics consumed by humans and animals are actually absorbed, with some 30–90% of antibiotics excreted unchanged and released into waste treatment facilities or directly into the environment (Sarmah et al., 2006). Antibiotics, along with heavy metals, disinfectants and genes conferring resistance are disseminated into the environment via human waste streams, agricultural run-off (Su et al., 2014) and effluent from antibiotic production factories (Li et al., 2009, 2010). Current waste treatment methods are often unable to remove these substances, and the water is either reclaimed (Wang et al., 2014) or released into the environment via rivers (Pruden et al., 2006; Storteboom et al., 2010), estuaries or the ocean (Lapara et al., 2011; Wang et al., 2014). The release of these substances into the environment should be thought of as a significant component of soil and water pollution.

Waste water treatment facilities and aquatic environments can become hotspots for the generation and acquisition of resistance. The presence of selective agents such as antibiotics, heavy metals and disinfectants, combined with genes conferring resistance, mobile elements such as transposons, plasmids, and integrons, and diverse microorganisms creates an ideal environment to generate resistance through mutation or lateral gene transfer.

Many studies have investigated the effect of clinical, or inhibitory levels, of antibiotics on the generation of antibiotic resistance. However, there is increasing evidence that sub-inhibitory levels of antibiotics may have significant effects on bacterial populations. A gradient of antibiotic concentration forms around human activities. Within the human microbiome there may be a gradient along the digestive tract, while dissemination of antibiotics via waste water will generate a gradient of antibiotic concentration spreading outwards from human population centers.

Sub-inhibitory levels of antibiotics are known to trigger the SOS response, a broad response to DNA damage that has been documented in many bacterial species. It may play a significant role in the generation of antibiotic resistance, as it can increase the rates of mutation and lateral gene transfer (Baharoglu and Mazel, 2014). It is triggered by the occurrence of single stranded DNA resulting from DNA damage, or inhibition of the processes involved in DNA replication. The SOS response is mediated by the LexA repressor. Under normal conditions, LexA prevents SOS genes from being expressed. Under stressful conditions, the protein RecA is recruited onto single stranded DNA where it stimulates cleavage of the LexA repressor, inactivating it and therefore allowing the expression of approximately 40 SOS genes. SOS genes are often involved in DNA repair (Laureti et al., 2013; Baharoglu and Mazel, 2014).

It is well documented that lethal concentrations of antibiotics can induce the SOS response in bacteria (Miller et al., 2004; Michel, 2005). It has also been suggested that sub-inhibitory levels of antibiotics, as those discussed above, may be more relevant to the problem of antibiotic resistance than lethal concentrations of antibiotics (Andersson and Hughes, 2012; Hughes and Andersson, 2012; Laureti et al., 2013). Lethal concentrations exert a strong selective pressure on bacteria, whereby they either die or they acquire mutations allowing them to survive. When exposed to sub-inhibitory levels of antibiotics, most bacteria survive with little effect on growth, and the SOS response is initiated. This, in turn, increases general rates of mutation and lateral gene transfer amongst all bacteria in a population, adding to any extant diversity upon which natural selection can operate. It is also thought that humans may be inadvertently selecting for lineages of bacteria with a greater ability to evolve through increased basal rates of mutation and lateral gene transfer (Gillings and Stokes, 2012).

Sub-inhibitory concentrations of antibiotics polluting areas surrounding human activity may be affecting: (i) the rates at which bacteria can generate variation; and (ii) the rates at which advantageous mutations fix in natural environments. However, there has been little or no empirical testing of these ideas.

In this study, two species of *Pseudomonas* were passaged as single colony transfers on media containing 1/10 their respective minimum inhibitory concentrations for three different classes of antibiotics. This experiment was designed to test the genotypic and phenotypic effects of realistic levels of antibiotic pollution.

Materials and Methods

Bacterial Isolates

Isolates of two species were selected for this study: *Pseudomonas aeruginosa* strain PA14; and *P. protegens* strain PF-5. These species were chosen as they encompass both clinical and environmental representatives of the genus. Both strains have been genome sequenced (GenBank: AY273869.1 GenBank: CP000076.1, He et al., 2004; Paulsen et al., 2005). *P. aeruginosa* PA14 is an opportunistic bacterium that causes infections in hospitals and cystic fibrosis patients. *P. protegens* PF-5 (formerly *Pseudomonas fluorescens* PF-5) is a common soil bacterium studied for its potential biocontrol properties (Loper et al., 2012).

