Lancaster University

Centre for Global Eco-Innovation

OCCURRENCE, FATE AND REMEDIATION OF VETERINARY ANTIBIOTICS IN SURFACE WATERS

Evangelia Tzelepi

This dissertation is submitted for the degree of Doctor of Philosophy

This project was supported by the Centre for Global Eco-Innovation and is part financed by the European Regional Development Fund.

November 2024

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Declaration

This dissertation is the result of my own work. It has not been previously submitted, in part or whole, to any university of institution for any degree, diploma, or other qualification.

Signed:

Date:

Acknowledgement

I would like to thank my supervisors Crispin Halsall and Keith Waterhouse for providing me with this opportunity to unravel the exciting world of environmental fate and for their continuous support over the years. I have gathered so much knowledge over the years that I was able to take it further and now have a job that I love helping companies release compounds into the market that really helps people while taking care of the environment.

I would also like to thank the Eden DTC team for making me briefly part of their team and help me with my environmental sampling work and also the lovely farmers at both sites that were so keen and welcomed every time I had a sampling event scheduled.

A huge thank you to Maria Snell and Jacky Parry that they both guided me through my algal work. Without them I wouldn't be able to move and implement this strange idea I had at that moment of using algae to treat antibiotics.

Finally, I want to thank my husband and my family that they supported me and motivate me through this long process of finishing this PhD thesis.

Abstract

Pollution from pharmaceuticals in the aquatic environment is now recognized as an environmental concern in many countries. There has been an increasing concern in recent years about the occurrence, fate, and adverse effects of pharmaceutical residues in the aquatic environment. Some of the most widely and frequently used drug classes are antibiotics as they can be used both in humans and animals. Pharmaceuticals end up in soil, surface waters and eventually in ground water, which can be used as a source of drinking water, after their excretion (in unmetabolized form or as active metabolites) from humans or animals via urine or faeces. The possible routes of antibiotics once they get into the aquatic environment are mainly three either they mineralise to carbon dioxide, sorb into the soil and sediment or for ends up in receiving waters (surface and ground water.

A fast and simple method for the analysis of 15 commonly used antibiotics in water samples deriving from a catchment area was developed. The method combines online solid phase extraction using a reusable online trapping column combined with analytical separation on a C18 analytical column and detection by a signle quadrupole mass spectrometer. The method was fully validated for detection and quantification limits as well as linearity, repeatability, and matrix effects. The method gave an excellent linear response ($r^2 > 0.99$) and detection limits for all compounds (1–50 ng L^{-1}) The method was used to monitor diffuse pollution from farm and WwTWs in a rural area. Most of the antibiotics were detected in the samples, except from cephalosporins, with maximum concentrations measure for sulfamethoxazole at 1659 ng L^{-1} at the discharge point of WWTP.

Once released into the aquatic environment, pharmaceuticals may undergo different degradation processes. Photodegradation is an important route of elimination for light-sensitive pharmaceuticals, such as antibiotics. In this study, the fate of two sulphonamides, one tetracycline and one fluoroquinolone was investigated in different matrices to establish possible degradation patterns. A comparison between laboratory acquired photolysis data and field data was made that identified an increasing need for more routine field work. Degradation under natural sunlight for more photosensitive compounds appeared in line with the laboratory results however there were big discrepancies between the laboratory obtained values and the ones derived from the outdoor experiment for the more persistent compounds.

Recently, microalgae based technology has been explored as a potential alternative for the treatment of wastewater containing antibiotics by adsorption, accumulation, biodegradation, photodegradation, and hydrolysis. In this role a primitive study was conducted to evaluate the removal rates and degradation of two sulphonamides in the presence of naturally grown algae. Both of compounds exhibited faster degradation in the presence of algal species. After a 5 day incubation period up to 79% of sulfadiazine and 68% of sulfamethoxazole were removed by algae mediated photolysis against 56% and 28% when compared to photolysis alone by using clean media.

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List of Abbreviations and Acronyms

AMX: Amoxicillin
CFL: Cefalonium
CFT: Ceftiofur
CFP: Cephapirin
CFQ: Cefquinome
DW: Drinking water
ENR: Enrofloxacin
ERY: Erythromycin
GW: Groundwater
LC/MS: Liquid chromatography tandem Mass spectrometry
LIN: Lincomycin
LOD: Limit of detection
LOQ: Limit of quantification
LW: Lake water
MAR: Marbofloxacin
min: minutes
mL: millilitre
nd: not detected
ng/g: nanogram per gram
ng/L: nanogram per litre
OECD: Organisation for Economic Co-operation and Development
OTC: Oxytertacycline
PEN: Penicillin G
TMP: Trimethoprim
DEFRA: Department for Environment, Food & Rural Affairs
PW: Pond water
ROS: Reactive oxygen species
RRT: Relative Retention Time
SDZ: Sulfadiazine
SFO: Single First Order
SMZ: Sulfamethoxazole
SNR: Signal to noise ratio
SPE: Solid Phase Extraction
SW: Surface water deriving by a river/ estuaries/ watersheds
TYL: Tylosin
VMD: Veterinary Medicines Directorate
VMD: Veterinary Medicines Directorate
EMA: European Medicines Agency
EFSA: European Food Safety Authority
ECDC: European Centre for Disease Prevention and Control
WHO: World Health Organisation
WW: Wastewater
WWTP: Wastewater Treatment Plants
µg/g: micrograms per gram
µg/L: micrograms per litre

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1 INTRODUCTION

1.1 Why is it important to understand antibiotic's presence in the environment?

In recent years, there is an increasing concern regarding the presence of pharmaceuticals in the environment and their potential impact on human health, not only from the scientific community but also from the media.

Over the years, organisations have set in place regulations to ensure public health. The Directive 2000/60/EC was the first mark in the European water policy, which set up a strategy to define high risk substances to be prioritized. A list of 33 priority substances/groups and the respective environmental quality standards (EQS) were presented in the Directive 2008/105/EC. However, it was only in 2011 that the World Health Organization (WHO) produced a literature review to assess the presence and implications of pharmaceuticals in drinking water. (WHO, 2011) In 2012 there was a number of reports from relevant organisations introducing the importance of monitoring the presence of pharmaceuticals in the environment. The first report was published from OECD showcasing the challenges and knowledge gaps regarding monitoring "emerging contaminants" (ECs) that arise from agriculture. The term ECs referred to a variety of product types including human pharmaceuticals, veterinary medicines, nanomaterials, personal care products, paints and coatings. (Alistair B.A. Boxall, 2012)The second report was a revision of the Directive on priority substances in the field of water quality. It was suggested amongst other compounds to also include the following three pharmaceutical substances: 17 alpha-ethinylestradiol (EE2), 17 beta-estradiol (E2) and Diclofenac. (Proposal for a Directive of the European Parliament and of the Council amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy, 2012) Three years later, three antibiotics were added to the priority

list (Erythromycin, Clarithromycin and Azithromycin).(European Commission, 2015) By 2018 two more antibiotics were added to the list (Ciprofloxacin and Amoxicillin). (European Commission, 2018a) The EU Commission recently proposed to amend the Directive on Environmental Quality Standards (EQS) with additional quality standards for groundwater priority pollutants, by including sulfamethoxazole (0.01 µg/L) and a sum-EQS for pharmaceuticals (0.25 µg/L) (Backhaus, 2023)

The need to effectively monitor pharmaceuticals and more specifically antibiotics in the environment had derived from a WHO report, published in 2014, with the title "Antimicrobial Resistance: Global Report on Surveillance", that indicated that a post-antibiotic era is a real possibility in the twenty-first century due to the alarming rate of development and spread of antibiotic resistance. ('WHO Regional Office for Europe (2014) Antimicrobial resistance. Copenhagen) This assumption was also validated by another joint report that showed overall a positive association between antimicrobial consumption in food-producing animals and occurrence of resistance in bacteria from such animals for most of the combinations investigated.) (ECDC/EFSA/EMA, 2017) In 2019, the World Health Organization included Antimicrobial Resistance as one of the ten threats to global health. EU Commission in 2022, following the revision of three major legislative frameworks, proposed ways to manage and monitor water pollution in groundwater and surface water related to AMR by implementing the "one substance, one assessment" idea. This was done so there is a consolidated approach on gathering and interpreting data across the world.

Many countries have set in place surveillance of antibiotic consumption in food-producing animals to tackle excessive and irrational usage. (WHO methodology for a global programme on surveillance of antimicrobial consumption, 2011) The quantity of authorised veterinary antibiotics sold throughout the UK has been reported to the Veterinary Medicine Directorate (VMD) by pharmaceutical companies since 1993 and this has been a statutory requirement since 2005. In the latest issue antibiotics for use in food producing animals equals to 193 tonnes (in mg/PCU). (UK-VARSS, 2022) In Europe, the harmonized Surveillance of Veterinary Consumption (ESVAC) report has been in place since 2005, and currently reports data from 31 countries in the European Union (EU). In the latest reports all the European countries apart from one, Poland, showed a significant decrease in sales of veterinary antimicrobial agents. (European Medicines Agency, 2022)

There is a definite link between the use of antibiotic drugs in animals and the risk to man from antimicrobial resistance.(Allel *et al.*, 2023) However, the mechanism and frequency by which this resistance may be transferred to man and the extent of the threat that this represents to human health is less clear. (TAFTAR, 2016) Resistance to commonly used antibiotics are in the genes of bacteria everywhere. (Jian *et al.*, 2021) A worldwide study of the gene sequences of bacteria, has found resistance across 71 environments, including oceans, soil and human faeces. There was a case where 30 % of total known antibiotic drugs resistance genes could be found in a single soil sample. (Joseph Nesme et al., 2014) (Singh *et al.*, 2022)showed that there is a high incidence rate of multiple antibiotic resistant bacteria in the spring water and suggests spring water are not suitable for drinking without prior treatment.

A publication from World Health Organisation presents a list of antibiotic-resistant "priority pathogens". The list prioritise 12 families of bacteria based on their likelihood to pose a threat to human health and is divided into three categories according to the urgency of need for new antibiotics: critical, high and medium priority. This list was updated in 2023 but the publication is forthcoming. The aim of this list was to encourage the research and development into new antimicrobials, diagnostics and vaccines, and inform public health action (Geneva: World Health Organisation, 2017)

Table 1-1: WHO priority pathogens list for R&D of new antibiotics. (Tacconelli et al., 2017)

Priority 1: CRITICAL
1. Acinetobacter baumannii, carbapenem-resistant 2. Pseudomonas aeruginosa, carbapenem-resistant 3. Enterobacteriaceae, carbapenem-resistant, ESBL-producing
Priority 2: HIGH
1. Enterococcus faecium, vancomycin-resistant 2. Staphylococcus aureus, methicillin-resistant, vancomycin-intermediate and resistant 3. Helicobacter pylori, clarithromycin-resistant 4. Campylobacter spp., fluoroquinolone-resistant 5. Salmonellae, fluoroquinolone-resistant 6. Neisseria gonorrhoeae, cephalosporin-resistant, fluoroquinolone-resistant
Priority 3: MEDIUM
1. Streptococcus pneumoniae, penicillin-non-susceptible 2. Haemophilus influenzae, ampicillin-resistant 3. Shigella spp., fluoroquinolone-resistant

The 2022 Global Antimicrobial Resistance and Use Surveillance System (GLASS) report highlights alarming resistance rates among prevalent bacterial pathogens. Median reported rates in 76 countries of 42% for third-generation cephalosporin-resistant *E. coli* and 35% for methicillin-resistant *Staphylococcus aureus* are a major concern. For urinary tract infections caused by *E. coli*, 1 in 5 cases exhibited reduced susceptibility to standard antibiotics like ampicillin, co-trimoxazole, and fluoroquinolones in 2020. This is making it harder to effectively treat common infections and such results reinforced the need to understand that by continuing the misuse of antibiotics and neglecting their proper disposal will hinder our ability to treat effectively infectious diseases in the future. Legal authorities, veterinarians, physicians, and farmers all have a role in "conserving the power of antibiotics" and ensuring the safety of our ecosystem. (Centner, 2016)

1.2 Which is the chemical behaviour and fate of antibiotics in the environment?

Once in the environment, the fate of antibiotics is influenced by several factors primarily correlated to their underlying physical properties such as water solubility, lipophilicity, volatility and sorption potential (Puckowski et al., 2015) and also external factors such as climatic conditions, pH, soil type and a variety of other factor (Sarmah *et al.*, 2006)

In wastewater treatment plants the varying chemical properties will influence the behaviour of the antibiotic through the treating processes and they will determine the possible mobility or persistence (Yao *et al.*, 2017a). In rural areas though along with the aforementioned parameters, the mode of action of these compounds and subsequently their excretion rate and route (faeces or urine) also needs to be evaluated to understand the full scale of their fate. Antibiotics undergoes substantial changed throughout their transfer from the ingestion by the animal to the environment (Escher and Fenner, 2011) There are an important number of biotransformation by-products (metabolites) excreted by animals. The degradation pathway of these molecules is complex and could result in bioactive compounds, stable, mobile in the environment with potentially higher toxicity than their parent. (Jacek *et al.*, 2014)

The metabolism of veterinary antibiotics can be divided into several stages. Primary metabolites result from the biotransformation of the parent compounds by the animal, whereas secondary metabolites result from the biotransformation by the bacteria present in the ecosystem. (Robles Jimenez *et al.*, 2019) All these compounds are then subjected to when released into the environment which further complicates the environmental fate of these substances.(Casi and Neri, 2012) In order to access the environmental fate of a substance the following parameters are taken into consideration (OECD, 2017)

Table 1-2: Factors affecting the environemtnal fate of antibiotics

Parameter	**Based on**	**Comments**
Mobility	water solubility soil-water partition coefficient Koc retardation factor R	Compounds with high solubility values are more prone to leach into groundwater. According to (McCall P.J *et al.*, 1981) compounds that have Koc values 0-50 L/kg show a very high mobility in soil with compounds with Koc>5000 being immobile. However, according to FAO a compound is considered immobile with Koc>100,000
Degradation	persistence half-life time DT50 biodegradation hydrolysis photolysis	Hydrolysis is associated strongly with the pH of the matrix (Xuan *et al.*, 2010a). A compound is considered readily degradable when it exhibits a DT_{50}<20days and it is considered a persistent pollutant when observed DT_{50} values are over 180 days. (FAO)
Bioaccumulation	octanol-water partition coefficient Kow	According to (Rogers, 1996) compounds with log Kow >4 are more likely to dissipate to sediments or accumulate in organisms where those with log Kow <2.5 are likely to remain in surface water or soil zone.

Appendix 1 summarizes different physical properties of the most common pharmaceuticals, indicating the dissociation constant (pKa), solubility, octanol-water partition coefficient (log Kow), organic carbon-based sorption coefficient (Koc) along with excretion rates of the different compounds.

Even though there is evidence that there is a correlation between log Kow and antibiotics metabolism this is not completely accurate as the exact percentages can be influenced also by the species. The exposure of substances to the environment depends on the metabolism and excretion values of the parent compounds. (Slana and Dolenc, 2013) Based on their excretion rates they can be classified as low excretion (≤5 %), moderately low (6–39 %), relatively high (40–69 %) and high excretion compounds (≥70 %).(Jjemba, 2006)

Tetracyclines are poorly metabolized in the digestive tract of animals and show high excretion rates in faeces and urine.(Anadón *et al.*, 1985) Sulfonamides are metabolised in the liver excreted either in its parent form (15-30%) or metabolites or even conjugated with acetic acid through faeces and urine (Lamshöft *et al.*, 2007). Fluroquinolones show high excretion rates as well with >90% of the administrated dose excreted as parent or metabolites. (Mizuki *et al.*, 1996)

Sulfonamides are polar compounds that exhibit high solubility and low chelating properties. Once in the environment they bind to soil organic matter through cation bridging and cation exchange mechanisms, but their sorption is relatively low and they can easily transfer to

water/sediment/groundwater. (Sukul *et al.*, 2008a) Even though they exhibit low sorption and hydrolysis ability (Białk-Bielińska *et al.*, 2012) studies have shown that they can persist in soils or sediment for many days. (Radke *et al.*, 2009) Direct photolysis can also positively influence the degradation/elimination of sulfonamides, however it was demonstrated that some of the metabolites can show higher photostability than the parent with conjugated excreted metabolites also transformed back to parent under sunlight.(Bonvin *et al.*, 2013)

Fluoroquinolones are highly polar compounds that show amphoteric characteristics with poor water solubility and high affinity to adsorption in soil by forming bonds of the carboxylic acids with the cations present in soil. (Wang and Wang, 2015) Due to their increased chelation properties biodegradation is soil is minimal. (Tolls, 2001) Also they are not affected by hydrolysis (Girardi *et al.*, 2011). However, photodegradation plays a key role in their removal/transformation in the environment (Sturini *et al.*, 2012) with some fluoroquinolones like enrofloxacin showing DT_{50} of minutes when they are under direct sunlight, even though these values are highly affected by external parameters (eg. pH, organic matter). (Babić *et al.*, 2013a)

Tetracyclines have been reported as non-biodegradable compounds that are easily adsorbed to treatment sludges, soils and sediments by binding to calcium and similar ions and forming stable complexes.(Zhang *et al.*, 2011) Once adsorbed tetracyclines demonstrate high persistency with oxytetracycline being detected even after 6 months in manure. (Loke *et al.*, 2003) It has also been shown that tetracyclines are susceptible to photodegradation due to the generation of reactive oxygen species (O_2^-, H_2O_2, etc.) arising from direct irradiation of tetracycline. (Chen *et al.*, 2008) However the decomposition rates are highly dependent on conditions such as water hardness, pH and the presence of other constituents (suspended solids, high organic matter). (Jiao *et al.*, 2008a)

Beta-lactams due to their low log Kow values show low elimination through biotransformation and soil sorption. Once in soils they appear to be moderately persistent with $DT_{50} < 49$ days in soils and < 5days in sediment. (Gilbertson *et al.*, 1990; Jiang *et al.*, 2010) Results also indicated that cephalosporins are not readily biodegradable, (Alexy *et al.*, 2004) but generally they appear to be relatively unstable in the environment due to their b-lactam ring. They are heavily influenced by abiotic processes such as hydrolysis under acidic/alkaline conditions, high temperatures or by reaction with weak nucleophiles. (Mitchell *et al.*, 2014) Elimination due to photodegradation appears to be also significant with DT_{50} values <7 hours (Jiang *et al.*, 2010; Timm *et al.*, 2019)

Macrolides are mild acids, lipophilic, and poorly water soluble that show low sorption capacity due to their high molecular weight. They appear to be relatively persistent in the environment with DT_{50} between 6 and 130 days in manure (Schlüsener *et al.*, 2006) and between 5 and 20 in the soil (Schlüsener and Bester, 2006) Direct photolysis does not promote the degradation of macrolides because they show low absorbance in the UV range however indirect photolysis in the presence of highly reactive species (nitrates, humic acid) promotes the degradation. (Tong *et al.*, 2011)

1.3 Which are the primary sources of antibiotics in the environment?

The occurrence of pharmaceuticals in the environment has become a matter of concern in the last decade, due to potential risks posed to non-target organisms and humans by the unintended exposure via the food chain or water.(Baralla *et al.*, 2021) Antibiotics are some of the most frequently prescribed drugs used in modern medicine and today's doctors are armed with a whole suite of them for the purposes of treating bacterial infections in both humans and animals. (Kümmerer, 2009) This extended use of antibiotics is the primary reason why they are frequently detected in a wide range of environmental samples, including sewage effluents, surface waters, groundwaters and drinking water, with their concentrations generally ranging from the low ppt to ppb levels. (Polianciuc *et al.*, 2020a)