P. aeruginosa PA14 was obtained from Professor Joyce Loper, Oregon State University and *P. protegens* PF-5 was obtained from Professor Ian Paulsen, Macquarie University. Bacteria were maintained on LB Agar plates (0.01% tryptone, 0.005% yeast extract, 0.005% sodium chloride, 0.015% Agar) at 25°C. A second isolate of *P. protegens* PF-5 was obtained that had been routinely maintained of 100 μg/ml ampicillin, which is a common laboratory practice. This isolate was examined to determine whether maintenance on ampicillin affects the resistance of *P. protegens* PF-5, and will be referred to as *P. protegens* PF-5A. Single colonies were re-suspended in equal parts 30% glycerol and M9 salts and held at -80°C for long term storage.

Antibiotic Treatments

Three antibiotics were selected for this study, each with different modes of action: kanamycin; tetracycline; and ciprofloxacin. Kanamycin is an aminoglycoside antibiotic which binds to the 30S ribosomal subunit and inhibits prevents protein synthesis (Misumi and Tanaka, 1980). Tetracycline is a polyketide antibiotic that is similar to kanamycin in that it binds to the 30S ribosomal subunit, however it prevents aminoacyl-tRNAs attaching to the ribosome, which in turn prevents addition of amino acids to growing polypeptide chains (Chopra and Roberts, 2001). Ciprofloxacin is a second generation fluoroquinolone used to treat a broad spectrum of infections. It inhibits DNA gyrase, which in turn prevents DNA replication (Lebel, 1988).

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) was determined for each isolate against the three antibiotics following established methodology (Wiegand et al., 2008). MICs were determined in microtitre trays containing a serial dilution of the relevant antibiotic in Luria-Bertani medium (0.01% tryptone, 0.005% yeast extract, 0.005% sodium chloride). Wells were inoculated with bacteria prepared from an overnight culture and diluted to an optical density of 0.01. The concentration of antibiotic in test wells ranged from 32 to 0.0156 mg/L for ciprofloxacin and 512–0.0156 mg/L for tetracycline and kanamycin. A growth control containing only the suspension of bacteria and a sterility control containing only medium were included on each plate. Plates were incubated at 25°C for 24 h and then the optical density was read on a Pherastar FS spectrometer at 540 nm. Relative optical density was plotted against antibiotic concentration to determine the MICs, which were defined as no visible growth in the wells.

To determine statistical significance of differences in MIC, a One-Way analysis of variance (ANOVA) was performed. Growth data were expressed as the ratio of growth in the presence of antibiotics against growth in the control. This standardized the data prior to the ANOVA.

DNA Extraction

DNA was extracted from bacterial cultures using a bead-beating method (Yeates and Gillings, 1998; Gillings, 2014). Briefly, a single, well isolated colony from an overnight culture was resuspended in a lysing matrix tube with sodium phosphate buffer and MT buffer (MP Biomedicals) or with CLS-TC buffer (MP Biomedicals). Preliminary testing indicated no significant difference between sodium phosphate/MT buffer and CLS-TC buffer, therefore CLS-TC buffer was used for the remainder of the study as it was more economical. Cells were physically lysed by treatment in a FastPrep FP120 (BIO 101 Savant) machine for 30 s at 5.5 m/s before being centrifuged in an Eppendorf 5417C, for 5 min at 14,000 g. Protein precipitation, binding, washing and subsequent elution of DNA in TE buffer were as previously described (Yeates and Gillings, 1998; Gillings, 2014). Purified DNA was stored at −20°C.

Repetitive Element PCR

DNA fingerprints were generated using ERIC-PCR, REP-PCR or BOX-PCR (Versalovic et al., 1991; Martin et al., 1992) with the modifications previously outlined (Gillings and Holley, 1997). One μL of DNA was mixed with 9 μL of Genereleaser™ (Bioventures Inc.) in a 0.5 mL PCR strip tube, and heated on high for 7 min in a 650 W microwave oven with a microwave sink. Tubes were then held at 80°C for 5 min in an Eppendorf Master Cycle Epigradient S PCR machine, before 40 μL of PCR master mix was mixed into each tube. The PCR master mix per reaction was as follows: 11 μL PCR water, 25 μL GoTaq® white (Promega), 2.5 μL 25 mM $MgCl_2$, 0.5 μL 1 mg/ml RNAse, 1 μL 50 μM of the relevant rep-PCR primer. Negative controls containing Genereleaser™ only and water only were included in each PCR. The appropriate PCR cycle was then performed (**Table 1**). BOX, ERIC, and REP primers were synthesized by Sigma-Aldrich Inc.