It is only due to the development and improvement of analytical instrumentation and methodologies that we are able to detect these chemicals in environmental samples at low concentration levels. However the detection is hindered by the elevated number of active ingredients available and also the complexity of the matrices.(Białk-Bielińska *et al.*, 2016) There are hundreds of different types of antibiotics but most of them can be broadly classified into six groups:

- Penicillins: widely used to treat a variety of infections, including skin infections, chest infections and urinary tract infections. They are the most frequently used antibiotics in humans and the second most used in animals.
- Tetracyclines: can be used to treat a wide range of infections caused by susceptible bacteria and microorganisms. It is the most common used class of antibiotics for animals.
- Cephalosporins: used to treat a wide range of bacterial infections, but some are also effective for treating more serious infections, such as septicaemia and meningitis
- Sulfonamides: are the basis of several groups of drugs and they have a broad spectrum of activity against a wide range of Gram-positive and Gram-negative organisms. They are usually used in combination with Trimethoprim
- Macrolides: can be particularly useful for treating lung and chest infections, or an alternative for people with a penicillin allergy, or to treat penicillin-resistant strains of bacteria
- Fluoroquinolones: are broad-spectrum bactericides that can be used to treat a wide range of infections (ECDC, 2016; Gulland, 2017; Geneva, 2001)

Unlike with the controlled usage of antibiotics for humans, the use of antibiotics in animals is not always intended for treating infections. Instead antibiotics can be used to prevent the development of an infection amongst the animals or in some countries as growth promoters to speed up the pace at which animals gain weight. (Rushton *et al.*, 2014) Both cases increases the possibility of trace amounts of antibiotics to reach the environment and accumulate in areas where intensive farming facilities are located. (Zhou *et al.*, 2013)

Antibiotics intended for human medicine are entering the sewage system in low concentrations on a daily basis, either via the excretion of metabolites and unchanged drug into the urine and faeces or by the irresponsible disposal of unused antibiotics.(Huerta *et al.*, 2012; Samrot *et al.*, 2023) Similarly the major route of elimination of antibiotics administrated to animals is via the excretion into the urine or the faeces, with some cases reaching up to 95% of the drug to be excreted in animal wastes unaltered or as metabolites.(Serrano *et al.*, 2021) As shown in **Figure 1-1**, the presence of antibiotics in the environment is mainly attributed to either inadequate/non-efficient wastewater treatment processes that releases antibiotics in the aquifers via effluents or application of treated sludge, or with the use of manure as fertilizers. (Jia *et al.*, 2023)

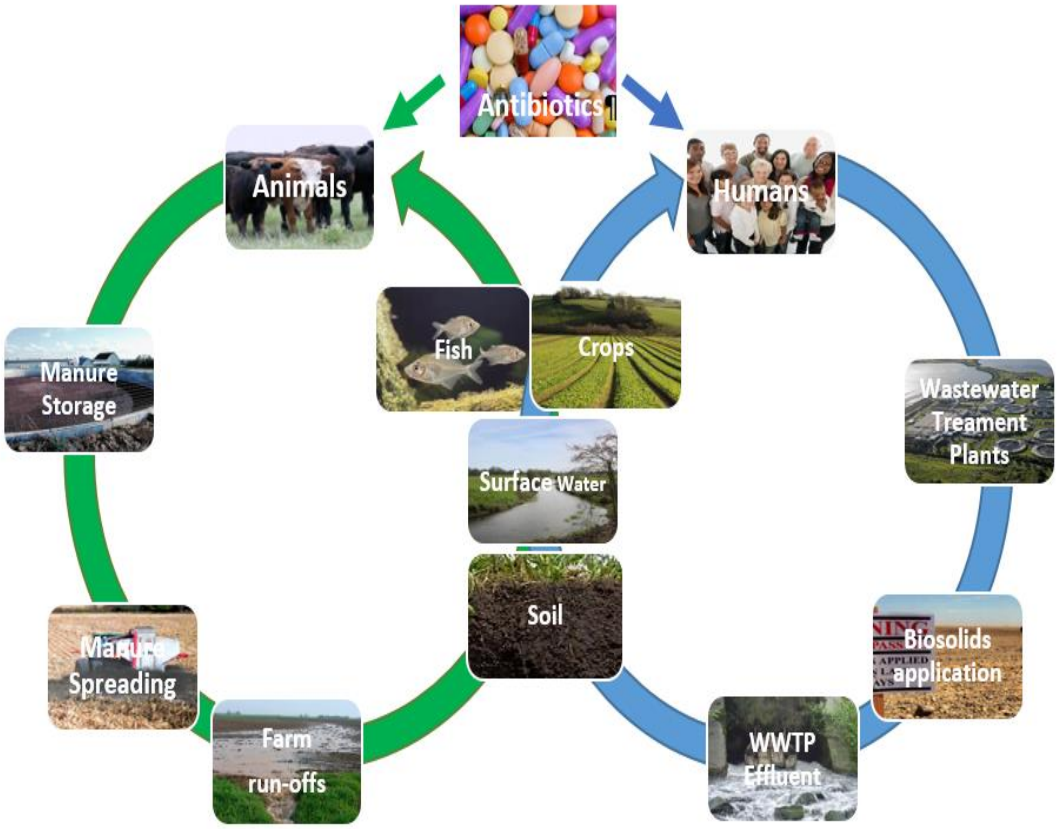


Figure 1-1: Anticipated environmental pathways for antibiotics used in humans and livestock animals. (adapted from (Carvalho and Santos, 2016a))

The exact distribution of pharmaceutically active compounds in the environment has been reported in a number of publications. There are studies that identify the wastewater treatment plants as the main source of these compounds.(Liu *et al.*, 1999; McClellan and Halden, 2010; Xu *et al.*, 2012; Michael *et al.*, 2013; Collado *et al.*, 2014; Rodríguez-Molina *et al.*, 2019; Mutuku *et al.*, 2022). However, there are studies that indicate the cumulative presence of these compounds in the environment can also be attributed to farming activities in combination with the anthropogenic.(Luo *et al.*, 2011; Berendonk *et al.*, 2015; Manaia *et al.*, 2016; Charuaud *et al.*, 2019a; Wu *et al.*, 2023).

The occurrence of antibiotics in surface water bodies and whether it is attributed to anthropogenic or livestock/agricultural activities is summarised below. The summary is based on studies from the past decade that include multiple analytes of interest (mainly >10) and multiple sampling events. **Table A2-1** is focusing on studies that showcase the correlation of WWTP effluents and the contamination of receiving water bodies where in **Table A2-2** the primary point of entry of the antibiotics in the environment is associated with livestock or agricultural practises (manure spreading).

All amounts of antibiotics are entering the water bodies via WWTPs effluents on a daily basis due to the insufficiently removal during the treating process. (Arun *et al.*, 2022) It was shown that older WWTP's (Dinh et al., 2017) or smaller ones (Osińska *et al.*, 2020) that use only physicochemical processes in contrast with the ones that used also biological processes are contributing more to the antibiotic impact on the environment. (Botero-Coy et al., 2018) Antibiotics released from WWTP were detected even 20 kilometres away from the discharging point. (Sabri *et al.*, 2020a) Levels of residues appeared interlinked with the flow of the water body indicating that smaller tributary rivers or estuaries are more prone to increased contamination. (Brown et al., 2015) However in some cases it is difficult to distinguish the source of the contamination especially in areas where both anthropogenic and agricultural activities are taking place (Collado *et al.*, 2014) due to the fact that some of the antibiotics are both being used for humans and animals. In some cases sewage markers can be used to solve this origin issue unfortunately though this is not always the case. (Murata *et al.*, 2011; Kaown *et al.*, 2021)

The prevalence of antibiotic concentrations associated with wastewater treatment plants is following a different pattern to those concentrations that are associated with non-point sources. (Kolok et al., 2014) However veterinary antibiotics are mostly measured episodically during periods of increased rainfall, runoff events and periods of manure spreading. (Charuaud *et al.*, 2019b; Dong *et al.*, 2021) There is a greater chance of veterinary antibiotics to reach the aquatic system in areas where concentrated animal feeding operations are taking place. This is due to the fact that larger volumes of waste are produced but also more antibiotics are consumed by the animals.(Sandoz *et al.*, 2018) This was also demonstrated by (Iglesias *et al.*, 2012) where sulfadiazine levels reached a maximum of 2979 ng/L in water samples collected from an area heavily influenced by large farms (eg. swine farm >100 animals).

The most common way of eliminating livestock waste is by spreading manure to agricultural fields as it is known to be a good fertiliser for enhancing crop production due to the nutrient-rich organic nature.(Rotz *et al.*, 2011). Even though manure composting or digestion might help reduce the presence of antibiotics in the animal waste, high concentrations are still present and in some cases concentrations observed are way above the PNEC levels (Solliec *et al.*, 2016; Chen *et al.*, 2018). (Zhao *et al.*, 2010) showcased that higher amounts of antibiotics were present in manures from industrial-scale farms compared to those belonging to smaller farmers with concentration levels being higher in pig manure followed by poultry and lastly by cattle manure. Fluoroquinolones, Tetracyclines and Sulfonamides are

the most represented classes of antibiotics present in manure (Van Epps and Blaney, 2016; Marutescu *et al.*, 2022). Enrofloxacin was found at concentration as high as 1421 mg/kg in a poultry litter in China (Zhao *et al.*, 2010) and sulfadiazine was detected at 91 mg/kg in turkey litter (Martínez-Carballo *et al.*, 2007) where oxytetracycline was found at 59 mg/kg in cattle manure. (Arikan *et al.*, 2007)

Following the spreading of manure, antibiotics depending on their physicochemical properties can be retained in agricultural soils with concentrations ranging from a few μg kg^{-1} to several mg kg^{-1}. (Li *et al.*, 2011a) found high antibiotic concentrations in vegetable farmlands at 94% of the soil samples affiliated with livestock farms and detected concentrations of tetracyclines, sulphonamides, and quinolones at nd-242.6, 33.3-321.4, and 27.8-1537.4 μg/kg, respectively.

Either antibiotics can be mobilized from these fields during heavy precipitation and enter surface water bodies via runoff events (Blackwell *et al.*, 2007). (Solliec et al., 2016) measured 17 veterinary antibiotics alongside some of their major degradation products in drainage water after manure application. Tetracyclines showed the higher abundance with measured concentrations appearing higher for the degradation products (eg. isochlortetracycline:3256 ng/L) over the parents (eg. chlortetracycline:29 ng/L). (Pinheiro *et al.*, 2013) detected high levels of chlortetracycline (max. 380.6 ng/L) in run off water up to 30 days after pig slurry application. Other studies also pointed out that antibiotics concentrations were growing exponentially with increased rainfall. (Kim *et al.*, 2016; Williams *et al.*, 2022) Through leaching antibiotics can dissipate into the sediment (Xu *et al.*, 2014) (Ok *et al.*, 2011) or even reach groundwater (Burke *et al.*, 2016; Spielmeyer *et al.*, 2017; Boy-Roura *et al.*, 2018) Environmental pathways that may allow antibiotics to be transported into groundwater include leakage from lagoons, leaching of manure applied to fields, and leaching from animal housing areas. (Watanabe *et al.*, 2010) Antibiotics present in shallow groundwater might originate from livestock waste, because animal waste from livestock farms usually accumulated in an open environment without adequate anti-seepage measures. (Han *et al.*, 2023)

Rural water bodies or wells are normally used as drinking water supplies. Studies indicated the presence of antibiotics close to drinking water plants (Iglesias *et al.*, 2014a) at concentrations as high as 56.3 ng/L (trimethoprim) or even tap water with florfenicol showing a residue of 211ng/L (Charuaud *et al.*, 2019a).

Elevated levels of antibiotics were also detected in farm water supply systems with levels ranging from hundreds ng/L to hundreds μg/L. A study contacted on the water supply for farms detected very high concentration of antibiotics. The presence of antibiotics was found in 52% of all the analysed water samples with enrofloxacin and tetracycline being the most frequently detected at a maximum concentration of 1670 and 1650 μg/L respectively (Gbylik-Sikorska *et al.*, 2015). This means that animals are constantly exposed to antibiotics that exist in their water possibly due to antibiotics accumulation in water supply systems because of limited or no cleaning. (Veiga-Gómez *et al.*, 2017)

reported that 57% of all the drinking water samples from milking facilities had at least one antibiotic present with sulfadiazine showing the highest concentration of 3941 ng/L.

Humans are exposed to small doses of antibiotics unintentionally every day via a plethora of sources, even via drinking water, this should also be considered when we assess the potential human health risks associated with antibiotics in the environment. (He *et al.*, 2016)

1.4 Can we eliminate them?

Wastes deriving from anthropogenic sources are gathered in wastewater treatment plants where they undergo further treatments before they are safe to be released back into the environment. There are many reports stating that there is an incomplete removal of pharmaceuticals even in WWTPs that meet the basic regulatory requirement for waste water treatment and only those with tertiary treatment appeared to be more effective. (Gao *et al.*, 2012).

Most WWTPs processes (see **Figure 1-2**) have been designed to eliminate nutrients and pathogens from wastewater through solid removal, biological processes and disinfection but there are many pharmaceuticals that are unaffected by these processes and end up in the effluent in concentrations sometimes equal or even higher than that measure in the influent. (Fatta-Kassinos *et al.*, 2011a). This could attributed to the fact that antibiotics when metabolised might form glucuronide conjugates, which essentially hinder the accurate measurement of the parent in the inlet, however during biological treatment the sugar moiety is detached releasing the parent to its free form.(Radjenovic *et al.*, 2007) The increased presence of these compounds after treatment might be also correlated with their reactive mode of action, for example erythromycin was found to be toxic to activated sludge. (Louvet *et al.*, 2010)

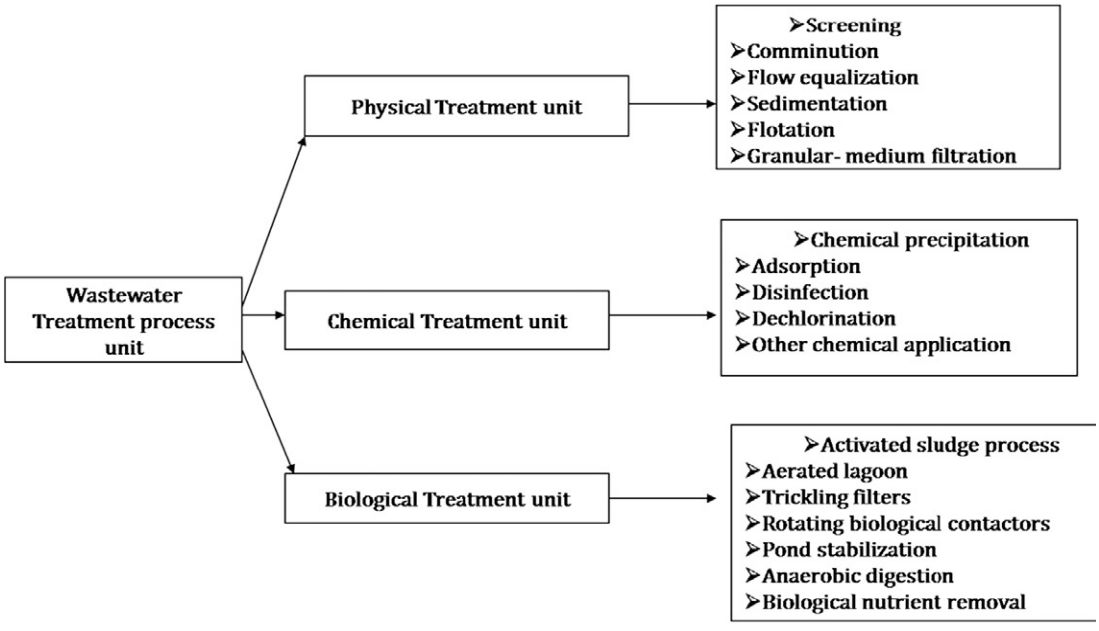


Figure 1-2: Wastewater treatment processes

The overall removal efficiency of antibiotics treated by Advanced Activated Sludge (A^2O), Activated Sludge (AO), and Membrane BioReactor (MBR) alone were not significantly different but significantly higher than the biological aerated filter (BAF) process alone. (Liu *et al.*, 2023a) The exact type of wastewater treatment process cannot explain 100% the capacity of antibiotic and antibiotic resistance genes removal. (Sabri *et al.*, 2020b) Operational conditions and external parameters involving the load or weather conditions can influence the removal efficiency. (Neef *et al.*, 2022)

(Comber *et al.*, 2018) performed an extensive screening of 19 pharmaceuticals and 4 metabolites in 45 UK WWTPs. Adequate removals rates were observed for most of the pharmaceuticals studied apart from, the macrolide antibiotics. This is in line with other studies by (Petrie *et al.*, 2014) and (Roberts and Thomas, 2006) that reported low removal of these compounds. Other antibiotics besides macrolides that show incomplete/low removal rates include ofloxacin (Dong *et al.*, 2016), tetracycline (Gulkowska *et al.*, 2008)

In contrast to the centralised treatment of the human wastes, animal wastes only undergo simple treatment process such as lagoons, digesters, tanks or even released untreated in the environment by the application of raw liquid manure.(Van Epps and Blaney, 2016) Disposal of wastes deriving from livestock is not as well-regulated as the ones derived from humans and it is not required to undergo any form of treatment before land disposal in countries like US or Korea. (Tasho and Cho, 2016) European union and UK has put in place a legislation (No.151, 2018) in order to minimise the effect of diffuse pollution in agriculture.

Animal manure management system is an integral part of the agricultural waste management system and every farm needs to adopt some form of animal waste management. (Malomo *et al.*, 2018) Recommended management options for the accumulated wastes included manure stockpiling, compositing and storage of slurry/manure in anaerobic lagoons. More effective practices that require greater management and effort include anaerobic digestion, high intensity composting and aerobic lagoons. (Agga *et al.*, 2022)

Composting is a bioremediation technology able to reduce or eliminate the residual concentrations of antibiotics present in sludge or manure before their application to agricultural fields. (Dolliver *et al.*, 2008) During sludge or manure composting, the antibiotics removals range between 17–100%. (Ezzariai *et al.*, 2018) Even though observed removal rates for some antibiotics are good, degradation appears to be chemical and manure type dependent. Sulfamethazine in turkey manure showed no change in the concentration, where in swine manure removal rates were between 82 to 100%. (Chen *et al.*, 2018)

Anaerobic digestion is a microbial decomposition process that takes place in an environment without oxygen and has traditionally been used to decrease the mass of wastewater treatment solids. (Martín *et al.*, 2015) Composting has been established as an effective method of reducing levels of

extractable and bioavailable antibiotics in manure and biosolids with calculated half-lives ranging from 0.9 to 16 days for most antibiotics. (Youngquist *et al.*, 2016)

Even though the above practises are showing a potential in eliminating the presence of antibiotics in animal wastes, antibiotics are commonly measured in manure samples originating from different farm practises in concentration that reach sometimes >100 mg/kg. (Zhang *et al.*, 2014)

Other processes that are applied to promote or enhance the degradation of these compounds alongside the conventional treatments are based on phytoremediation or algae. Constructed wetlands can be used to mitigate the release of antibiotics from livestock facilities. Studies have demonstrated high removal rates(>90%)(Carvalho *et al.*, 2013) for some antibiotics such as tetracyclines and fluoroquinolones. Algae is another constituent that has shown good potentials in promoting the degradation of livestock wastes. Culturing of algae utilising the livestock manure appears to be a viable alternative over land spreading.(Wang *et al.*, 2010) One more benefit from using algae as a remediation technique is that the resulting algal biomass can be further utilised for biofuel production. (Houser *et al.*, 2011)

1.5 Aims and objectives

The aims of this thesis are to develop an analytical method, incorporating Liquid Chromatography coupled to a Mass Spectrometer, to measure antibiotics and their key transformation products in targeted environmental surveys and to research laboratory-based chemical degradation pathways in order to develop novel sampling and removal technologies.

The basic objectives of the thesis are:

1. Develop an analytical methodology to enable the measurement of a targeted list of antibiotics in surface water samples from a catchment area.
2. Understand the effect of sunlight in combination with other natural water constituents to selected antibiotics and their pathways of degradation
3. Investigate a possible mitigation technique utilising algal species to eliminate antibiotics from areas with high farming activity.

1.6 Thesis structure and outline

This thesis is organised into chapters with each chapter being representative of the objectives mentioned above. The first chapter includes general information and a brief literature review about the occurrence, fate and remediation of antibiotics and also presents current views on antimicrobial resistance.

The second chapter details findings from Objective 1. It is focused primarily on developing a sensitive method to overcome the instrumental limitations and determine concentrations of antibiotics from surface waters deriving from an area with intense farming activities.

Chapter 3 contains the details on the concentration of measured antibiotics in surface water samples that were collected from water bodies near farms, that are part of a catchment area and analysed by LC/MS coupled with online sample preparation. Furthermore, the effectiveness of mitigation features that were already in place for diffuse pollution management is also evaluated by comparing the measured concentration of antibiotics present in water before and after the mitigation feature during a storm event.

Chapter 4 is based on establishing a better understanding on the degradation behaviour of four commonly used antibiotics under natural and simulated sunlight. Sulfadiazine, Sulfamethoxazole, Enrofloxacin, Oxytetracycline were tested in varying aqueous media in order to establish a correlation between water constituents and degradation pathways. Also features a comparison between lab- and field-based photolysis experiments to investigate whether values obtained in control environment are representative to the ones in the real world. A tentative attempt has been also made to identify some of the major photodegradation products in different aqueous matrices.

Chapter 5 utilises a mixture of algal species cultivated from natural water sources to investigate the degradation effect that might have on selected antibiotics. Different conditions are tested to evaluate the extent of degradation including high and low algal concentrations, presence or lack of nutrients and presence or absence of UV irradiation.

2

A FULLY AUTOMATED ONLINE SPE-LC-MS METHOD FOR THE DETERMINATION OF 15 ANTIBIOTICS IN SURFACE WATER.

2.1 Introduction

The misuse and overuse of antibiotics is responsible for the development and rapid spread of antibiotic-resistant bacteria (ARBSs) and antibiotic-resistant genes (ARGs). (Samreen *et al.*, 2021) There are concerns and an uncertainty on how environmental residues of antibiotics can contribute in the selection and spread of antibiotic resistance. (Hanna *et al.*, 2023) Antibiotics can enter the environment through various pathways such as the discharge of wastewater treatment plants (Mutuku *et al.*, 2022), manufacturing plants (González-Plaza *et al.*, 2019), farms (Martin *et al.*, 2015), and landfill leachates of antibiotic disposal.(Chung *et al.*, 2018) Other sources may include runoff from agricultural fields following application of livestock manure(Zalewska *et al.*, 2021) or aquaculture ponds.(Afonso-Olivares *et al.*, 2013) Following their discharge into the environment antibiotics and their transformation products can reach concentrations that range from ng L^{-1} to µg L^{-1}.(Carvalho and Santos, 2016b)

The systematic surveillance of antibiotic use and antibiotic presence in the environment is imperative for managing antimicrobial resistance. (Prestinaci *et al.*, 2015) The need for expanding systematic surveillance to all parts of the world has been increasingly recognized. Surveillance, prevention and mitigation requires a collaborative and transdisciplinary approach, a so-called “One Health Perspective” which intends to preserve human, animal, and environmental health. (Prata *et al.*, 2022) However, many low-income countries currently face substantial challenges in building national surveillance systems due to a lack of infrastructure and resources, resulting in a shortage of systematic data (Huijbers *et al.*, 2019; WHO *et al.*, 2021).

Unfortunately, currently it seems that there is no standard analytical methodology for analysis of antibiotics and their transformation products in the environment. In order to carry out consistent assessments of studies within a regulatory framework, validated and harmonized methods and more realistic experimental scenarios are needed at international level. (Polianciuc *et al.*, 2020b) The majority

of studies targeting antibiotic residues analysis tend to use techniques such as LC-MS/MS with a trend to develop multi-analyte techniques. (Mirzaei *et al.*, 2017)

There are a lot of methods reported for the analysis of antibiotics in different matrices, however the majority of these methods are unsuitable for monitoring studies as they employ sample enrichment techniques that require high sample volumes and are time consuming. (Kim *et al.*, 2018). Because of these reasons, great effort is going into the development of fast, cost-effective and environmental friendly alternative methods for environmental analysis that require less solvents, materials and minimise the sample transport. (López-Serna *et al.*, 2010) During online/automated analysis the whole sample is analysed and typically using higher injection volume which lead to lower detection limits In addition, on-line solid phase extraction (SPE) require less solvent than the conventional extraction regimes which also lead to decreasing running costs and waste disposal. (Pozo *et al.*, 2006)

The aim of this study was to develop and validate a quick and robust analytical method to verify residues of veterinary antibiotics in areas with intensive farming activities and evaluate the effectiveness of mitigation features that serve to contain farm runoff from entering water bodies. This analytical method describes the procedure for the determination of amoxicillin, cefalonium, cephapirin, cefquinome, ceftiofur, enrofloxacin, erythromycin, lincomycin, marbofloxacin, oxytetracycline, penicillin G, sulfadiazine, sulfamethoxazole, trimethoprim and tylosin in surface water.

2.2 Experimental

2.2.1 Reference items for calibrations and fortification

Amoxicillin (AMX), Cephapirin (CFP), Cefalonium (CFL), Ceftiofur (CFT), Cefquinome (CFQ), Enrofloxacin (ENR), Erythromycin (ERY), Lincomycin (LIN), Marbofloxacin (MAR), Oxytetracycline (OTC), Penicillin (PEN), Sulfadiazine (SDZ), Sulfamethoxazole (SMX), Trimethoprim (TMP) and Tylosin (TYL) were purchased from Sigma Aldrich (UK) and were of VETRANAL grade (purity >99%). As internal standards Caffeine-$^{13}C_3$ (Sigma Alrich, UK) and sulfamethoxazole-d_4 (QMX laboratories, UK) were used. See Appendix 1 for exact structure and physicochemical parameters of each compound.

Methanol and formic acid were of LC/MS (Optima) grade (Fisher, UK) and water was purified on a Milli-RO plus 30® and a Milli-Q purifiers (Millipore, USA). Individual stock solutions were prepared at a concentration of 500 µg/mL in methanol apart from amoxicillin, cefquinome, penicillin and cephapirin where the stock solutions were prepared in ultra-pure water and kept in the dark at >-20ºC for up to a month.

A 10 µg/mL mixed fortification solution was prepared by dilution of 200 µL of each stock standard solution in 10 mL of methanol. Subsequent dilutions were performed accordingly to achieve 1, 0.1 and 0.01 µg/mL accordingly. Solutions used for calibration were prepared fresh on the day by taking appropriate aliquots from the fortification solutions and diluting them to a final volume of 1 mL

with 0.1% formic acid in water. Calibration solutions were not stored. Recovery samples (QC samples) were prepared in surface water and acidified surface water (pH 3) to check the matrix effect and method efficiency.

2.2.2 Sample collection and pre-treatment

Surface water used for method validation and as a control was collected from a natural water body located in Lancaster University. The water was collected by large bucket and stored in glass 5L bottles in the fridge (4-8ºC) for up to a month to ensure viability of the test system. Prior to use in the study the water was screened to ensure there is no residue of the analytes of intertest and deemed suitable for use to prepare matrix matched standards and instrument recovery samples.

2.2.3 HPLC/MS analysis

The following instrumentation and conditions have been found to be suitable for this analysis. The method has been developed for use on an Agilent 1100 HPLC system linked to an Agilent MSD 6100 single quadrupole mass spectrometer.

Final determination by online SPE-LC-MS with a single transition is not considered to be highly specific and so in cases where uncertainty was high or when residues were measured the method of standard addition was used to verify results.

Table 2-1: Typical Liquid Chromatography Operating Conditions

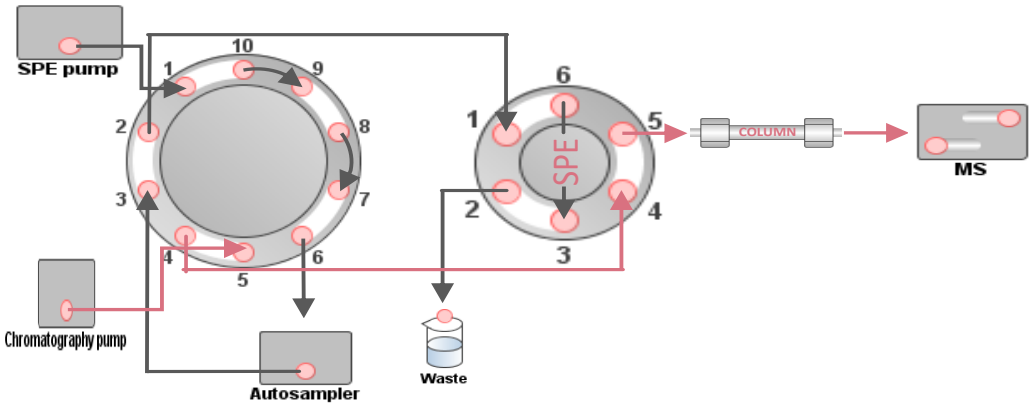
Instrumentation:	Agilent 1100 HPLC system with two pumps and an external switch valve
Pre-column:	Poroshell C18 fast guard (2.1 x 5, 2.7µm)
Column:	Poroshell 120 EC-C18 (2.1 x 100mm, 2.7µm)
SPE cartridge	PLRP-S, 2.1 × 12.5 mm, 15-20 µm
Column Temperature:	40°C
Autosampler temperature:	4°C
Typical Injection Volume:	9 µL for direct injection
	900 µL for online SPE injection

The chromatographic separation was performed using an Agilent 1100 binary pump (Chromatography pump-A) equipped with a vacuum degasser, a thermastated column oven set to 40 °C with a 6-port two position switching valve, and an Agilent Poroshell EC C18 column (2.1 × 100 mm, 2.7µm) attached to an UHPLC Poroshell C18 fast guard (2.1 x 5, 2.7µm) . For loading and enrichment, an Agilent 1200 series quaternary pump with build in degasser (SPE pump-B) coupled to an Agilent

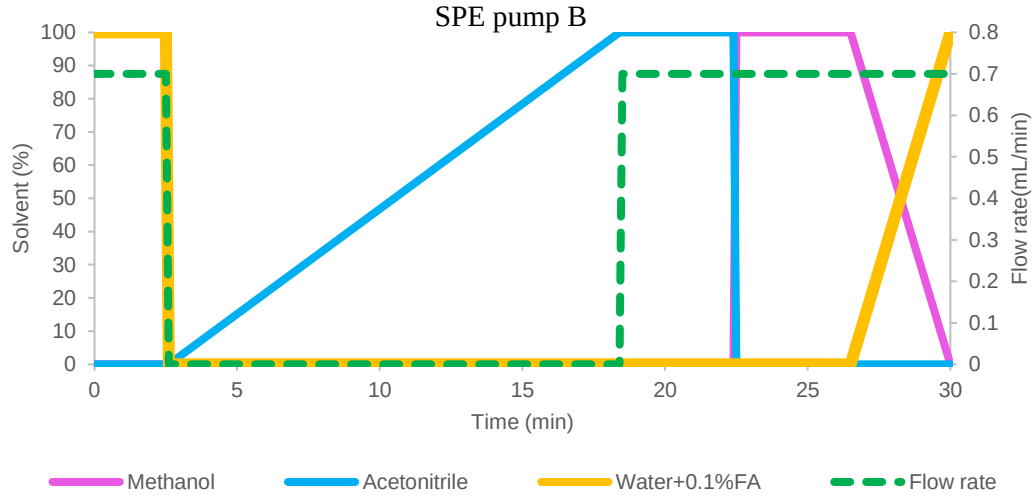
1110 autosampler with a 1400-µl injection loop was used. A reusable Agilent online trapping column with rigid macroporous styrene/divinylbenzene (PLRP-S) phase and dimensions of 2.1 × 12.5 mm (15–20µm) was used for samples clean-up.

Figure 2-1: Schematic Conditions for online SPE clean up

Switching valve configuration for conditioning and loading SPE cartridge – Configuration 1



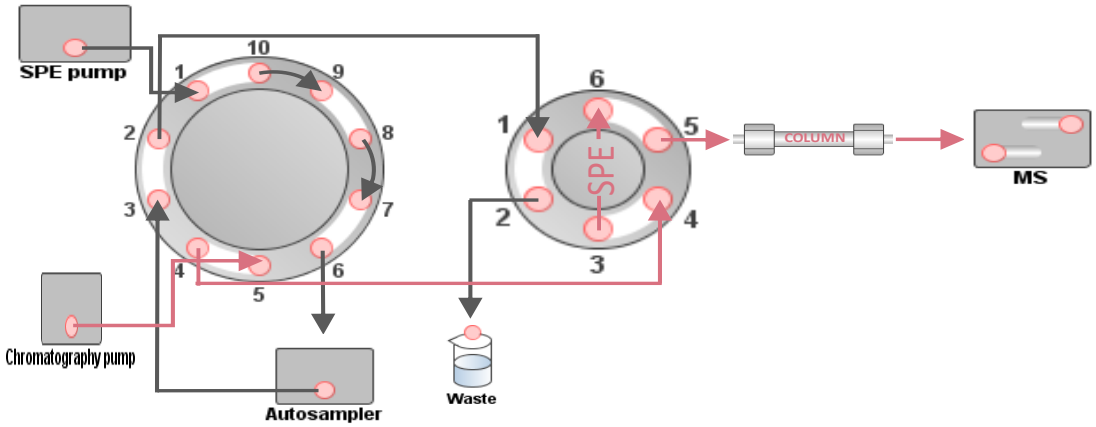
Composition of solutions (% Solvent) used for conditioning and eluting the SPE cartridge at 0.7 mL/min



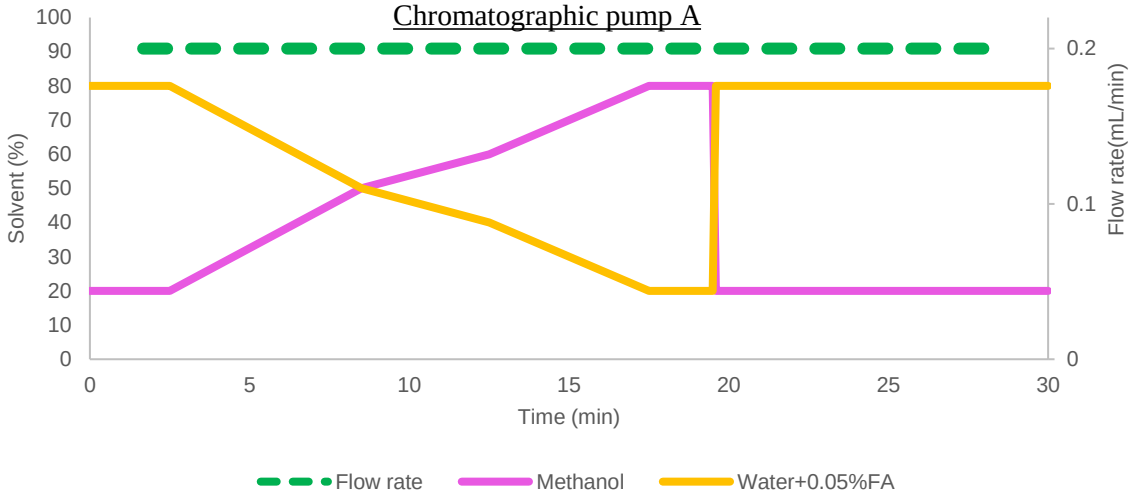
The two systems were connected through a two position nine-port valve in order to facilitate also direct injection, and a six-port switching valve located in the column compartment that was used for the sample clean up and were able to switch automatically from loading (configuration 1) to injection (configuration 2) (Figure 2-1). The mobile phases For the SPE pump 0.1% formic acid in water (B1) was used to deliver the sample to the trapping column with acetonitrile (B2) and methanol (B3) used to clean and condition the cartridge respectively. A multi-draw method was used for the injection of 0.9 mL of sample, which was then loaded onto the trapping column using 100% eluent B1 for 2.5 minutes from pump B and using a flow rate of 0.7 mL min^{-1}.

Figure 2-2: Conditions for chromatographic separation

Switching valve configuration for eluting to column (valve switch at 2.5 min) – Configuration 2



Composition of solutions (% Solvent) used for chromatographic separation at 0.2 mL/min



For the chromatographic pump the mobile phases were 0.05% formic acid in water (A1) and methanol (A2). The six-port valve was then switched to configuration 2 (Figure 2-2), and pump A was running in a three step gradient starting with 20% A2 and going up to 80% A2 over 17.5 min and held there for 2 minutes before returning to initial conditions.

The total sample analysis time was 26.5 minutes with a column equilibration time of 8 minutes. During the run, the eluent composition in pump B was also set at 100% B2 (from 18.5 to 22.4 minutes) to flush the lines and prevent cross contamination and then switch to 100% B3 (from 22.5 to 26.5 minutes) to condition the cartridge followed by 100% B1 up to 30 minutes. Pump A was run at 0.2 ml min^{-1} and pump B was run at 0.7ml min^{-1}. Blank injections with pure methanol were interspaced within the run to make sure that the needle was also kept clean to avoid cross contamination.

Table 2-2: Typical Mass Spectrometry Operating Conditions

Ionisation mode:	Positive ion electrospray
Capillary voltage:	4000V
Nebuliser pressure:	40 psig
Drying gas temperature:	300^{o}C
Drying gas flow	9 L/min
Scan ranges:	Select Ion mode (SIM)

Compound	Mass (m/z)	Retention time – SPE (min)	Retention time – direct injection (min)
Amoxicillin	366	8.4	3.2
Sulfadiazine	251	8.5	2.9
Cephapirin	424	8.7	3.5
Cefquinome	529	9.9	4.8
Lincomycin	407	10.5	5.5
Cefalonium	459	10.8	5.8
Trimethoprim	291	11.3	6.9
Marbofloxacin	363	11.6	8.2
Oxytetracycline	461	12.5	11.6
Sulfamethoxazole	254	13.2	12.6
Enrofloxacin	360	12.9	16.0
Penicillin G	335	13.7	20.9
Ceftiofur	524	16.7	23.4
Tylosin	916	19.8	24.6
Erythromycin – H_2O	716	20.6	25.3
Erythromycin	734	20.6	24.6

2.3 Results and discussion

2.3.1 Optimisation of chromatographic separation

Due to the low selectivity and sensitivity of the mass spectrometer it was important to develop a method that sufficiently separates the analytes of interest. Even though in some cases baseline resolution was not achieved for some of the analytes this did not hinder the identification as compounds with similar masses were well separated (Figure 2-3).