Agarose Electrophoresis

PCR products were separated on 2% agarose gels poured in Tris-Borate-EDTA (TBE) buffer (Russell and Sambrook, 2001). DNA samples were loaded with one quarter volume of bromophenol blue loading dye (0.45 M Tris-borate, 0.01 EDTA, 40% sucrose, 0.25% bromophenol blue). A 100 base pair ladder (Crown Scientific) was included on each gel. Gels were run in TBE at 110 v for 50–80 min. Gels were stained with GelRed™ (Biotium) and DNA visualized under UV light. Gel images were captured using a Gel logic 2200 PRO camera and Carestream MI computer software.

Serial Plating Experiments

A single, well isolated colony of each species was chosen to (as far as possible) eliminate any extant variation amongst cells. This single colony was then used to inoculate the control LB agar plates; LB plates containing 1/10 the MIC for kanamycin; LB plates containing 1/10 the MIC for tetracycline; and LB plates containing 1/10 the MIC for ciprofloxacin, each in triplicate

TABLE 1 | Thermal cycling programs and primers used to generate DNA fingerprints using Rep-PCR.

Rep PCR	Primers	Thermal cycle
BOX	BOXA1R: 5′CTACGGCAAGGCGACGCTGACG	94°C 3 min 94°C 30 s × 35 52°C 30 s × 35 68°C 8 min × 35 68°C 15 min × 35 4°C hold
ERIC	ERIC1R: 5′ATGTAAGCTCCTGGGGATTCAC ERIC 2: 5′AAGTAAGTGACTGGGGTGAGCG	94°C 3 min 94°C 30 s × 35 52°C 30 s × 35 68°C 8 min × 35 68°C 15 min × 35 4°C hold
REP	REPR: 5′TTCGCYGGCAAGCCRGCTCC REP F: 5′GGCTTGCCRGCGAARRGGCC	94°C 3 min 94°C 30 s × 35 65°C 30 s × 35 72°C 8 min × 35 72°C 15 min × 35 4°C hold

TABLE 2 | Concentrations of antibiotics used in serial plating experiments, corresponding to 1/10 the experimentally determined MIC.

Antibiotics	Bacterial isolates		
	PA14 (mg/L)	PF5 (mg/L)	PF5A (mg/L)
Kanamycin	25.6	0.8	0.8
Tetracycline	25.6	25.6	25.6
Ciprofloxacin	0.0125	0.025	0.025

(**Table 2**). Plates were incubated at 25°C for 48 h, referred to here, for convenience, as one generation.

After incubation for 48 h, a single well-separated colony from each plate was used to continue the serial plating. After five generations, three single colonies were randomly selected from each plate for DNA extraction and PCR analysis, the first of these three would also be used to continue the serial plating. Repetitive Element PCRs were carried out to monitor changes in DNA patterns and to monitor for possible contamination of the cultures.

DNA Banding Analysis

Images captured of the gels were analyzed to identify changes in the banding patterns, indicative of changes in the genome of the sample. Changes were scored against a control profile to calculate the similarity coefficient (F) using the formula devised by Nei and Li (1979):

$$F = 2N_{xy} / (N_x + N_y)$$

Where Nx and Ny are the number of bands in lane x and lane y respectively and Nxy is the number of bands that lane x and lane y share. Samples with an *F*-value of 1 are identical while a value of 0 indicates no similarity. Scoring of the bands was carried out blind by an individual not involved in the Rep-PCR process, to remove the possibility of bias. The *F*-values for the various antibiotic treatments were plotted as a scatter graph to illustrate the spectrum of variation.

Changes in Colony Morphology

To examine colony morphology at the end of the experiment, colonies of all lines from generation 40 were streaked onto LB agar plates and incubated for 48 h at 25°C. Images of single colonies were captured using a Motic BA300 compound microscope with a 4x lens, mounted with a Moticam 2 2.0MP camera and were analyzed using DigiLabII-C and Motic Images Plus 2.0 computer programs.

Results

Colony Morphology Changes

Images captured of colonies at generation 40 show significant morphological changes between treatment groups. The three control lines of *P. aeruginosa* PA14 displayed no significant changes, kanamycin line 2, tetracycline lines 2 and 3, and ciprofloxacin line 3 exhibited significant changes to their colony morphology (**Figure 1**). The three control lines of *P. protegens* PF-5 displayed no significant changes, kanamycin line 3 and tetracycline line 3 exhibited significant morphological differences. The three ciprofloxacin lines were relatively unchanged (Figure S1). The three control lines and three tetracycline lines of *P. protegens* PF-5A had similar colonies. All three kanamycin lines had significantly changed colonies, as had lines 2 and 3 of the ciprofloxacin treatment (Figure S2).