The need to develop a quick and robust method also required a short analysis time. Keeping the total analysis times below 30 minutes while maintain a good chromatographic separation and adequate sensitivity was tricky. The method developed facilitated the chromatographic separation of the following classes of antibiotics: tetracyclines, b-lactams (penicillins, cephalosporins), sulphonamides, lincosamides, macrolides, fluoroquinolones and aminoglycosides. However, during online SPE

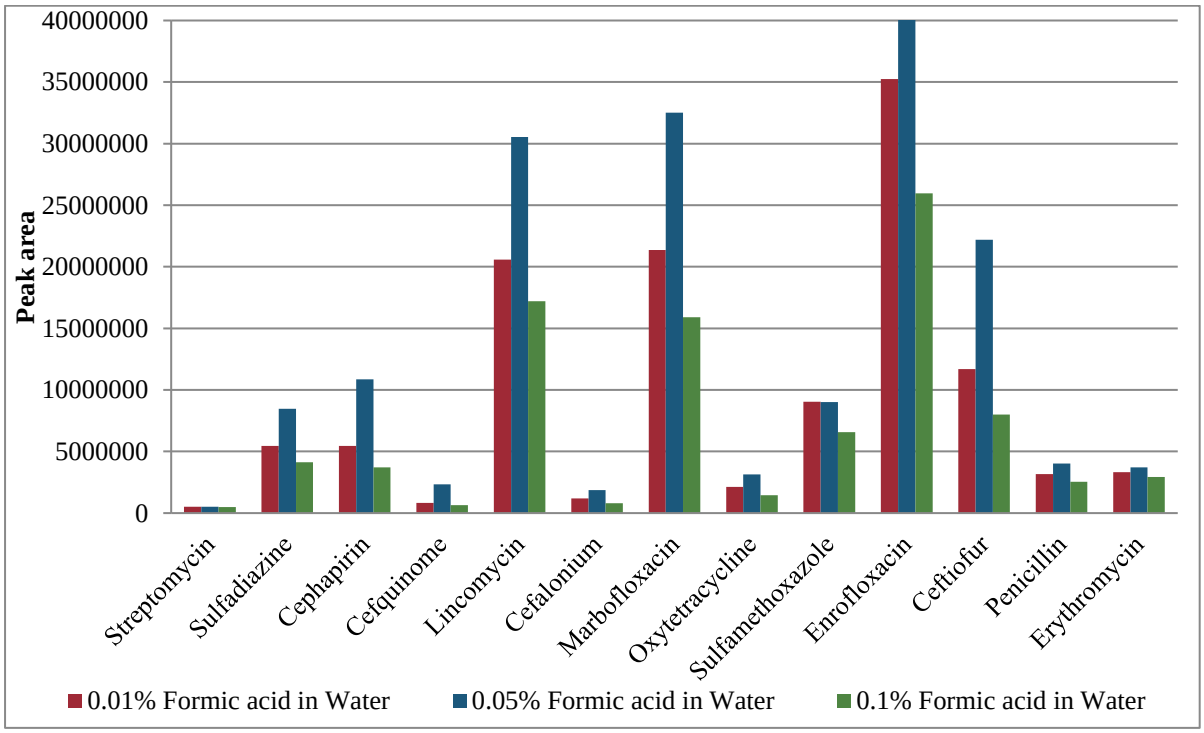
streptomycin was showing poor retention and excluded from the final analytical method. Different classes of antibiotics showcase different physicochemical parameters with pKa showing a significant impact on detection and extraction of antibiotics. Most compounds are ionized to different forms in solution according to their pKa and the pH of the solution according to their functional groups. Hence, the pH of the sample and the mobile phase significantly affects the ionisation and detection efficiency respectively. (Petrović *et al.*, 2005)

The initial testing set up included Water and Acetonitrile as mobile phases. Even though sharpen peak shapes and good chromatographic separation was an interfering peak with sulfamethoxazole that was causing issues with accurately measuring the analyte concentration, so taking this into consideration Methanol was chosen as the organic eluent. Then, the influence of the following parameters was tested:

1. Flow rate (0.2–0.5 mL min^{-1})
2. Column temperature (15–50ºC).
3. Mobile phase pH: acidic with the addition of formic acid (0.01-0.1%)
4. Keeper solution pH with the addition of formic acid (0.01-0.1%)

Higher flow rates were showing high back pressure and weren't particularly successful in achieving a better resolution so 0.2 mL min^{-1} was chosen. Lower column temperatures were resulting in sharper peaks however the column pressure was getting higher the lower the temperature, so 40 ºC were chosen to achieve a balance between optimal separation and operating conditions.

Figure 2-3: Effect of mobile phase acidity on sensitivity



The addition of formic acid is known to improve ionisation in positive electrospray mode.(Snoble *et al.*, 2008) Better sensitivity was achieved by using 0.05% formic acid in Water as mobile phase A

Figure 2-3). Overall better detection sensitivity and better peak shapes (less tailing) for most compounds was observed at pH=3 with the addition of 0.1% formic acid (Figure 2-4).

Penicillin showed lower sensitivity in acidic condition, which is attributed to the rapid decomposition of the b-lactam ring and it has been observed also and in other studies. (Gros *et al.*, 2013; Li *et al.*, 2008) Erythromycin was detected in pure MilliQ water however under acidic conditions was converted to anhydroerythromycin through a loss of a water molecule, in medium acidic pH's this transformation was incomplete and lead to both forms being present. (Paesen *et al.*, 1995)

Figure 2-4: Effect of keeper solution acidity on sensitivity

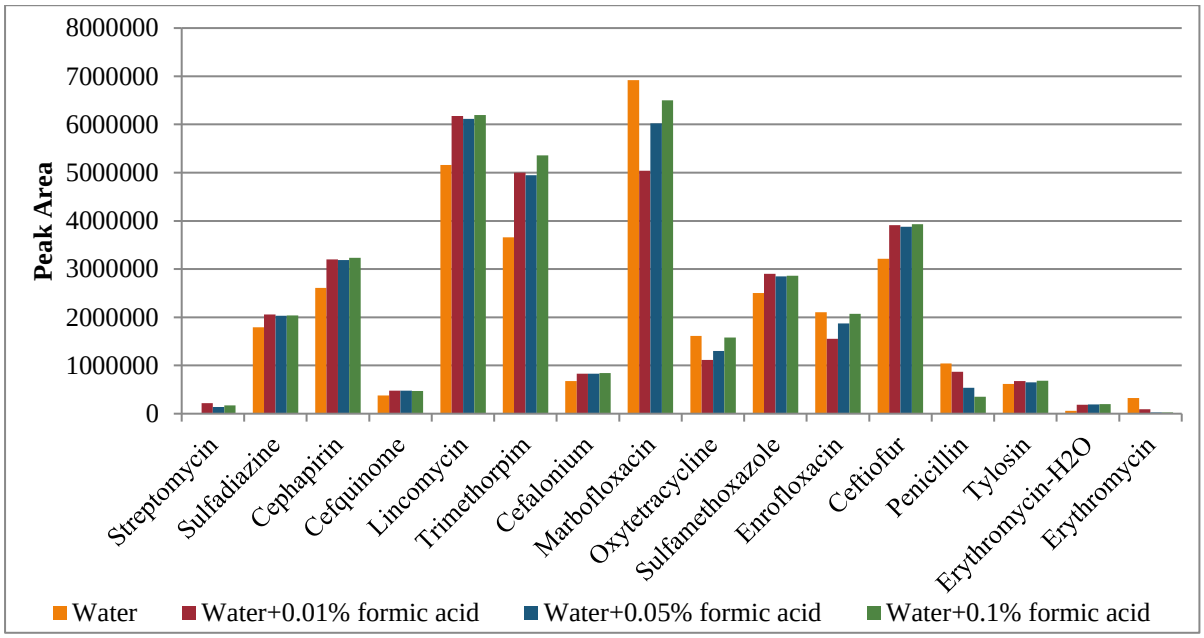
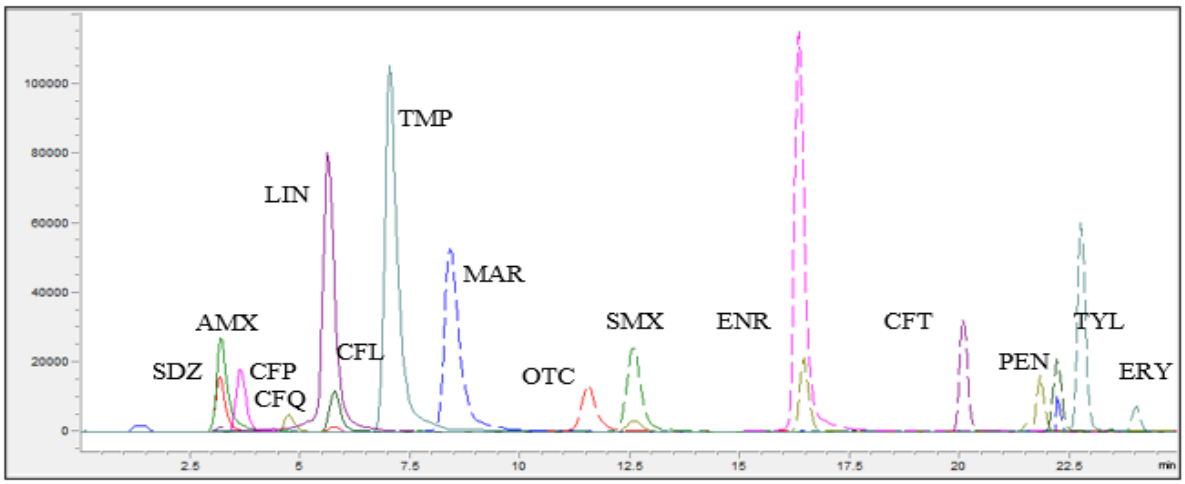


Figure 2-5: Example chromatogram – optimised conditions



2.3.2 Optimisation of online Solid Phase Extraction

Once the optimal chromatographic conditions were achieved and a good peak shape, sensitivity and analyte separation was demonstrated the online SPE extraction step was added. The mobile phase used to load the sample into the cartridge was kept the same as the keeper solution to minimise any changes in the sample pH. Given the diversity of analytes involved, developing a viable method that could simultaneously extract and determine these analytes was challenging due to the varying individual analytical requirements of the compounds.

Streptomycin, an aminoglycoside, is hydrophilic, extremely polar, basic and difficult to be retained in a normal C18 column (Farouk *et al.*, 2015). Poroshell C18 column seemed to be able to retain streptomycin during direct injection with a retention time of 2.1 minutes. Unfortunately, during SPE streptomycin was not detected. It could be due to poor retention on the cartridge as they are normally extracted using cation exchanging cartridges or due being strongly bind to extraction sorbent as it has been reported that strong solvents were required to achieve elution. (Mokh *et al.*, 2015)

Na_2EDTA is a chelating agent that is commonly added to samples to improve extraction of compounds like tetracyclines that have the tendency to bind to metals, cations or other matrix constituents. (Gros *et al.*, 2013) However this method was not preferred for analysis as it can cause undesirable ion source contamination and ion suppression, and the current instrumentation setting is not very robust.

The selection of the online SPE cartridge is critical to the success of the entire analysis. Presently, the availability of different online SPE cartridges is significantly lower than conventional offline cartridges. (Anumol and Snyder, 2015)

2.3.2.1 Sample loading flow rate

The influence of sample loading flow rate and loading duration was investigated over the range 0.4–0.7mL min^{-1} and 2.5-5.5 minutes, respectively, by injecting 0.9 mL of recovery samples at 100ng L^{-1}. Higher flowrates than 0.7mL min^{-1} were not tested because even though the overall recoveries were better with the increased flow rate the peak shape was getting too wide due to column overloading. Lower flow rates accompanied with longer loading times were resulting in increased matrix, especially for oxytetracyline and enrofloxacin, while lower loading times showed poor retention. A 0.7mL min^{-1} flow rate with a 2.5 minutes loading times demonstrated the lower matrix interferences while maintain acceptable recoveries (70-120%).

2.3.2.2 Cartridge conditioning and clean-up

The presence of organic substances in the matrix can lead to interferences that hinder the accurate analysis especially in complex water matrices. Hence a cartridge clean-up step is often required after the sample has been loaded into the cartridge. Both acetonitrile and methanol were evaluated for

their efficiency in removing any carryover. Different washing timings and combinations were tested ranging from 2 to 5 min. Adding a washing step without proper conditioning of the cartridge with aqueous solution (B1) prior to injection were resulting in poor retention of the early eluting compounds. Cartridge clean up only with methanol resulted into poor matrix elimination especially for the last eluting compounds that had also the higher molecular weight. Acetonitrile followed by methanol each for 4 minutes showed best recoveries for most of the target analytes.

2.3.3 Method performance

The linearities and linear ranges of the calibration curves, limit of detection (LODs)/ limits of quantification (LOQs), precisions, matrix effect and relative recoveries were evaluated in order to validate the performance of the developed method.

2.3.3.1 Linearity

At least six standard solutions were prepared over a range of concentrations. The detector response for LC/MS was plotted against standard concentration. The lowest concentration injected was at 50 ng/L and the highest concentration was at 10000 ng/L.

A calibration curve was prepared by plotting peak area versus concentration expressed in ng/L. Using appropriate regression analysis, the equation of the line and the correlation coefficient for each analyte was determined. Example calculation for when using a straight line equation, generate the following equation:

$$y = mx + c$$

Where: x = concentration (ng/mL)
y = peak area
m = slope
c = intercept (c = 0 when the curve is forced through zero)

The response of the detector was linear using regression analysis (>0.995) over the range 50 to 10000 ng/L. Varying sensitivity was observed due to the number of compounds, so the lowest concentration had to be adjusted accordingly. Linearity plots and peak areas are shown in Appendix 3 Appendix 3 Appendix 3 Appendix 3 Appendix 3 Appendix 3 .

2.3.3.2 Specificity

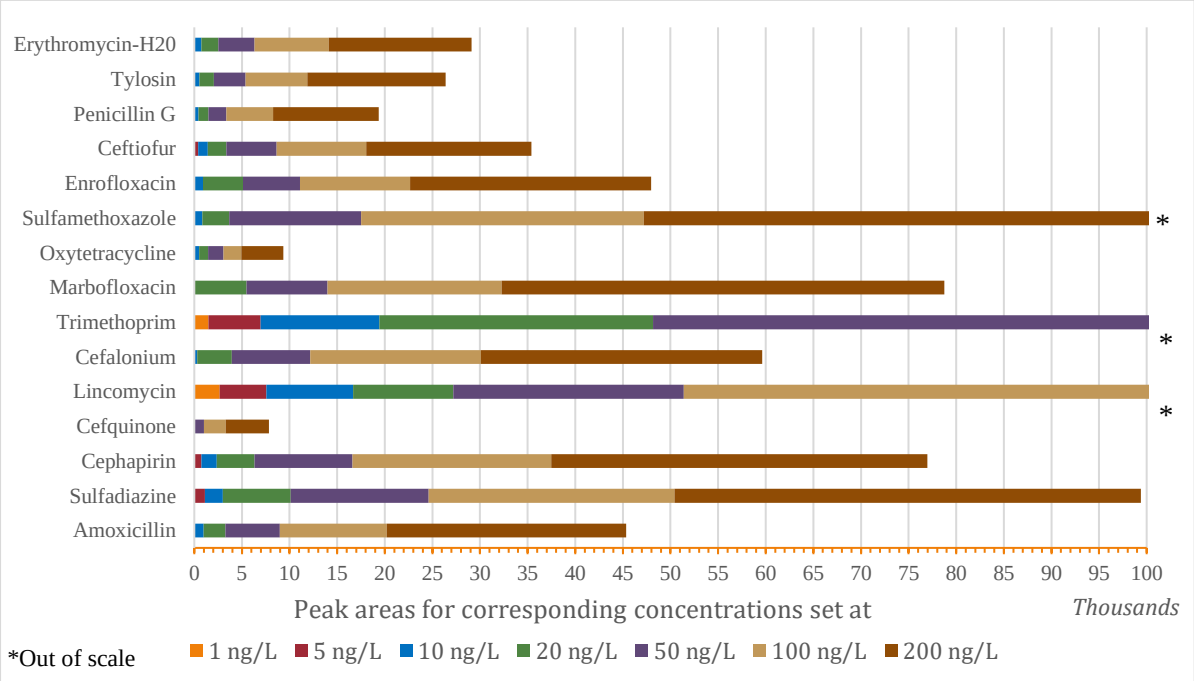
Due to the instrument low specificity great effort and time was invested into creating a gradient that can separate several compounds with varying chemical properties. There were no components present in the control surface that interfered with the analysis. During the analysis of the environmental samples for any antibiotic that showed a positive response the standard addition method was utilised to increase specificity and verify results. Example chromatogram can be found in **Figure A4-3**.

2.3.3.3 Limit of detection and quantification

The instrument limit of detection (LOD) was based on an analyte signal to noise ratio (SNR) greater than three and for the limit of quantification greater than 10. A set of standards at 1, 5, 10, 20, 30, 40 and 50 ng L^{-1} were analysed to determine the LOD by online SPE and 50, 75, 100, 150, 200, 250, 300 ng L^{-1} for the direct injection. **Figure 2-6** demonstrates the range of limit of detection and quantification per analyte. It shows the corresponding peak area to the injected concentration. Lincomycin and trimethoprim appeared to be the most sensitive compounds with limits of detection down to 1 ng L^{-1}. LOD ranges from 1 to 50 ng $L^{-1}$for online SPE. See Appendix 4 for example chromatography.

These values are comparable with other studies that used more sensitive instrumentation but similar settings (Panditi *et al.*, 2013; Axel *et al.*, 2017a) The limit of detection for Amoxicillin and Erythromycin (10 ng L^{-1}) is even lower than the minimum acceptable LOD (68 and 19 ng L^{-1} , respectively) as set by Directive 2008/105/EC for continuous monitoring of substances included in the watch list. (European Commission, 2018b). Due to varying levels of sensitivity a universal appointed LOQ level of 100 ng L^{-1} was set for all compounds for recovery testing.

Figure 2-6: Ranges of LOD and LOQ after online SPE (in acidified water)



2.3.3.4 Matrix effect assessment

Matrix effects were evaluated using the following equation:

$$Matrix\ effect\ \% = \left[\frac{Peak\ area\ of\ analyte\ in\ matrix}{Peak\ area\ of\ analyte\ in\ solvent} - 1\right] x\ 100$$

Matrix effect is commonly occurred during LC/MS analysis, positive values indicate signal enhancement and negative values represent signal suppression due to the sample matrix. (Gros *et al.*, 2012) Matrix effects were investigated at LOQ level. Surface water (pH= 8.1) from natural water body was used as matrix control. The matrix was tested against batch standards (prepared in acidified water at pH=3). Lowering the pH of the surface water to 3 seemed to also lower the matrix effect for most of the compounds expect for oxytetracycline. This is expected as oxytetracycline has three ionization equilibriums and the four protonation states so pH changes affect significantly the response.(Jiao *et al.*, 2008b) For this reason, matrix matched standards were used for further quantification.

Table 2-3: Matrix effect in acidified and non acidified surface water

	Surface water (pH 8.1)	**Surface water (pH 5)**	**Surface water (pH 3)**
Sulfadiazine	-33.4	-9.8	-0.3
Amoxicillin	-126.5	39.8	0.1
Cephapirin	-14.1	-0.3	0.2
Cefquinone	-70.8	54.6	0.3
Lincomycin	4.8	-76.9	-0.8
Cefalonium	-6.5	-47.8	-0.5
Trimethoprim	-38.9	52.2	0.7
Marbofloxacin	52.3	16.6	-0.3
Oxytetracycline	-84.1	-1.2	-21.4
Sulfamethoxazole	-2.0	-57.5	0.0
Enrofloxacin	63.1	-36.1	-0.5
Ceftiofur	-93.8	29.5	0.6
Penicillin G	-39.9	14.0	-0.8
Tylosin	-8.7	6.9	-2.9
Erythromycin-H_20	0.0 *	24.8	0.1

*Erythromycin (m/z 734) was only detected

2.3.3.5 Matrix spiked recoveries

The extraction of antibiotics from river water samples was evaluated by performing recovery experiments on samples taken from a reference site (Lake Carter), spiked at LOQ and 10xLOQ. Good overall recoveries were achieved regardless of the fortification level (range 73.1–115.2%) Precision was defined as the relative standard deviation (RSD) of quintuple analysis of river water samples (adjusted at pH 3). Higher concentrations showed better reproducibility, however all analytes showed acceptable %RSD <20% in both fortified levels (Table 2-4). Depending on the analyte and the sample matrix, such recoveries are similar to those achieved by others, using online SPE with similar sorbents.(Axel *et al.*, 2017a)**.** Representative chromatograms at LOQ level after SPE analysis are presented in Appendix 4Appendix 4Appendix 3 Appendix 3 **.**

Table 2-4 : Recovery data

Analyte	**LOQ (100 ng L^{-1})**		**10xLOQ (1000 ng L^{-1})**		**LOD (ng L^{-1})**	**LOD (ng L^{-1})**
	Mean Recovery (%) (n=5)	**%RSD**	**Mean Recovery (%) (n=5)**	**%RSD**	**Online SPE**	**Direct injection**
Sulfadiazine	73.1	12.1	107.9	1.8	5	50
Amoxicillin	75.3	12.1	79.3	6.3	10	50
Cephapirin	113.5	4.6	107.2	1.0	5	50
Cefquinone	79.2	9.9	96.5	4.9	50	100
Lincomycin	99.3	4.5	99.6	0.9	1	25
Cefalonium	103.1	4.5	107.1	5.4	10	250
Trimethoprim	96.3	6.0	108.4	0.7	1	25
Marbofloxacin	99.3	16.6	88.5	4.4	20	50
Oxytetracycline	105.1	14.8	115.2	7.3	20	250
Sulfamethoxazole	92.6	6.1	112.2	1.3	10	50
Enrofloxacin	108.3	13.7	88.8	9.1	10	50
Penicillin G	102.1	13.0	97.2	4.3	5	50
Ceftiofur	110.6	2.0	82.5	5.3	10	250
Tylosin	90.1	9.5	107.0	6.1	10	250
Erythromycin-H_20	74.6	17.7	109.7	11.6	10	100

2.3.4 Environmental results

The developed method was applied to the determination of the concentrations of target antibiotics in environmental water samples located in a catchment area. Out of the 15 analytes included in the method, 12 of them were detected in at least one sample with ceftiofur, cefalonium and cefquinome being the ones that were not detected. This might be due to the fact that generally cephalosporins which are part of the b-lactams group show low detection in environmental matrices because of the instability of the beta lactam ring.(Junza *et al.*, 2014) Higher observed concentrations were associated with sampling location that received secondary treated wastewater effluent and adjoined two grazing fields. Concentrations reached 1659 ng/L for sulfamethoxazole and 931 ng/L for oxytetracycline. However, the presence of marbofloxacin, an antibiotic that is only licensed for veterinary use, indicated that the release of veterinary antibiotics to receiving water through animal excreta is another possible route.

3

OCCURRENCE OF VETERINARY ANTIBIOTICS IN FARMYARD RUN OFF AND STREAM WATER IN LIVESTOCK FARMING AREA

3.1 Introduction

Antibiotics are some of the most frequently prescribed drugs used in modern medicine and today's doctors are armed with a whole suite of them for the purposes of treating bacterial infections in both humans and animals. In 2019, a total of 706.3tonnes of antibiotic active ingredients was dispensed in the United Kingdom for human and veterinary use. Out of the 706.3 tonnes, 68% was for use in people and 32% for use in food-producing animals and companion animals combined. Of the 68% prescribed for human use, approximately 52% was used in the community and 16% in hospitals. (Veterinary Medicines Directorate, 2023a) Of the 32% sold for use in animals, 77% was for use in food-producing animals only and 6% for use in horses and companion animals and 17% for combined use in food and non-food producing animals. (Veterinary Medicines Directorate, 2020)Comparing the sales of antibiotics intended for animal use to 2014, which the first year the UK- VARSS report was published, there is a 59% drop observed. (Veterinary Medicines Directorate, 2023b)

Predominant farm types in the North West region are Grazing Livestock which accounted for 61% of farmed area and Dairy which covered a further 19% of farmed area with a total number of livestock animals close to 22 million. (Defra, 2018).

It is still unclear which are the predominant factors contributing to the occurrence of antibiotics in surface waters in agricultural watersheds. While land use is one important aspect, parameters such as soil type, slope and seasonality strongly affect the occurrence of veterinary pharmaceuticals in run-off and surface water (Jaimes-Correa *et al.*, 2015) An additional possible source that influence the presence of antibiotics in surface waters apart from the release of antibiotics through manure application or directly excreted by the animal, are the "dirty water" deriving from the washing of the stalls that generally contain <3% dry matter, and are made up of water contaminated by manure, urine, silage run off, milk, other animal products and cleaning materials. (Aga *et al.*, 2016) Normally this water is

removed by and disposed directly to grassland. Unlike slurry that only can be applied to grassland on specified occasions dirty water disposal can be performed all year round. (Minogue *et al.*, 2016)

Whilst many sampling studies have been performed primarily in Asia (Wei *et al.*, 2011; Ostermann *et al.*, 2014; Wang *et al.*, 2018; Liu *et al.*, 2021a) and America (Jaimes-Correa *et al.*, 2015; Washington *et al.*, 2018), that link directly the farming practises directly to the occurrence of antibiotics this is not so widely investigated in European surface water systems. To investigate the potential relationship between antibiotic presence in the environment and their possible sources, monitoring strategies are needed to be put in place with sensitive enough analytical methods to detect these low concentrations. (Liguori *et al.*, 2022) Strategic monitoring is needed in order to establish the baseline data on antibiotics, residues, and correlation to antibiotic resistance genes (ARGs). (Pruden *et al.*, 2013).

There are a lot of methods reported for the analysis of antibiotics in different matrices, however the majority of these methods are unsuitable for monitoring studies as they employ sample enrichment techniques that require high sample volumes and are time consuming. (Kim *et al.*, 2018). Because of these reasons, great effort is going into the development of fast, cost-effective and environmental friendly alternative methods for environmental analysis that require less solvents, materials and minimise the sample transport. (López-Serna *et al.*, 2010) There is an increase in fast, sensitive, and cost-efficient online SPE LC-MS/MS methods for the analysis of a number of pharmaceuticals and personal care products (PPCPs). (Goh *et al.*, 2016; Axel *et al.*, 2017b; Tang *et al.*, 2022; Hilawie Belay *et al.*, 2022) During online/automated analysis the whole sample is analysed and typically using higher injection volume which lead to lower detection limits. In addition, on-line solid phase extraction (SPE) require less solvent than the conventional extraction regimes which also lead to decreasing running costs and waste disposal. (Pozo *et al.*, 2006)

The selection of the targeted antibiotics has been influenced by discussions with the farmers and literature review of the most persistent and detected antibiotics in rural areas.

Amoxicillin is a β-lactam antibiotic drug belonging to penicillins and it is considered as an essential medicine by the World Health Organisation due to its pharmacological properties capable to treat pneumonia, pharyngitis, sepsis, sinusitis or bacterial meningitis. It has become a major antimicrobial substance primarily in pig medicine and also in chickens, turkeys and dugs. (Burch and Sperling, 2018). In the UK it was detected in concentrations up to 245 ng/L. (Kasprzyk-Hordern *et al.*, 2007)

Cefalonium and cephapirin are first generation cephalosporins they are used for providing treatment to bovine mastitis caused by Gram-positive bacteria including staphylococci (Harada *et al.*, 2020) or intrauterine treatment preceding a timed artificial insemination protocol in lactating dairy cows with purulent vaginal discharges (PVDs). (Tison *et al.*, 2017) Cephapirin has been detected in surface water at a concentration of 9 ng/L.(Cha *et al.*, 2006)

Ceftiofur is a third-generation cephalosporin antibiotic, which is administered to cattle and swine for. control of bacterial infections of the respiratory tract. (Wagner *et al.*, 2011) Cefquinome is another fourth-generation cephalosporin that is licensed in the United Kingdom for use in foals with septicemia and horses with respiratory tract disease.(Lee *et al.*, 2020) No studies were found detecting cefalonium, ceftiofur and cefquinome in the aquatic environment and it could be attributed to possible hydrolytic cleavage of the lactam ring (Naderi Beni *et al.*, 2020)

Enrofloxacin and marbofloxacin are third-generation fluoroquinolone widely used in veterinary medicines as an antimicrobial drug. Enrofloxacin is used in the livestock and poultry industries to combat a variety of infectious diseases caused by *E. coli*. (Liu *et al.*, 2021b) Marbofloxacin is an injectable third-generation fluoroquinolone used in adult cattle for bovine respiratory diseases (BRD) and Escherichia coli acute mastitis. (Kroemer *et al.*, 2012) In water samples, the concentrations detected for enrofloxacin and marbofloxacin in surface water varied from 0.2 to 164.5 ng/L and 3.6 to 20.1 ng/L respectively. (Hanna *et al.*, 2018; Iglesias *et al.*, 2014b)

Erythromycin is a macrolide antibiotic and is primarily used to treat mastitis in lactating cows and as against infectious diseases from gram-positive bacteria in cattle, sheep, swine, and poultry. (Anadón and Reeve-Johnson, 1999) Tylosin is another macrolide antibiotics and is commonly used to reduce the incidence of liver abscesses in cattle. (Cazer *et al.*, 2020) Erythromycin and tylosin have been detected in surface water samples with maximum concentrations reaching 1600 and 61 ng/L, respectively. (Yao *et al.*, 2017b) In the UK erythromycin was detected at 32 to 790 ng/L. (White *et al.*, 2019)

Lincomycin is a broad-spectrum lincosamide antibiotic, particularly active against Gram-positive bacteria and mycoplasmas. It is frequently used for the control of respiratory infections in pigs, poultry and sheep.(Skoufos *et al.*, 2006) In the UK lincomycin was detected at 2.1 to 7.1 ng/L in surface waters. (White *et al.*, 2019)

Oxytetracycline is a broad-spectrum tetracycline antibiotic active against a range of both Gram-positive and Gram-negative micro-organisms that cause gastrointestinal and respiratory system diseases in sheep, goat, pigs and cattle. (Laven, 2012) Oxytetracycline was observed in stream waters in the UK in concentrations up to 4.49 µg/L.(Boxall *et al.*, 2006)

Penicillin G is used as an injectable narrow spectrum β-Lactams antibiotic for the treatment of cattle and sheep for bacterial pneumonia; swine for erysipela; and horses for strangles. (Li *et al.*, 2014)

Sulfadiazine is used primarily to treat urinary tract infections and as an adjunct for the treatment of a few parasitic diseases in pigs, chickens and sheeps (Baert *et al.*, 2003; Batzias *et al.*, 2005; Lamshöft *et al.*, 2007) Maximum levels of sulfadiazine observed in UK streams reached 4.13 µg/L. (Boxall *et al.*, 2006) Trimethoprim and sulfamethoxasole are commonly, but not always, used in combination because of their claimed synergistic effects; and used for the treatment of respiratory tract infections, urogenital, gastrointestinal and skin infections in pigs and poultry (broilers).(Petritz *et al.*, 2023) Trimethoprim,

and sulfamethoxazole have also been included in the the European Surface Water Watch List. (European commision Decision 2022/1307). In the UK sulfamethoxazole and trimethoprim has been detected at a maximum level of 0.035 and 0.35 µg/L respectively. (White *et al.*, 2019)

The aim of this study was to use a quick and robust analytical method to verify residues of veterinary antibiotics in areas with intensive farming activities and evaluate the effectiveness of mitigation features that serve to contain farm runoff from entering water bodies. This analytical method describes the procedure for the determination of amoxicillin, cefalonium, cephapirin, cefquinome, ceftiofur, enrofloxacin, erythromycin, lincomycin, marbofloxacin, oxytetracycline, penicillin G, sulfadiazine, sulfamethoxazole, trimethoprim and tylosin in surface water.

3.2 Experimental

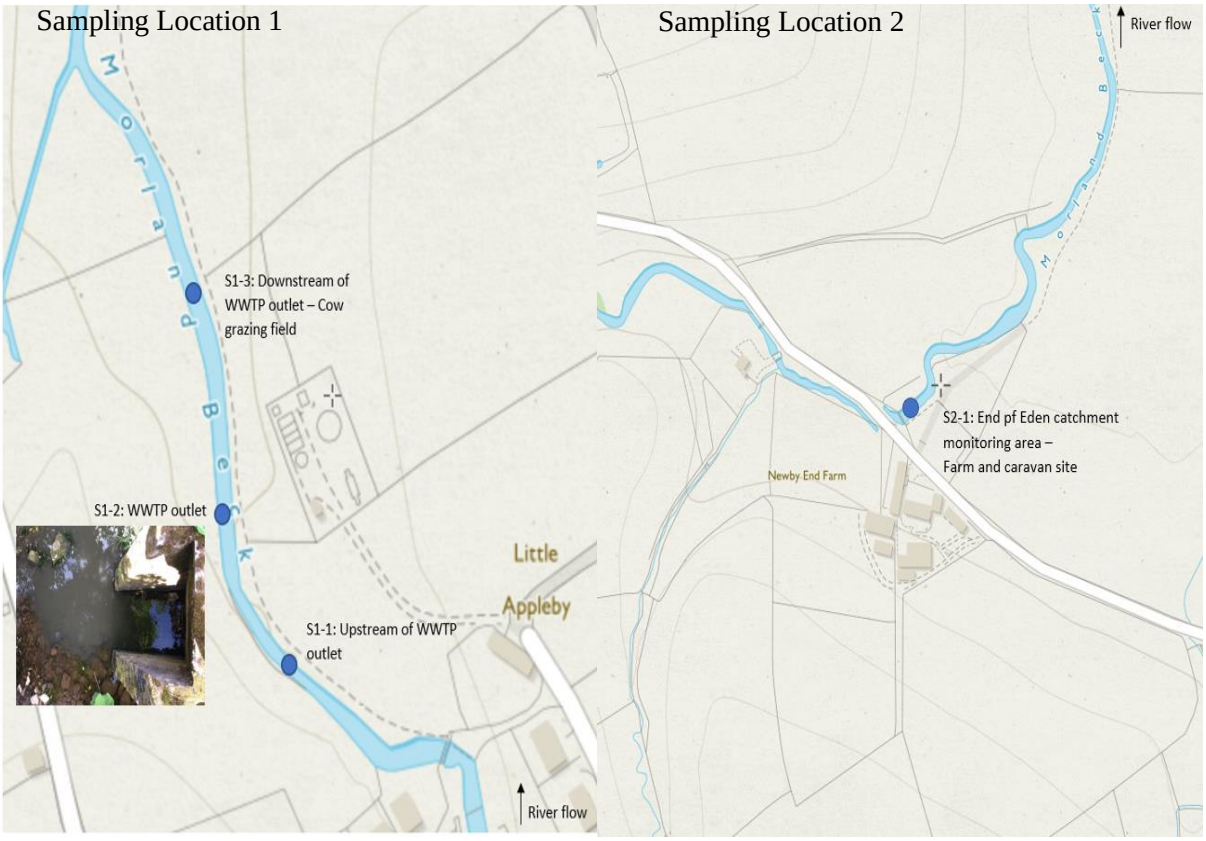
3.2.1 Sample locations and sampling information

Samples collected for residue screening originated from water bodies (river, streams, settling ponds and run off) within the Morland catchment, which form parts of the Defra (Department for the Environment and Rural Affairs)-funded Demonstration Test Catchments (DTC) programme. This is a catchment-scale research platform testing measures for addressing the effects of diffuse pollution from agriculture on stream ecosystems (Owen *et al.*, 2012). The Morland catchment contains a large proportion of improved grassland (83%), with just 10 per cent rough grazing and 3 per cent arable land. The predominant farming types encompass a mixture of dairy and meat production. Within the catchment, mitigation features were incorporated to enable waste management plans to diffuse water pollution from agriculture. Mitigation features included the collection and storage of dirty water in runoff attenuation features (RAFs) such as bunds, drain barriers, runoff storage features, buffer strip and willow barriers. (Wilkinson *et al.*, 2014) Samples were also collected from areas that receive effluents from a wastewater treatment plant. The WWTP offers secondary filtration with chemical phosphorus removal. Exact locations and sample information can be found below in **Table 3-1** and **Figure 3-1** and in Appendix 5.

Table 3-1 Description of the Morland catchment sampling sites.

Sampling point	Sampling Area	Site/Sample Description
S1-1	Wastewater treatment plant (WWTP)	Upstream of WWTP
S1-2		WWTP outlet
S1-3		Downstream of WWTP outlet – Cow grazing field
S2-1	End of catchment monitoring area	Farm and caravan site
S3-1	Dairy farm	Two streams combined passing through a dairy and sheep farm
S3-2 / S3-3		Strem passing through a cow grazing field
S3-4		Settling pond collecting farm yard run off
S3-5 /S3-6		Emergency overflow stream collecting run-off from manure and slurry storage tank. Samples collected before and after mitigation feature
S4-1/S4-5	Pig and sheep farm	Emergency overflow stream collecting run-off from farm yard – Contains a mitigation feature
S4-6 / S4-7		Settling pond collecting farm yard run off. Samples collected before and after mitigation feature

Figure 3-1: Sampling locations within Morland focus catchment along the flow of the river