Detectable Genome Changes

BOX, ERIC, and REP-PCRs were carried out to detect genome changes. The basis of these PCRs is explained in Gillings and Holley (1997), but, in brief, relies on amplification of regions between two random, but reproducible priming sites. Consequently, amplicons are sensitive to both mutations in the priming sites and indels across the amplified regions. After testing both species with ERIC, REP and BOX primers, BOX-PCR was determined as the best method to examine changes. BOX-PCRs were conducted on triplicates of all lines every five generations. Experimental lines often exhibited changes in banding patterns, while the control lines remained the same, indicating that the changes were due to exposure to 1/10 MIC antibiotics (**Figure 2**). Changes were apparent after as few as five passages (evidence not presented), and increased in frequency as the experiment progressed, until they were present in the majority of experimental lines after 40 passages (**Figure 2**, Figures S3–S6).

Two features were notable in the lines exposed to sub-inhibitory antibiotic concentrations. In general, polymorphisms were commonly exhibited in experimental lineages, and often, replicates from single lineages exhibited diversity, demonstrating an ongoing instability within each generation. Further, similar changes to banding patterns were often observed in independent lineages, suggesting that similar events (such as transpositions or prophage activation) were being promoted within independent lines by the antibiotic treatment (**Figure 2**).

To determine the degree of polymorphism amongst the individual experiments, F statistics were calculated. A scatter plot of the F-statistics shows that control lines maintained a uniform BOX-PCR pattern across all three bacterial isolates (PA14, PF-5, and PF-5A) for the 40 generations of the experiment (**Figure 3**). Amongst the lineages treated with sub-inhibitory antibiotic concentrations, only the kanamycin treatment of PA14 maintained a stable BOX-PCR pattern. All other treatments generated polymorphic banding patterns in at least some of the replicates. The approximate degree to which polymorphisms were generated was in the order of Kan < Tet < Cipro (**Figure 3**).

Changes in the MIC

The MIC of each line was determined at passage 40 in order to detect any significant differences in MICs from the control line and from the starting MIC. There were no significant differences in the MIC of *P. aeruginosa* PA14 for any of the treatment lines. In contrast, there were some significant differences in the MIC of *P. protegens* PF-5 and *P. protegens* PF-5A. A representative sample of MIC graphs are displayed in **Figure 4**. **Figure 4A** shows

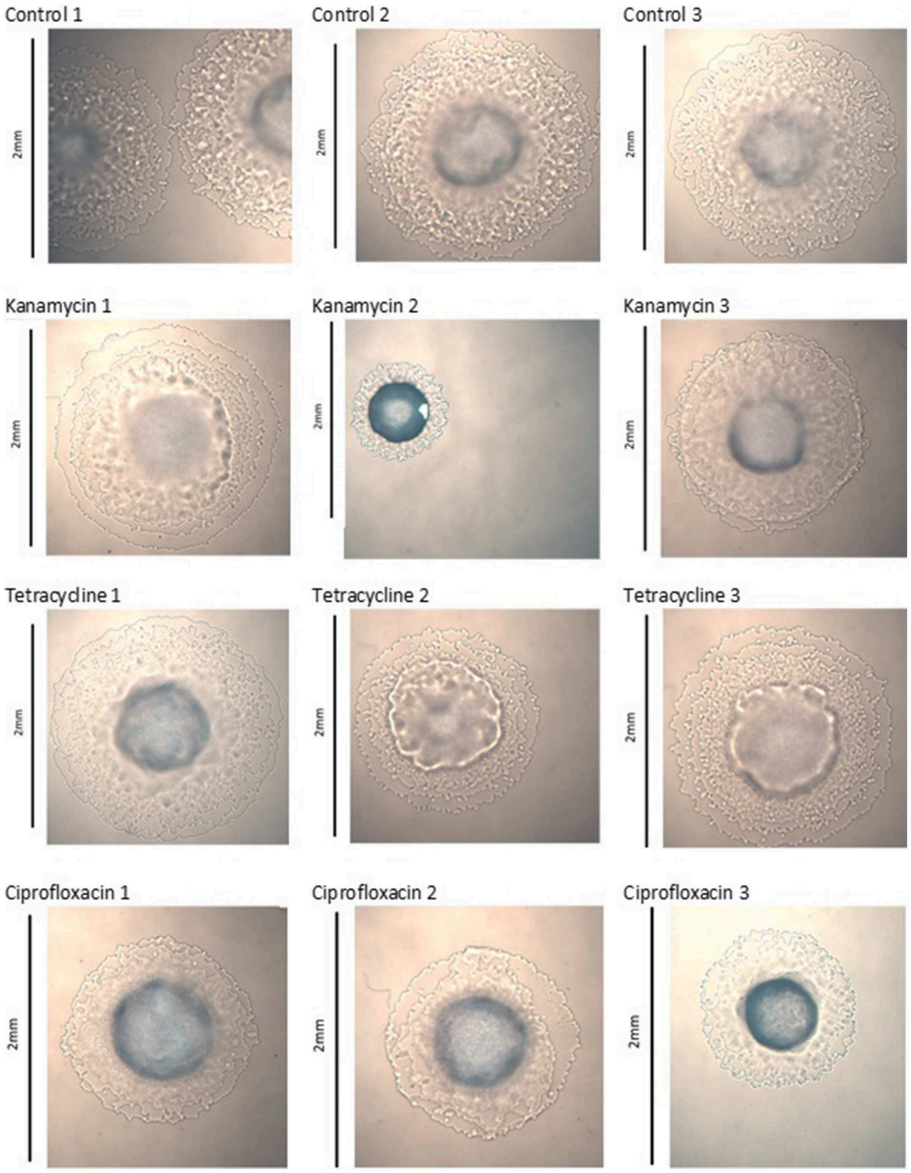

FIGURE 1 | ***Pseudomonas aeruginosa*** **PA14 colony morphology at generation 40.**

the MIC for ciprofloxacin for all control and experimental lines of *P. protegens* PF5. One line of *P. protegens* PF5 that had been exposed to 1/10 the MIC of ciprofloxacin over the serial plating experiment exhibited a 10-fold increase in MIC for ciprofloxacin ($DF = 11$, $F = 11.94$, $P < 0.0001$). A similar phenomenon was seen in *P. protegens* PF5 (**Figure 4B**) and *P. protegens* PF5A (**Figure 4C**) when tested on kanamycin. All six lines that had been exposed to kanamycin over the serial plating experiment had four to eight fold increases in their MIC for kanamycin ($DF = 11$, $F = 1.96$, $P > 0.05$ and $DF = 11$, $F = 46.04$, $P < 0.0001$ respectively). Similar tests conducted on a subset of the kanamycin treated lines at passage 20 did not detect any elevation in MIC.

Discussion and Conclusions

The role of antibiotics as environmental pollutants is attracting more attention, as more concern is being raised about their effects at sub-inhibitory concentrations (Gillings and Stokes, 2012; Andersson and Hughes, 2014). Specific issues include their potential effects on environmental microorganisms, and their potential for triggering complex interactions with the environmental resistome, thereby generating new opportunistic pathogens of relevance to human health (Gillings, 2013). Here we set out to test whether sub-inhibitory concentrations of antibiotics affect the genotype and phenotype of representative clinical and environmental pseudomonads.