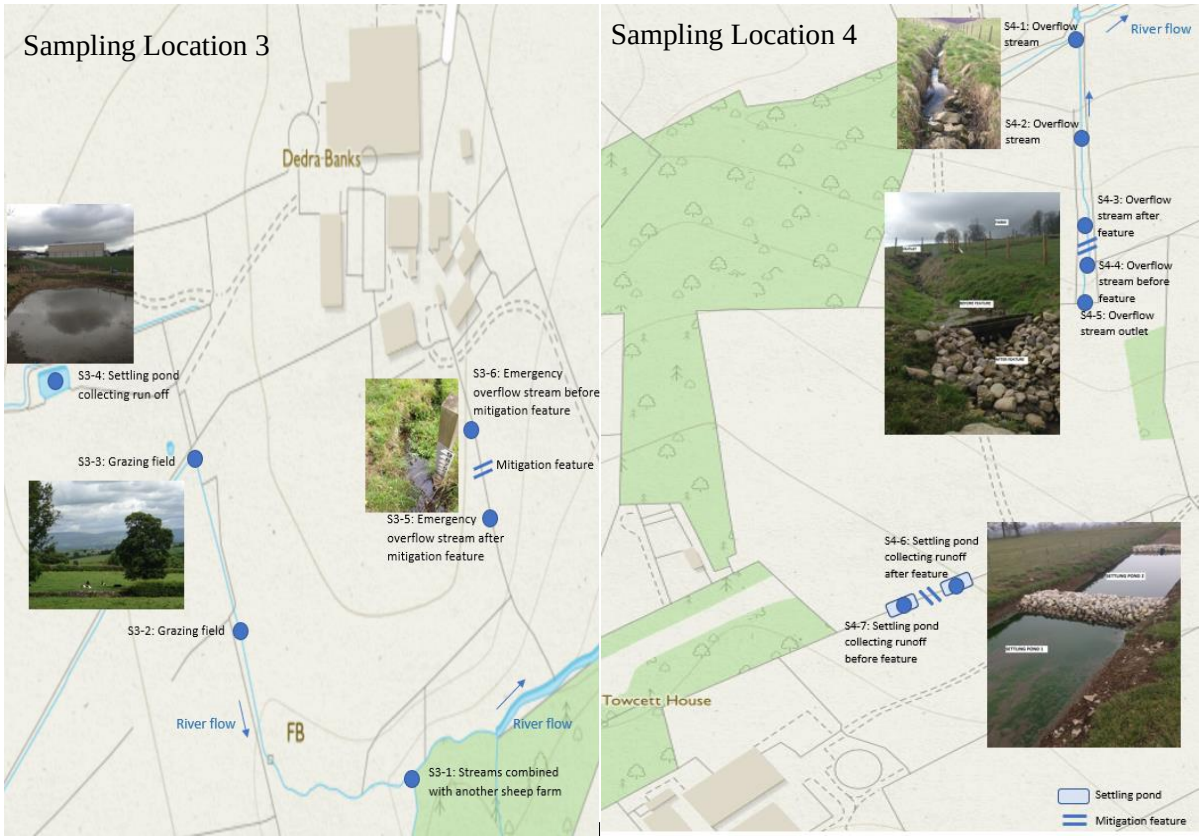


Figure 3-2: Clear trail of farm run off leading to mitigation feature

3.2.1.1 March sampling and storm event

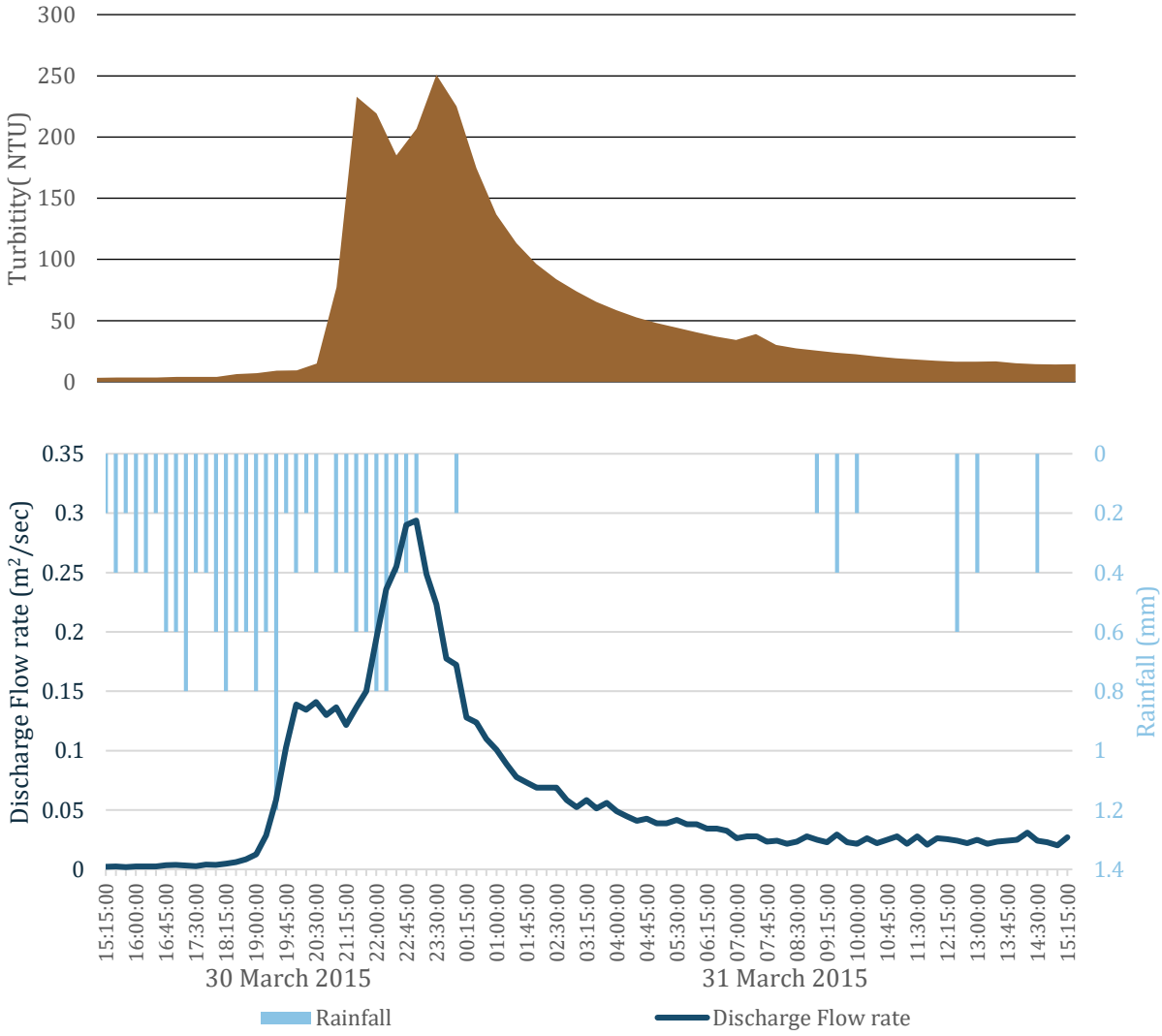
The weather during March sampling was wet and it was raining for the past two weeks prior to sampling. In sampling location 4 there was an evident run off path from the farm leading to mitigation feature. (**Figure 3-2**). Samples were collected from all locations except sampling location 1.

A period of extended heavy rainfall occurred during March which triggered a storm event. Samples originated from two ISCO 3700 C Portable Automatic Water Samplers located at a farmyard overflow outlet. The ISCO samplers were bracketing the mitigation feature with one positioned

upstream and one downstream of the mitigation feature. (**Figure 3-3**) Six samples deriving from S3-3 and S3-4 sampling locations were gathered over a period of increased water run-off. Actual timing of the samples taken cannot be allocated as the autosamplers were triggered once a predefined water-level threshold has been crossed (normally 0.45m). Rainfall, water flow and turbidity were monitored during the event through automated weather stations located in the area. (**Figure 3-4**)

Figure 3-3: Farmyard overflow outlet sampling specifications

Figure 3-4: Parameters monitored during storm event



3.2.1.2 April sampling

During April sampling the weather was dry with intensive sunshine for the past 7 days leading to the sampling and only a 15 minutes rain event happened the day before sampling with total rainfall observed at 17.8mm. Samples were collected from most sampling locations, however as the level of some of the streams were dependent on the receiving water from emergency overflow outlets they appeared to be dry.

3.2.1.3 May sampling

During May there was light rain during sampling and some rainfall has occurred over the 7 days prior to sampling. Samples were collected from all the sampling locations. To the sampling survey an additional sampling location (Sampling location 1) was added that was receiving effluent from a small wastewater treatment works which employed secondary treatment processes.

3.2.1.4 June sampling

June was a very dry month with high temperatures. Artificial streams around the farms(sampling locations 3 and 4) and mitigation features were dry and overtaken by growing vegetation as seen in **Figure 3-5** , so sampling occurred at the sampling points with natural water sources.

Figure 3-5: Streams and mitigation features during June sampling in Sampling locations 3 and 4

3.2.2 Sample Collection and handling

For sampling events occurred during March, April, May and June 2015, grab samples were collected in duplicate in amber glass bottles and stored in portable cooler until arriving at the laboratory later on the same day. The samples that were collected using the ISCO autosamplers during the March storm event were transferred to the laboratory within 24 hours and 10 mL subsamples were removed from each of the six polypropylene autosampler bottles and stored frozen until analysis.

The pH of each of the samples was measured and found to be between 6.79-8.65, prior to analysis the sample pH was adjusted to pH~3 with the addition of formic acid and samples that showed evident suspended solids were ultra-centrifuged at 24000 rpm or filtered through a PES syringe filter, if centrifugation did not produce a clear solution, prior to analysis. Water samples were fortified with the internal standard solution mix and analysed as detailed in Section 2.2.2 using the conditions outlined. Analysis was initiated on the day of sampling. The rest of the collected samples were stored frozen in case repeat analysis was required.

In samples that showed a positive response for any antibiotic an aliquot of a standard mixture of all analytes was added at a final concentration of 100 ng/L and the sample was re-analysed. This procedure was done to verify results due to the low specificity of the mass spectrometer that was used.

Surface water used for method validation and as a control was collected from a natural water body located in Lancaster University that is not influenced by any anthropogenic factors. The water was collected by large bucket and stored in glass 5L bottles in the fridge (4-8^{o}C) for up to a month to ensure viability of the test system. Prior to use in the study the water was screened to ensure there is no residue of the analytes of intertest and deemed suitable for use to prepare matrix matched standards and instrument recovery samples in order to check the method's repeatability and reproducibility.

3.2.3 Reference items for calibrations and fortification

Amoxicillin (AMX), Cephapirin (CFP), Cefalonium (CFL), Ceftiofur (CFT), Cefquinome (CFQ), Enrofloxacin (ENR), Erythromycin (ERY), Lincomycin (LIN), Marbofloxacin (MAR), Oxytetracycline (OTC), Penicillin (PEN), Sulfadiazine (SDZ), Sulfamethoxazole (SMX), Trimethoprim (TMP) and Tylosin (TYL) were purchased from Sigma Aldrich (UK) and were of VETRANAL grade (purity >99%). As internal standards Caffeine-$^{13}C_3$ (Sigma Alrich, UK) and sulfamethoxazole-d_4 (QMX laboratories, UK) were used. See Appendix 1 for exact structure and physicochemical parameters of each compound.

Methanol and formic acid were of LC/MS (Optima) grade (Fisher, UK) and water was purified on a Milli-RO plus 30® and a Milli-Q purifiers (Millipore, USA). Individual stock solutions were prepared at a concentration of 500 µg/mL in methanol apart from amoxicillin, cefquinome, penicillin and cephapirin where the stock solutions were prepared in ultra pure water and kept in the dark at >-20^{o}C for up to a month.

A 10 µg/mL mixed fortification solution was prepared by dilution of 200 µL of each stock standard solution in 10 mL of methanol. Subsequent dilutions were performed accordingly to achieve 1, 0.1 and 0.01 µg/mL accordingly. Solutions used for calibration were prepared fresh on the day by taking appropriate aliquots from the fortification solutions and diluting them to a final volume of 1 mL with 0.1% formic acid in water. Calibration solutions were not stored. Recovery samples (QC samples) were prepared in surface water and acidified surface water (pH 3) to check the matrix effect and method efficiency.

3.2.4 HPLC/MS analysis

Samples were analysed using an Agilent 1100 binary pump (Chromatography pump-A) equipped with a vacuum degasser, a thermostated column oven set to 40 °C with a 6-port two position switching valve, and an Agilent Poroshell EC C18 column (2.1 × 100 mm, 2.7µm) attached to an UHPLC Poroshell C18 fast guard (2.1 x 5, 2.7µm) . For loading and enrichment, an Agilent 1200 series

quaternary pump with build in degasser (SPE pump-B) coupled to an Agilent 1110 autosampler with a 1400-µl injection loop was used. A reusable Agilent online trapping column with rigid macroporous styrene/divinylbenzene (PLRP-S) phase and dimensions of 2.1 × 12.5 mm (15–20µm) was used for samples clean-up.

The two systems were connected through a two position nine-port valve and a six-port switching valve located in the column compartment that was used for the sample clean up and were able to switch automatically from loading to injection. The mobile phases for the chromatographic pump were 0.05% formic acid in water (A1) and methanol (A2). For the SPE pump 0.1% formic acid in water (B1) was used to deliver the sample to the trapping column with acetonitrile (B2) and methanol (B3) used to clean and condition the cartridge respectively. A multi-draw method was used for the injection of 0.9 mL of sample, which was then loaded onto the trapping column using 100% eluent B1 for 2.5 minutes from pump B. The six-port valve was then switched and pump A was running in a three step gradient starting with 20% A2 and going up to 80% A2 over 17.5 min and held there for 2 minutes before returning to initial conditions.

During the run, the eluent composition in pump B was also set at 100% B2 (from 18.5 to 22.4 minutes) to flush the lines and prevent cross contamination and then switch to 100% B3 (from 22.5 to 26.5 minutes) to condition the cartridge followed by 100% B1 up to 30 minutes. Pump A was run at 0.2 ml min^{-1} and pump B was run at 0.7ml min^{-1}. Blank injections with pure methanol were interspaced within the run to make sure that the needle was also kept clean to avoid cross contamination.

The analysis was completed using an Agilent MSD 6100 single quadrupole mass spectrometer with an Electrospray ionization (ESI) source and Select Ion mode (SIM) mode. Nitrogen (99.9999%) was used as dry gas, sheath gas, nebulizer gas, and collision gas. The fragmentor voltage and collision energy were optimized for every compound individually. An accelerator voltage of 3 V was used for all compounds. Other mass spectrometric conditions were optimized manually for the entire method. Drying gas was held at 9 L min^{-1} and heated to 300 °C. The nebulizer pressure was set to 40 psig. A capillary voltage of 4000 V. All of the compounds were run in positive ionization mode (ESI+). The total sample analysis time was 26.5 minutes with a column equilibration time of 8 minutes.

Final determination by online SPE-LC-MS with a single transition is not considered to be highly specific and so in cases where uncertainty was high or when residues were measured the method of standard addition was used to verify result For this purpose an aliquot of a standard mixture containing all the analytes was added in the HPLC vial to achieve a final concentration of 100 ng/L and the sample was reanalysed. The final concentration was calculated as indicated below

$$\text{Concentration of analyte in unknown} = \frac{\text{Peak area of unknown}}{\text{Peak area of mixture}} \text{ x Concentration of analyte + standard in mixture}$$

3.3 Results and discussion

3.3.1 Environmental results

The developed method was applied to the determination of the concentrations of target antibiotics in environmental water samples located in a catchment area, the results of which are listed in Appendix 2 (**Table A5-1:** . Results that are below the universally set 100 ng L^{-1} LOQ limit but above the individual LOD are reported in brackets to show the uncertainty of the method as concentrations below 100 ng L^{-1} were not properly validated. A number of compounds did not show adequate sensitivity to allow for a lower universal LOQ to be established. Out of the 15 analytes included in the method, 12 of them were detected in at least one sample with ceftiofur, cefalonium and cefquinome being the ones that were not detected, even though these three antibiotics were used in the farms. This might be due to the fact that generally cephalosporins which are part of the b-lactams group show low detection in environmental matrices because of the instability of the beta lactam ring.(Junza *et al.*, 2014)

Rainfall-triggered runoff is a major driver of pesticide input in streams (Fardin Sadegh-Zadeh *et al.*, 2017) in this case the occurrence of veterinary antibiotics was monitored. The most frequently detected antibiotics during the storm event were oxytetracycline and marbofloxacin. (**Figure A5-2: Figure A5-3**) It appeared that residues before the mitigation feature were higher and that is because it was the first point of entry for the farmyard overflow. However, there was a breakthrough point halfway through the event were concentrations for both analytes appeared higher after the mitigation feature. The exact timings of the samples are not known but **Figure 3-4** shows a peak in the flow rate of the stream that might coincide with the breakthrough point, or it could be attributed to the increase in turbidity that carried absorbed analytes over the feature and subsequently collected in samples. Samples prior to analysis where centrifuged to remove suspended solids, this action might also have helped some of the adsorbed analytes to be released into the aqueous phase. This was a scenario that was also noticed by (Kim *et al.*, 2016) that samples collected during rainy season demonstrated higher concentrations for tetracyclines and fluoroquinolones

The high detection of these compounds contradicts a soil sorption study that indicated that oxytetracycline, a major member of tetracyclines, is strongly adsorbed in soil regardless of soil type and thus only weakly mobile. (Li *et al.*, 2010) However, in an overland flow study, oxytetracycline concentration in runoff from a manure-applied field was detected to be 71.1 μg/L. (Kay *et al.*, 2005) illustrated that overland flow is a possible route for oxytetracycline to be transported to surface water. This has shown that compounds with high sorption coefficients are not necessarily immobile. To what degree we can use the Koc values of a metabolite to address its soil mobility is quite unclear. This assumptions is further validated by (McCall P.J *et al.*, 1981) and (FAO) which are both used as references to classify soil mobility but both assume that a compound is considered immobile with a Koc>5000 or >100,000 respectively. (Solliec *et al.*, 2016) measured high concentrations of tetracyclines along with some of their major metabolites in drainage water around swine farms further establishing

that were also detected in drainage. Compounds with high solubility values are more prone to leach into groundwater.

Event-triggered or flow proportional samples give a better understanding on exposure levels than grab water sampling at individual time points which are only capturing a snapshot of environmental concentrations. However automated sampling like this might underestimate the exposure as it requires immediate transfer of the samples to be analysed to avoid compound degradation.(Fernández *et al.*, 2014) The detection patterns of environmental concentration of veterinary pharmaceuticals varied according to discharge sources, hydrological factor (flowrate), and particularly meteorological element (rainfall). Wetter months March and May showed an increase in antibiotics detection where drier months show no to limited detections. This can be also linked with the fact that the points of sampling along the farms were associated with measures to access diffuse pollution that derived from farm run offs during the wet season. Higher concentrations were observed in sampling point 4 which was associated to a large pig farm and was down to the higher use of antibiotics/head. (Jaffrézic *et al.*, 2017) also demonstrated that detection of antibiotics was animal specific.

Concentrations related to the wastewater works effluent were higher than the ones detected around the farms. Samples were taken directly from the discharge point and where there was limited flow and the water appeared static as it was protected by cemented wall. During both sampling events a constant flow discharge could be noticed. The presence of three antibiotics which are only intended for veterinary use and not used in humans (marbofloxacin, tylosin and cephapirin) at high concentrations was unexpected. However, it could be influenced by the presence of two cattle grazing fields alongside the discharge point. Cow faeces were apparent by the water edge as cows were using the stream as a drinking water source.

The wastewater treatment facility was described as a secondary filtration site with chemical phosphorus removal. It has been demonstrated by other studies that simpler/older WWTP are unable to eliminate most of the antibiotics. Erythromycin concentrations were comparable to those found by (Kay *et al.*, 2017) who conducted a study in 7 WWTPs discharge points along the River Aire and Calder catchments. Penicillin was also detected in high concentrations before, at and after the discharge point. Even though penicillin is easily degraded, proper storage and quick analysis can improve stability and detection. The presence of penicillin derives from a combination of the WWTP effluent and the animal direct releases in the water. (Li *et al.*, 2008) have also detected at WWTP effluent at a maximum concentration of 310 ng L^{-1}.

4

A BETTER UNDERSTANDING ON HOW ANTIBIOTICS PHOTODEGRADE

4.1 Introduction

Antibiotics are widely used not only by humans but also animals and due to this they present a diverse class of aquatic contaminants with significant quantities entering the environment daily and once they enter surface waters they can cause a broad range of responses in non-target organisms. (Nesme *et al.*, 2014) The issue of antimicrobial resistance and how to eliminate the threat has gained a lot of interest the past years. (Puvača *et al.*, 2022) A better understanding is required on how these chemicals are transported and entering the environment and their fate once they are part of it in order to evaluate better the extent of their impact. (Aslam *et al.*, 2018) In surface waters, the main removal processes are biodegradation, sorption, and photodegradation.(Bavumiragira *et al.*, 2022) Some pharmaceuticals have been designed to be resistant to biodegradation, thereby inhibiting one of the major elimination mechanisms. (Patel *et al.*, 2019)

Typically compounds that contain aromatic rings and various functional groups tend to absorb light and show an overlap on their adsorption spectra with the visible light wavelengths and thus being susceptible to photodegradation.(Fatta-Kassinos *et al.*, 2011b) However to what degree they can be affected by photochemical processes is totally dependent on the compound.(Challis *et al.*, 2014) Photodegradation includes direct photodegradation and indirect photodegradation. Direct photolysis is easier predicable as it involves a direct photolysis rate constant under a given irradiation source. Indirect photolysis has a trickier pathway to understand as it involves interaction with naturally occurring photo-generated transient species. (Lastre-Acosta *et al.*, 2019) There are studies that has indicated some of these mechanisms to involve species such as triplet excited dissolved organic matter (^{3}DOM), singlet oxygen ($^1O_2$) or hydroxyl radicals ($^{\cdot}$OH) (Vione *et al.*, 2006), (Bahnmüller *et al.*, 2014a), (Ge *et al.*, 2015).

Most studies evaluating the photodegradation of a chemical are performed in a laboratory setting using light sources such as mercury lamps (monochromatic or polychromatic) of filtered Xenon arc lamps that mimics the sunlight irradiance spectra. Results obtained in a laboratory are easily

manageable and reproducible, as most of the parameters involved are controlled by the analyst (temperature, irradiance). However there very few studies accessing the photolysis of a chemical under natural sunlight.