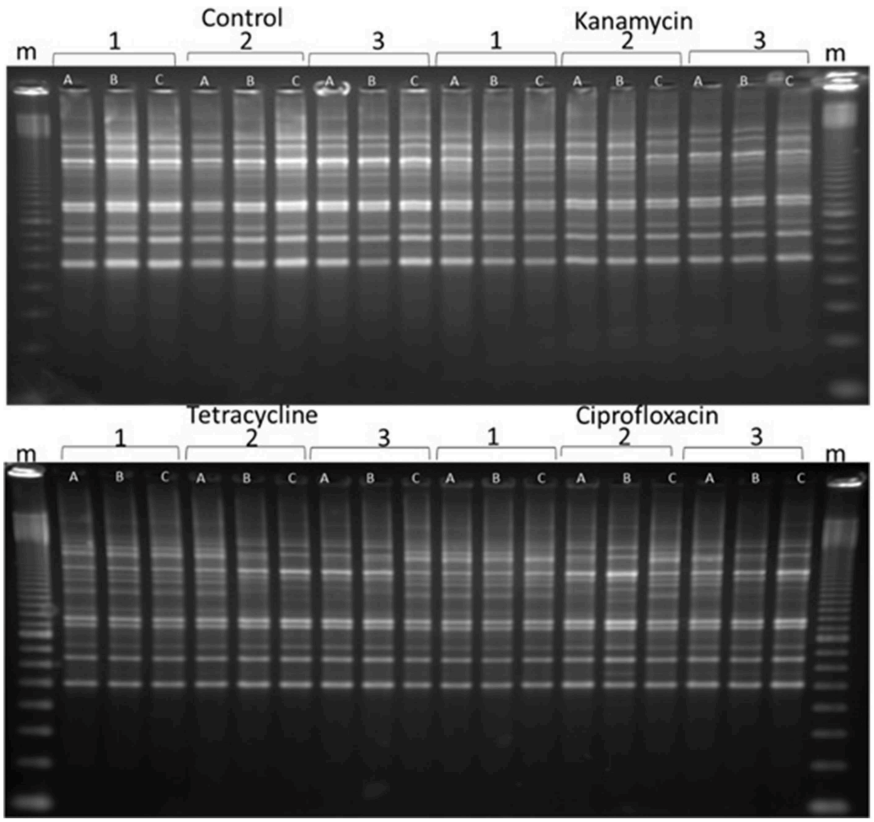

FIGURE 2 | A representative sample of BOX-PCR products. BOX-PCR was performed on generation 40 *Pseudomonas protegens*. Lanes are labeled as follows: $m = 100$ bp ladder. Antibiotic treatments are noted as independent lines within each treatment (1, 2, or 3). Three colonies were tested from each line. For further examples see Supplementary Material (Figures S3–S6).

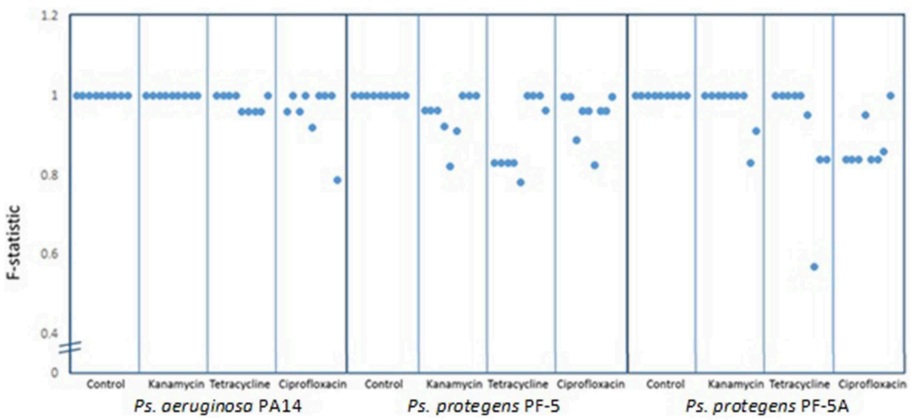

FIGURE 3 | Similarity co-efficient (F) of BOX patterns from experimental lines at generation 40 [F = 2Nxy/(Nx + Ny)] compared to control lines. Dots represent the averge F-statistic of three samples from each line and each box contains nine lines for each treatment. Scores below 1.0 indicate consistent polymorphisms in BOX-PCR patterns.

P. aeruginosa and *P. protegens* were serially plated on agar containing 1/10 the experimentally determined MIC for representatives of three antibiotic classes. This antibiotic concentration was chosen because it induces maximum transcriptional activity (Davies et al., 2006). Exposure to 1/10 the MIC for the panel of antibiotics tested had significant genotypic and phenotypic effects.

Effects on the genomes were immediate and readily detectable. Changes to rep-PCR DNA fingerprints could be detected after as few as five serial transfers on sub-inhibitory antibiotic

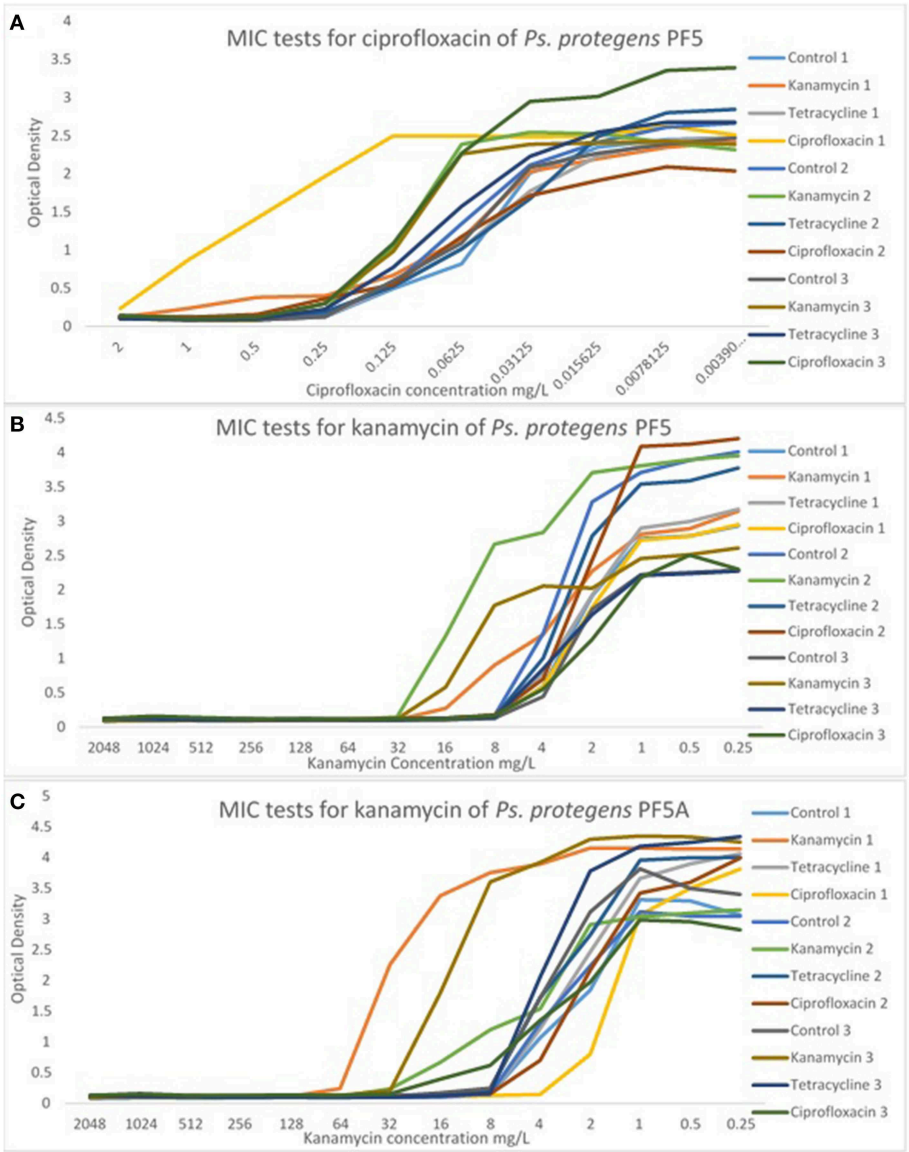

FIGURE 4 | Representative graphs displaying the MIC of selected antibiotics for the experimental and control lines. MIC tests for *Ps. protegens* strains are shown for PF5/ciprofloxacin **(A)**, PF5/kanamycin **(B)**, and PF5A/kanamycin **(C)**. For more examples see Supplementary Material (Figures S7–S9).

concentrations. This result is even more remarkable, since BOX-PCR is a fairly insensitive measure of genomic variation, although it generates highly reproducible DNA fingerprints. In this series of experiments, the BOX assay sampled between 15 and 20 kb of DNA, amounting to less than 0.3% of the ~7 Mb pseudomonad genome. If the genome changes are similar in the un-sampled portion of genome, sub-inhibitory antibiotic concentrations are having a widespread and significant effect on DNA sequence, genome architecture, or both.

Sub-inhibitory antibiotic concentrations also induced phenotypic changes. After 40 generations of serial transfer, many of the experimental lines exhibited changes in colony morphology. Perhaps of most significance, all six lines of *P. protegens* maintained on 1/10 the MIC for kanamycin showed up to eight-fold elevation in their MICs for kanamycin by 40 generations. Similarly, one line held on 1/10 MIC for ciprofloxacin also showed an elevated ciprofloxacin MIC by 10-fold. Sub-lethal ciprofloxacin exposure has previously been shown to induce resistance in hypermutable strains of *P. aeruginosa* (Jørgensen et al., 2013). Whether the resistance observed in our experiment is also mediated by mutations in *gyrA* or *gyrB* will have to await sequence analysis.

The changes in MIC we observed are not likely to be the result of selection on pre-existing mutations in the single colony we used to initiate each experiment. A suspension of a single, well isolated colony was used as inoculum for both control and experimental lines of the three pseudomonads tested (PA14, PF-5, and PF-5A). All six kanamycin treated lines (three each

for the two independent strains of *P. protegens*) showed elevated MICs for kanamycin by the 40th passage. One line from the ciprofloxacin treatments also showed a ten-fold increase in MIC. For these outcomes to have arisen from pre-existing mutants in the generation zero colony, each of the three kanamycin lines for both strains of *P. protegens* must have been the recipient of an appropriate mutant cell, as must have been the ciprofloxacin lineage. All of these putative mutations must have arisen during the growth of the time zero colonies from a single cell, which seems unlikely. Further, testing of a subset of the lines at passage 20 did not detect any increase in MIC in the kanamycin treated, or any other lines. By passage 20, any pre-existent mutant should have gone to fixation. The most parsimonious explanation for our results is that the changes in MIC were due to *de-novo* mutation.