In this study the photolysis of four antibiotics (sulfamethoxazole, sulfadiazine, oxytetracycline and enrofloxacin) that are frequently detected in natural water bodies was investigated.(Charuaud *et al.*, 2019b) In order to determine how these chemicals are behaving once they reach natural water and are subjected to photolysis, experiments using natural and simulated sunlight were conducted. Parameters such as water pH, presence of well-known photosensitising agents (nitrate and dissolved organic matter) and natural surface water were evaluated. An attempt was made to identify some of their major metabolites during this process and determine whether degradation kinetics obtained in a laboratory are representative of the real environment.

4.2 Experimental

4.2.1 Materials and methods

4.2.1.1 Materials and chemicals

Standards of sulfamethoxazole, sulfadiazine, enrofloxacin and oxytetracycline, were of PESTANAL® grade and purchased from Sigma-Aldrich (UK). Standard stock solutions (500 μg/mL) of all the compounds were prepared in methanol and stored in the dark at nominally -20ºC.

Treatment solutions of mix standards and mix calibration standards were prepared by dilution of the stock solutions in ultrapure water:methanol (50:50 v/v). Ultrapure water was produced in the laboratory using a Milli-RO plus 30® and a Milli-Q purifiers from Millipore (UK) Methanol (Optima grade or equivalent), formic acid and Whatman™ nylon membranes filters were obtained from Fisher Scientific (UK) and PES Captiva syringe filters were purchased from Agilent (UK).

Fulvic acid was obtained from the International Humic Substances Society (IHSS), batch: Suwannee River II, 2S101F. The elemental composition of the fulvic acid is 52.34% C, 42.98% O, 16.9% H_2O, 4.36% H, 0.67% N, 0.46% S, 0.004% P and 0.58% ash and was selected to represent dissolved organic matter due to its high aqueous solubility. For the preparation of the buffer solutions potassium nitrate, potassium biphthalate, sodium hydroxide, monopotassium phosphate, boric acid and potassium chloride were purchased from Sigma Aldrich.

4.2.1.2 Preparation of test system

Surface water was collected from Lake Carter, a natural lake located in Lancaster University that is not affected by any agricultural, industrial or domestic inputs. Surface water was characterised prior usage. Surface water was mostly used on the day of sampling. In occasions the collected surface water was stored overnight chilled and aerated prior to use to maintain its viability.

MilliQ water was used to prepare the rest of the tested matrices. Originally the pH of the solution was adjusted with sodium hydroxide or hydrochloric acid depending on the required level, however during the irradiation test fluctuation in the pH values were observed, so buffer solutions at the required pHs were used instead as described below.

Table 4-1: Characteristics of lake water

Parameter	Value [a]	Parameter	Value [a]
pH	7.39 – 7.47	Dissolved organic carbon	25.28 – 43 mg L^{-1}
Conductivity	394.2 – 429 μS	Calcium (Ca^{+})	34 – 83.4 mg L^{-1}
Nitrate (NO_3-N)	1.7-6.9 mg L^{-1}	Magnesium (Mg^{2+})	2.5 – 4.8 mg L^{-1}
Phosphorus (PO_4-P)	0.04-0.09 mg L^{-1}	Bacterial abundance [b]	4.18 e^6 cells/mL
Dissolved oxygen	7.9-8.2 mg L^{-1}	Bacterial abundance [b] (filtered)	2.59 e^6 cells/mL

[a] Values obtained from three different water batches
[b] Measured only in one replicate from June sampling to ensure surface water bacterial viability

4.2.1.2.1 Buffer solutions

1L of each buffer solution were prepared as described in (OECD 111, 2004) hydrolysis guidance document.

- 0.1M Potassium biphthalate buffer, pH 4.0 was prepared by adjusting potassium biphthalate solution (0.1 M) to pH 4.0 by addition of sodium hydroxide solution (0.1 M).
- 0.1M Monopotassium phosphate Buffer, pH 7.0 was prepared by adjusting monopotassium phosphate solution (0.1 M) to pH 7.0 by addition of sodium hydroxide solution (0.1 M).
- 0.1M Boric acid Buffer, pH 8.0 was prepared by adjusting 0.1 M boric acid in 0.1M potassium chloride solution (0.1 M) to pH 8.0 by addition of sodium hydroxide solution (0.1 M).

4.2.1.2.2 Elevated nutrients

Known for producing reactive oxygen species when irradiated the effect of the presence of humic acid and nitrate at different levels was studied.(Xu *et al.*, 2011) Prior to use the humic acid was dried in a desiccator until a constant weight was reached. Two 1L solutions were prepared each containing humic acid at a final concentration of 0.5 and 5 mg L^{-1}. Dissolved organic carbon was measured in the two solutions and were found to be at 4 mg L^{-1} and 48 mg L^{-1} for the 0.5 and 5 mg L^{-1} concentrations respectively. Potassium nitrate solutions were prepared at concentration levels of 0.2 and 2 mM. These levels were chosen based on the analysis of available lake water.

4.2.1.2.3 Surface water

Surface lake water was used both filtered through a GF filter and unfiltered to evaluate how suspended solids affect the photodegradation process. It has been suggested that suspended solids have a significant influence on the photo transformation of antibiotics in water. Thus, their role need to be

considered in accessing the transformation and fate of pollutants in aqueous environments. (Cheng *et al.*, 2021)

4.2.1.3 Method of irradiation

The simulated sunlight test was performed in a Suntest accelerated exposure machine (Atlas Suntest CPS + Solar Simulator, Germany) fitted with a Xenon arc lamp (1,500 W). The radiation from the xenon lamp was filtered to remove light below 290 nm to give ultraviolet and visible light with a spectral distribution, which closely approximates to natural sunlight. Samples were continuously irradiated in glass vials, with quartz glass lids, at a temperature of 25 ± 2°C as indicated in OECD 316 guideline(OECD, 2008), which was maintained by circulating chilled water around the vials with the aid of a Thermoquest water bath (Thermo Finnigan UK). The light intensity was measured using a SR9110-V7 Spectroradiometer (Macam UK) fitted with a fibre optic probe with a spectral range of 240–800nm. The spectral properties and intensity of the lamp were measured at the height of the buffer surface and in the position of each irradiated unit. Measurements were made at 1 mm bandwidths before the irradiation period using a spectroradiometer. For the experiments, the xenon lamp was set at a medium intensity level (500 W/m^2). Slight variations in the irradiance readings across the chamber positions were recorded with the total irradiance values ranging from 466 W/m^2 to 531W/m^2.

For the test performed under direct natural light, all the borosilicate conical flasks were exposed to natural light on daytime and night-time for a duration of five days in Lancaster UK (54°N) during summer months (June-July-August 2014). The spectral distribution of sunlight compared with that obtained from the xenon lamp is compared in **Figure 4-1** and **Figure 4-2**.

Figure 4-1: Spectral irradiance of the Xenon Arc lamp measured on the base of the Atlas Suntest chamber

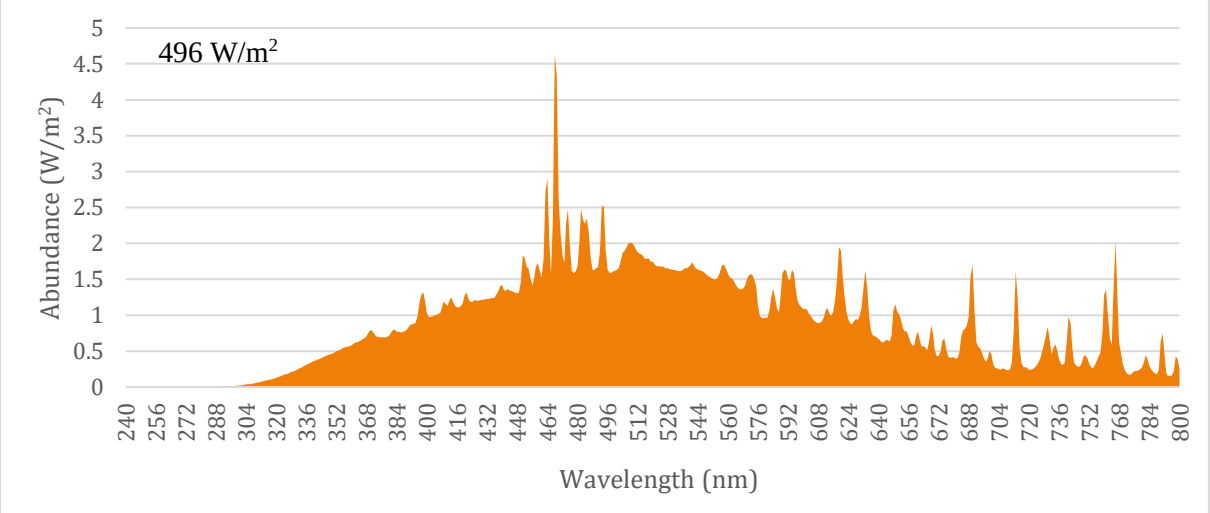
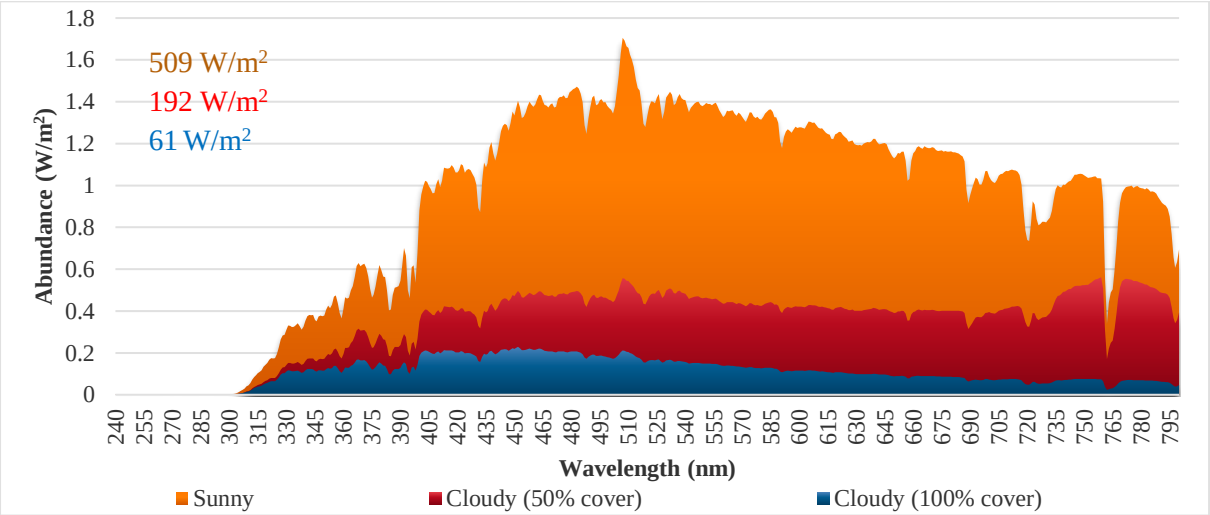


Figure 4-2: Spectral irradiance of natural sunlight measured in June 2014



4.2.1.4 Analytical methodology

An Agilent MSD single quadrupole mass spectrometer MS equipped with an electrospray ionization source (ESI) and connected to an Agilent 1100 HPLC system (Agilent, UK) was used for sample analysis. Separations were achieved on a Poroshell 120 EC-C18 column (2.1x100 mm, 2.7 μm particle size) equipped with a safe guard column, both purchased from Agilent. The pump was operated at a flow rate of 0.22 mL/min. The column oven temperature was 30 °C, and the injection volume was 20 μL.

The separation was performed using a simple binary gradient mobile phase consisting of 0.1% formic acid in water (A) and methanol (B). Exact gradients used for analysis per analyte are summarised in **Table 4-2**. The LC-MS was operated in positive ion mode. The drying gas temperature was 300 °C, and the capillary voltage was 4.0 kV. Nitrogen was used as a carrier gas at a flow rate of 9 L/min nebuliser pressure was set at 40psig. Analysis was performed at a full scan range of 200-800 m/z.

Table 4-2: Chromatographic conditions for metabolite identification, per analyte.

Sulfadiazine		**Sulfamethoxazole**		**Oxytetracycline**		**Enrofloxacin**	
minutes	%B	minutes	%B	minutes	%B	minutes	%B
0	5	0	10	0	25	0	10
15	15	3	10	5	40	20	90
15.1	5	6	90	10	60	20.1	10
20	5	9	90	13	70	25	10
		10	10	15	25		
		15	10	25	25		

4.2.2 Experimental design

4.2.2.1 Laboratory set-up

20 mL aliquots of each matrix were dispensed in quartz-glass cylindrical reaction vessels and the analyte was added. In order to facilitate the elucidation and detection of possible transformation products the test was performed at 10 μg L-1 and at 1 μg L-1 for calculating the rate constant, as it represents a more realistic approach to environmental matrices. Samples were continuously irradiated in glass vials, with quartz glass lids, at a temperature of 25 ± 2°C (maintained by circulating chilled water around the vials by means of a cooling block). The samples were irradiated for 12 hours continuously to mimic a full day as set by (OECD No. 316, 2008). Vials were covered with aluminium foil and placed alongside the irradiated samples in the chamber to access any potential losses through hydrolysis.

1mL aliquots were removed at representative timepoints. Aliquots were taken from the vessels at 0, 30, 60, 120, 180, 240, 300, 420 and 720 minutes. To monitor the quicker degradation of enrofloxacin and ensure that there are enough data points, extra aliquots were taken from the vessels containing enrofloxacin at 10 and 20 minutes. The pH of the samples was monitored at each timepoint using a three-point calibrated Orion Star 5 plus pH metre (Thermo Finnigan, UK) Each experiment set up ran in quintuples.

4.2.2.2 Natural sunlight set up

500mL of each of the matrices, expect the fulvic acid, were added to 1L borosilicate (3.3) conical flasks. The flasks were covered with transparent glasshouse film to eliminate evaporation. After dosing (1 μg L^{-1}) the flasks were placed outside in an area where they will get undisturbed natural sunlight for the whole duration of the day. (**Figure 4-3**) Dark controls run alongside the irradiated samples covered in aluminium foil to assess possible analyte degradation due to other sources (eg. heat, hydrolysis). Experiments were conducted over summer 2014 for 5 days. The duration was chosen based on observed half-life values during the June experiment. Irradiance readings were taken at least once a day (midday) using a Spectroradiometer with values ranging from 67 to 267, 46 to 509 and 66 to 459 W/m^2 for June, July and August respectively. Daily observations about the temperature, minutes of sunshine and total sunshine were taken from Hazelrigg Weather Station, a meteorological station situated in Lancaster University. (**Table 4-3**) During August the highest total sunshine was observed followed by June and then July. Even though in July the highest minutes of sunshine were observed it was probably during the hours of the day when the light intensity was not at its highest. Temperatures were ranging from 11 to 27^{o}C.

Table 4-3: Daily observations during outside photolysis experiments and comparison to Atlas Suntest

Date	Total Sunshine (kW/m^2) [1]	Total sunshine (Minutes)	Min Temperature (^oC)	Max Temperature (^oC)	Day length (Minutes)
			June		
13-Jun-14	3.7	83	15	27	1021
14-Jun-14	13.7	457	12	25	1022
15-Jun-14	4.6	0	14	20	1023
16-Jun-14	15.9	490	11	23	1024
17-Jun-14	12.0	285	12	25	1025
Total	49.9	1315			
			July		
09-Jul-14	7.8	314	13	23	1008
10-Jul-14	11.3	946	11	26	1006
11-Jul-14	11.1	875	11	25	1004
12-Jul-14	5.9	350	12	27	1002
13-Jul-14	10.4	597	13	21	999
Total	46.5	3082			
			August		
11-Aug-14	8.2	242	13	20	907
12-Aug-14	13.1	303	14	18	903
13-Aug-14	9.9	202	16	17	900
14-Aug-14	10.5	163	13	18	896
15-Aug-14	13.7	435	12	19	892
Total	55.4	1345			
			Atlas Suntest		
-	35.2 [2]	720	23	27	720

[1]Weather station measurement of light intensity was between 305 to 2800 nm. Correction applied to individual measurements to account only for the 305 to 800 nm range. (Modenese *et al.*, 2018)

[2]Average irradiance reading deriving from all the 15 Atlas positions is 482.1 W/m^2

Figure 4-3: Natural sunlight photolysis set-up

Mid-day **Sunset**

4.2.3 Photo-degradation rates in different matrices

Sunlight plays an important role in the persistence and environmental fate of antibiotics. However, intensity of natural sunlight depends on latitude and overcast conditions. Because of this, experiments conducted with sunlight take longer and may pose a challenge in comparing the data among different studies. The SunTest XLS produces continuum of wavelengths from 300 nm to 800 nm by using a properly filtered Xenon lamp on the top of the exposure chamber. (Batchu *et al.*, 2013) The xenon lamp was used at its medium intensity (500 W/cm^2) in order to match a midday summer irradiance reading obtained at Lancaster (Latitude 54º).

Prior to experimental start the absorbance spectra for each antibiotic in the studied matrices were obtained using a UV-vis spectrophotometer. (Appendix 6) Oxytetracycline, enrofloxacin, sulfadiazine and sulfamethoxazole all absorb in the UV range which makes them liable to degradation by photolysis.

Computer Assisted Kinetic Evaluation (CAKE) v3.3, by Tessella was used to generate degradation kinetics. The degradation followed the single first order (SFO) kinetic. Results were calculated using Single First-Order Rate Model (SFO)

$$C_t = C_0 e^{-kt}$$

Where, C_t = concentration at time t
C_0 = initial concentration or percent applied radioactivity
e = base e
k = rate constant of decline 1/hours
t = time (hours)

The results were evaluated based on the error (χ) observed which was <15% for all matrices tested and the t-test or confidence intervals which were <0.05. r^2 values were >0.99.

Degradation showed that it follows a pseudo-first-order kinetics. Example CAKE output file can be found in Appendix 9.

Table 4-4: Direct photolysis rate constant and calculated half lives for Sulfamethoxazole

Simulated sunlight – 10ppm	k (hours^{-1})	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 4	0.1927	0.9907	3.05	5.37E-23	3.6	12
pH 7	0.1780	0.9406	2.18	3.54E-13	3.89	12.9
pH 8	0.0370	0.8973	2.41	2.36E-13	18.7	62.1
0.2mM nitrate	0.2285	0.9935	2.49	9.46E-25	3.03	10.1
2mM nitrate	0.2272	0.9972	2.11	4.15E-29	3.05	10.1
0.5mg/L fulvic acid	0.2099	0.9928	2.12	1.27E-24	3.3	11
5mg/L fulvic acid	0.8360	0.9924	2.38	3.49E-24	3.78	12.5
Simulated sunlight – 1ppm	k (hours^{-1})	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 7	0.2066	0.9925	1.1	6.33E-21	3.36	11
0.2mM nitrate	0.2313	0.9892	2.88	3.66E-21	2.65	8.81
2mM nitrate	0.2472	0.9804	3.88	9.22E-19	2.8	9.32
0.5mg/L fulvic acid	0.2538	0.9963	1.38	1.04E-26	2.73	9.07
5mg/L fulvic acid	0.0879	0.9649	2.36	4.68E-17	7.88	26.2
SW filtered	0.0352	0.883	1.28	6.84E-15	19.7	65.5
SW unfiltered	0.0345	0.9209	0.746	7.03E-16	20.1	66.7
Natural sunlight June	k (hours^{-1})	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 7	0.0017	0.9421	10.4	4.68E-09	40.5	133
2mM nitrate	0.0161	0.96	7.17	7.64E-12	43.1	143
SW filtered	0.0031	0.8934	2.97	6.91E-10	226	751
SW unfiltered	0.0026	0.7635	3.4	1.49E-06	268	889
Natural sunlight July	k (hours^{-1})	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 7	0.0176	0.9689	10.1	5.18E-05	39.3	131
2mM nitrate	0.0195	0.9486	13.9	2.55E-04	35.5	118
SW filtered	0.0012	0.7101	5.95	1.99E-04	575	1910
SW unfiltered	0.0012	0.7734	4.78	7.03E-04	603	2000
Natural sunlight August	k (hours^{-1})	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 7	0.0150	0.9413	10.4	1.65E-08	46.3	154
2mM nitrate	0.0148	0.9519	9.16	3.46E-09	46.9	159
SW filtered	0.0060	0.8306	8.57	1.29E-06	115	384
SW unfiltered	0.0055	0.8012	7.68	5.90E-06	125	416

In the dark samples Sulfamethoxazole appears hydrolytically stable in buffered solutions with removal rates ranging from -10.3% to +20.4% of the applied concentration. The positive removal rate indicates either slight evaporation of the solution or changes in instrument response during the analysis. The highest observed removal rates were observed in surface water dark solutions where rates reached a maximum of -14.3% by the end of the study.