If our findings are generally applicable, it suggests that similar phenotypic and genotypic changes will occur in all environments where antibiotics reach concentrations of 1/10 the MIC, and that these effects will potentially apply to all members of the environmental microbiota.

The effects of sub-inhibitory antibiotic concentrations observed in our experiments might be driven by the bacterial SOS response, which is known to induce processes that increase mutation, transposition and recombination rates (Gillings, 2013; Andersson and Hughes, 2014). Certainly, ciprofloxacin is a potent inducer of the SOS response, and generated the most extreme changes in BOX fingerprints observed in our experiments. Aminoglycosides such as kanamycin also induce the SOS response, but here tetracycline had an even greater effect on genomic architecture as assessed by BOX-PCR. However, there is no evidence that the diversity we observed is entirely due to the SOS response. The advantage of the approach we have taken here is that all mechanisms that generate variation, including the SOS response, and other potentially novel mechanisms, can be captured.

The concentrations of antibiotics used here may represent typical of levels of antibiotic pollution. There is limited knowledge about the concentrations of antibiotics found in the environment, however it is now known that antibiotics can persist in the environment longer than previously thought. The time that an antibiotic can persist in the environment differs depending on the class of antibiotic and the environmental conditions. Closed bottle tests provide a simple way to measure the biodegradability of antibiotics and indicate whether or not the antibiotic will readily degrade. Classes of antibiotics such as the β-lactams, tetracyclines, macrolides, lincosamides, penicillin, aminoglycosides, carbapenems, nitroimidazoles, polyene-antimycotics, quinolones, sulphonamides, and glycopeptides have all been found to persist over a 28 day testing period (Al-Ahmad et al., 1999; Alexy et al., 2004). High temperatures and exposure to UV light can cause degradation of some antibiotics. Fluoroquinolone antibiotics can degrade in sunlight, however they are readily absorbed onto sediments, where they have been documented persisting up to 80 days with less than 1% of degradation (Marengo et al., 1997). It would be convenient if resistant organisms destroyed or inactivated antibiotics, however the mechanisms that usually allow resistance involve mutation of binding sites or efflux pumps, meaning that antibiotics are not physically altered and may persist in the environment (Levy, 2002). Following one application of manure, antibiotics and antibiotic resistance genes can persist in the soil for approximately 6 months, depending on environmental conditions, during which time it could be dangerous to consume products that have had direct contact with the soil (Marti et al., 2014). Given the significant time frame in which antibiotics can persist in the environment it is highly likely that they will exist at concentrations near 1/10 the MIC.

The concentration at which antibiotics may occur in the environment is affected by several factors: substrate, proximity to source of antibiotics, environmental conditions and the antibiotics themselves. Testing of rivers and oceans have detected the presence of antibiotics, most notably sulphonamides and quinolones which were found at high concentrations in a number of environments. Sulphonamides were detected in water (0.86–1563 μg/L) (Hirsch et al., 1999; Luo et al., 2011; Li et al., 2012; Zhang et al., 2013) and quinolones were detected in sediments and plants (65.5–1166 and 8.37–6532 μg/kg, respectively) (Li et al., 2012). The antibiotic concentration of 1/10 the MIC easily falls into the ranges of antibiotic pollution detected in these waterways, indicating that the results of this study are likely to represent the rates of mutation and recombination taking place in environments polluted by antibiotics.

Very small concentrations of common antibiotics can induce significant genotypic and phenotypic changes in bacterial species. Given the huge quantities of antibiotics that are entering the environment, it is likely that this antibiotic pollution is generating antibiotic resistant organisms that may be a source of newly emerging opportunistic pathogens. These may then pose significant threats to human and animal life. Changes need to be made at every level of antibiotic use, by the individual, in medical practice, in pharmaceutical production, government monitoring and waste treatment, otherwise modern medicine is at a risk of facing a post antibiotic era where infections are harder, and in some cases, impossible to treat.

Acknowledgments

We would like to gratefully acknowledge Marita Holley and Hilal Varinli for their technical support and assistance throughout the course of this study.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00803

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.