Table 4-5: Direct photolysis rate constant and calculated half lives for Sulfadiazine

Simulated sunlight – 10ppm	k (hours^{-1})	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 4	0.0172	0.8157	3.45	8.86E-07	40.4	134
pH 7	0.0253	0.8122	5.01	1.13E-04	27.4	91.1
pH 8	0.0332	0.8179	6.94	1.04E-06	20.9	69.3
0.2mM nitrate	0.0454	0.8368	7.51	5.53E-08	15.3	50.7
2mM nitrate	0.0299	0.8138	1.37	1.62E-05	23.2	76.9
0.5mg/L fulvic acid	0.0343	0.8335	2.67	6.55E-11	20.2	67.1
5mg/L fulvic acid	0.0295	0.9548	1.67	6.88E-18	23.5	78.1
Simulated sunlight – 1ppm	k (hours^{-1})	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 7	0.0312	0.8825	4.21	9.03E-08	22.2	73.6
0.2mM nitrate	0.0542	0.8542	5.75	7.35E-11	12.8	42.5
2mM nitrate	0.0402	0.8677	2.42	3.51E-08	17.2	57.2
0.5mg/L fulvic acid	0.0863	0.9457	3.07	4.08E-15	8.04	26.7
5mg/L fulvic acid	0.0641	0.8809	5.06	1.15E-11	10.8	35.9
SW filtered	0.0577	0.9226	3.93	4.84E-13	12	39.9
SW unfiltered	0.0559	0.9587	2.31	5.72E-16	12.4	41.2
Natural sunlight June	k (hours^{-1})	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 7	0.0087	0.9339	7.07	8.79E-08	79.8	265
2mM nitrate	0.0090	0.944	6.66	3.61E-08	77	256
SW filtered	0.0136	0.9313	12	1.32E-06	50.9	168
SW unfiltered	0.0143	0.9464	10.9	3.86E-07	48.6	162
Natural sunlight July	k (hours^{-1})	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 7	0.0080	0.8994	13.9	1.88E-04	86.8	288
2mM nitrate	0.0106	0.9349	13.4	5.77E-05	65.2	217
SW filtered	0.0130	0.9721	11.1	5.97E-06	53.4	177
SW unfiltered	0.0123	0.9753	9.94	3.34E-06	56.5	188
Natural sunlight August	k (hours^{-1})	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 7	0.0108	0.8825	11.6	1.45E-06	64.1	213
2mM nitrate	0.0116	0.8822	12.5	2.99E-06	59.6	198
SW filtered	0.0205	0.9145	14	1.10E-06	33.8	112
SW unfiltered	0.0193	0.9345	11.7	1.57E-07	35.8	119

In the dark samples Sulfadiazine appears hydrolytically stable in buffered solutions with removal rates ranging from -8.5% to +13.6% of the applied concentration. The positive removal rate indicates either slight evaporation of the solution or changes in instrument response during the analysis. The removal rates observed in surface water dark solutions reached a maximum of -12.7% by the end of the study. (Baena-Nogueras *et al.*, 2017) also observed minimal degradation in the dark samples for both sulfamethoxazole and sulfadiazine.

Table 4-6: Direct photolysis rate constant and calculated half lives for Oxytetracycline

Simulated sunlight – 10ppm	k (hours^{-1})	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 4	0.1453	0.9772	4.77	1.66E-18	4.77	15.8
pH 7	0.1568	0.9828	4.42	1.80E-13	4.42	14.7
pH 8	0.8659	0.9835	13.4	1.40E-15	0.80	2.66
0.2mM nitrate	0.2210	0.9839	3.58	8.13E-20	3.14	10.4
2mM nitrate	0.2365	0.9617	8.96	6.07E-15	2.93	9.74
0.5mg/L fulvic acid	0.2294	0.9885	3.28	1.01E-18	3.02	10
5mg/L fulvic acid	0.2279	0.978	6.86	5.13E-18	3.04	10.1
Simulated sunlight – 1 ppm	k (hours^{-1})	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 7	0.1884	0.9769	3.01	5.69E-11	3.68	12.2
0.2mM nitrate	0.4551	0.9689	7.35	4.98E-13	1.52	5.06
2mM nitrate	0.6028	0.9885	4.17	2.25E-16	1.15	3.82
0.5mg/L fulvic acid	0.5799	0.9795	2.62	4.29E-14	1.20	3.97
5mg/L fulvic acid	0.4909	0.9894	3.11	3.01E-17	1.41	4.69
SW filtered	1.2370	0.9331	6.92	9.05E-06	0.56	1.86
SW unfiltered	1.3790	0.997	2.32	2.73E-12	0.50	1.67
Natural sunlight June	k (hours^{-1})	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 7	0.1012	0.9423	2.05	0.04565	6.85	22.8
2mM nitrate	0.2219	0.9001	9.41	0.03092	3.12	10.4
SW filtered	Fully degraded in <1hour					
SW unfiltered						
Natural sunlight July	k (hours^{-1})	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 7	0.5249	0.9125	2.87	0.02845	1.32	4.39
2mM nitrate	0.5916	0.9028	2.79	0.02552	1.17	3.89
SW filtered	Fully degraded in <1hour					
SW unfiltered						
Natural sunlight August	k (hours^{-1})	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 7	0.2111	0.8892	16	0.001745	3.28	10.9
2mM nitrate	0.1982	0.8726	12.1	0.003188	3.50	11.6
SW filtered	Fully degraded in <1hour					
SW unfiltered						

In the dark samples Oxytetracycline appears hydrolytically stable in buffered solutions with removal rates ranging from -15.2% to +12.9% of the applied concentration. The positive removal rate indicates either slight evaporation of the solution or changes in instrument response during the analysis. The removal rates observed in surface water dark solutions reached a maximum of -4.8% by the end of the study. This is in line with the findings by (Zhong *et al.*, 2022) who reported oxytetracycline hydrolysis half-life at 66 hours,

Table 4-7: Direct photolysis rate constant and calculated half lives for Enrofloxacin

Simulated sunlight	k ($hours^{-1}$)	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 4	0.1652	0.9883	3.66	2.00E-14	4.20	13.9
pH 7	0.2551	0.9766	6.54	2.42E-06	2.72	9.03
pH 8	1.7020	0.9828	7.51	7.32E-12	0.41	1.35
0.2mM nitrate	0.7964	0.9926	6.45	1.74E-22	0.87	2.89
2mM nitrate	1.1090	0.9847	2.94	1.53E-12	0.62	2.08
0.5mg/L fulvic acid	0.7460	0.9859	8.52	6.57E-19	0.93	3.09
5mg/L fulvic acid	0.9277	0.9983	3.15	4.28E-33	0.75	2.48
1ppm	k ($hours^{-1}$)	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 7	0.7221	0.9951	6.03	9.18E-07	0.96	3.19
0.2mM nitrate	1.9930	0.9785	7.6	2.74E-09	0.52	1.72
2mM nitrate	1.4330	0.986	7.41	2.05E-10	0.48	1.61
0.5mg/L fulvic acid	0.8810	0.9892	4.01	5.08E-12	0.79	2.61
5mg/L fulvic acid	1.5960	0.9812	9.1	7.67E-08	0.43	1.44
SW filtered	1.3730	0.9936	2.59	5.00E-19	0.51	1.68
SW unfiltered	1.5800	0.9806	9.59	4.70E-14	0.44	1.46
Natural sunlight June	k ($hours^{-1}$)	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 7	Fully degraded in <1hour					
2mM nitrate	0.2219	0.9514	9.41	1.09E-04	3.12	10.4
SW filtered	0.5558	0.9222	6.51	1.22E-04	1.25	4.14
SW unfiltered	0.5575	0.9389	5.59	5.86E-04	1.24	4.13
Natural sunlight July	k ($hours^{-1}$)	r^2	χ	t-test	DT_{50} (hours)	DT_{90} (hours)
pH 7	1.9820	0.8921	7.02	2.82E-04	0.35	1.16
2mM nitrate						
SW filtered	Fully degraded in <1hour					
SW unfiltered						
Natural sunlight August	k ($hours^{-1}$)	r^2	χ	t-test	DT_{50} (hours)	DT_{50} (days)
pH 7	1.3370	0.9907	2.35	1.85E-05	0.52	1.72
2mM nitrate	1.3370	0.9158	6.71	7.31E-04	0.52	1.72
SW filtered						
SW unfiltered	Fully degraded in <1hour					

In the dark samples Enrofloxacin appears hydrolytically stable in buffered solutions with removal rates ranging from -13.8% to +4.0% of the applied concentration. Highest removal rates were observed in the solutions containing fulvic acid. The positive removal rate indicates either slight evaporation of the solution or changes in instrument response during the analysis. The removal rates observed in surface water dark solutions reached a maximum of -12.5% by the end of the study. Minimal degradation in the dark was also observed by (Álvarez-Esmorís *et al.*, 2022)

4.2.3.1 The effect of concentration, pH, nitrate and fulvic acid

4.2.3.1.1 Effect of sample concentration

The photodegradation rate constants for all four compounds generally decreased with an increase in their initial concentrations. This was observed for all the matrices tested (buffered solution at pH 7, high and low concentration of nitrate and fulvic acid enriched solutions). The effect of concentration on the degradation rates was more prominent in the solutions with the added constituents. When the initial concentration was 1 $mg \cdot L^{-1}$ at pH 7 buffer, the degradation constants of sulfamethoxazole, sulfadiazine, oxytetracycline and enrofloxacin were 0.2066 h^{-1}, 0.0312 h^{-1}, 0.1884 h^{-1} and 0.7721 h^{-1} respectively, and the degradation rates of these four pollutants decreased when the concentration increased 10 $mg \cdot L^{-1}$ to 0.1780 h^{-1}, 0.0253 h^{-1}, 0.1568 h^{-1} and 0.2551 h^{-1}. This was mainly because, when the initial concentration was low, the pollutants could fully absorb photons and react with the active groups in the reaction system. (Chen *et al.*, 2023) (Biošić *et al.*, 2017) also observed a strong negative linear relationships of $\ln(C/C_0)$ with photolysis time (t) and the photolysis rate constant (k) decreased with the increase of the sulphonamides concentration, which also agrees with (Jiao *et al.*, 2008c) for the case of oxytetracycline and enrofloxacin (Li *et al.*, 2011b)

4.2.3.1.2 Effect of pH

(Boreen *et al.*, 2004) indicated that five membered sulphonamides in natural sunlight degrade faster due to pH dependent direct photolysis. This was also observed in this study where sulfamethoxazole showed a higher degradation rate in pH 4 than in pH 8 with degradation constants decreasing from 0.1927 h^{-1} to 0.037 h^{-1}. At pH 4 sulfamethoxazole exists mainly in neutral form, which have demonstrated stronger light absorption and higher photochemical reactivity leading to shorter half-lives and higher degradation efficiency while at pH 8 is negatively charged and appears more stable. (Niu *et al.*, 2013a) For sulfadiazine at pH 4 the main form is neutral (SD^0), but changes to its anion form of SD^- when pH > 6.5. (Şanli *et al.*, 2010) The photolysis rate of sulfadiazine was greater than 50% in different pH environments and the photolysis rate of sulfadiazine was the fastest under acidic condition, followed by neutral and alkaline conditions (acidic > neutral > alkaline). (Li *et al.*, 2020) Also (Bian and Zhang, 2016) found that solution acidity is playing a less significant role in the degradation of sulfadiazine.

Increased degradation rate was observed for oxytetracycline in higher pH. With the increase of pH from 4 to 8, the photolysis rate constants increased from 0.1453 to 0.8659 h^{-1}. Oxytetracycline molecules exist in a neutral and positively charged form at pH 4 and negatively charged oxytetracycline molecules are dominant in a solution of pH 8. (Jin *et al.*, 2017) The degradation results show that the photolysis is markedly inhibited in positively charged form and promoted in the negatively charged form.(Jiao *et al.*, 2008c) because the anionic form of oxytetracycline is capable of generating reactive oxygen species (singlet oxygen) that plays an important role on photolysis. (Zaranyika *et al.*, 2015).

Enrofloxacin act as zwitterions and the pH values greatly influences their photolysis (Dolar *et al.*, 2013).Enrofloxacin exist in its anionic form in pH 8 and cationic form in pH 4. The photolysis rate constants of enrofloxacin increased from 0.1652 h^{-1} to 1.702 h^{-1} with the decrease of the pH from 4 to 8. (Wammer *et al.*, 2013) also observed the same behaviour in enrofloxacin solution however their degradation rates were faster. This might be due to the fact that the irradiation intensity was higher than the one used in this study and also the pH was only regulated by a buffer solution.

4.2.3.1.3 Effect of nitrate and fulvic acid

The addition of nitrate and fulvic acid were also investigated by using a low and high concentration level of the nutrients but comparable to that found in natural surface water. Previous studies have demonstrated that dissolved organic matter (fulvic or humic acid) could either promote pollutant photodegradation as a photosensitizer or inhibit photodegradation as a light masking agent or radical scavenger, closely related to its composition. (Niu *et al.*, 2013b) Low-concentration of dissolved organic matter induce the production of reactive oxygen species to promote indirect photolysis, whereas high-concentrations could compete with compounds that absorb in the same UV region for photons and decrease light penetration via light shielding to inhibit direct photolysis greatly. (Leal *et al.*, 2015) (Chen *et al.*, 2023) Some nitrogenous compounds, such as nitrate and nitrite, are known as effective inorganic photosensitizer, which can be excited by solar light to generate reactive species(Liu *et al.*, 2023b)

In this study the presence of these nutrients did not significantly enhance the degradation of the compounds on the contrary in some cases especially when the tested concentration was 1 ppm it slightly hinders it. Results from the irradiation of oxytetracycline in the presence of nitrate or fulvic acid suggested that direct photolysis was more efficient than its indirect photolysis.(Li *et al.*, 2018).

For sulfadiazine photo transformation rate constants for all the enriched water solutions appear higher when comparing to ultrapure water solution, indicating that indirect photo transformation is likely to be a significant process. For the solutions containing nitrate it showed that by increasing the concentration of nitrate from 0.2 mM to 2 mM led to a decrease to the sulfadiazine photolysis rate irrespectively from the analyte concentration. This result was consistent with investigations by (Biošić *et al.*, 2017) and (Li *et al.*, 2020) In the presence of fulvic acid, the half life of sulfadiazine (conc. 10ppm)_increased from 20.2 hours to 23.5 hours, indicating that dissolved organic matter had a negative effect on the photolysis process. The same effect was observed also with the fulvic acid enriched solutions containing 1 ppm of sulfadiazine The k value decreased from 0.0863 h-1 to 0.0641 h-1 with the increasing humic acid concentration. This can be attributed to the screening of light by the fulvic acid. (Lee *et al.*, 2022a)

In contrast to sulfadiazine and in line with previous studies the depletion rate constant of sulfamethoxazole is almost independent of nitrate or fulvic acid concentration with half lives being in the range of 3 hours for both concentration levels tested. (Bahnmüller *et al.*, 2014). However, for the lower concentration of sulfamethoxazole (conc. 1 ppm) the fulvic acid demonstrated an inhibiting effect

by increasing the photolysis half live to 7.88 hours. A similar trend was also observed by (Niu *et al.*, 2013c)

Photolysis rates reported in this study are generally slightly higher than the ones reported in literature. However, most studies even though they specify the light sources used they do not mention what vessels were used for the irradiation experiments. In the present study quartz vessels (internal diameter × height = 3.5 × 4.3 cm) were used equipped with a quartz lid on top. The exact set up only allowed light to enter through the top of the lid and not through the sides which limits the amount of irradiation the solution received when compared to quartz tubes (internal diameter × height = 1.8 × 20 cm) as used by (Loureiro dos Louros *et al.*, 2020). When comparing the current study with the one by (Loureiro dos Louros *et al.*, 2020) half-lives in simulated solar radiation (DT_{50} (h)) were 22.2 h and 6.76 h respectively. However, a direct comparison cannot be made as sample concentrations are differing by a factor of two and the pH studied was 7.3 instead of pH 7, it is evident that experimental condition can affect the final outcome.

4.2.3.1.4 Effect of natural surface water

The water matrix of actual surface water is more complex than that of pure water. Natural organic matter and inorganic ions are commonly present in natural water environments. Bahnmuller et al. (2014) have shown that sulfamethoxazole and sulfadiazine undergo both direct and indirect photolysis in natural waters, depending on the type and concentration of dissolved organic matter and the spectrum of light used for photolysis.

In natural waters sulfamethoxazole appeared to be more stable than sulfadiazine. The pH of the surface water was >7 so this result appears in line with the results obtained when testing the clean system. In the dark control experiment, there was a statistically non-significant decrease in the sulfadiazine concentration with time, indicating that the hydrolysis of sulfadiazine or sulfamethoxazole without UV radiation in the presence of natural water was weak. (Li *et al.*, 2020)

Previous work has shown that direct photolysis, rather than indirect photolysis involving interactions with natural water constituents, is likely to be the dominant photolysis process for enrofloxacin in most natural waters (Lam and Mabury, 2005) The degradation rates observed in natural surface water was similar to those observed in the clean water system.

For oxytetracycline the degradation rates in natural surface water when compared to that in pure water appear increased, which was probably attributed to the existence of natural organic matter (NOM) and inorganic ions. (Lee *et al.*, 2022b)

Whether the presence of different nutrients in surface waters will promote the degradation is not clear and dependent on the studied compound. However, it appears that when in the same media there are compounds that absorb in the same UV region then there is a competition over the irradiation availability.

4.2.3.2 The effect of natural sunlight

The effect of natural sunlight on the photodegradation of antibiotics was studied in order to get a better understanding on how the laboratory generated photolysis data and kinetics can be associated with field values. The kinetics of these compounds can be affected by non-continuous solar exposure, water depth, and other aqueous constituents that may attenuate or absorb sunlight, respectively. (Ge *et al.*, 2018) Comparing the results of the controlled photolysis experiment with the one outside we observe quite a significant difference on the photodegradation rates especially for the abiotic systems (flasks containing pH 7 buffer and nitrate enriched water). Sulfadiazine and sulfamethoxazole in pH 7 buffer solutions during the simulated sunlight experiment showed a half-life (DT_{50}) of 22.2 and 3.4 hours respectively where under natural sunlight these values were significantly higher at an average of 76.9 and 42 hours respectively. This could be attributed to the light intensity during the day varies and during the outdoor experiment cloudy and rainy days were recorded. Whereas in the solar simulator the light was continuous for 12 hours with an intensity of 500W/m^2, same intensity as a summer midday. Oxytetracycline showed a similar pattern with the outdoor constant rates being a bit higher.

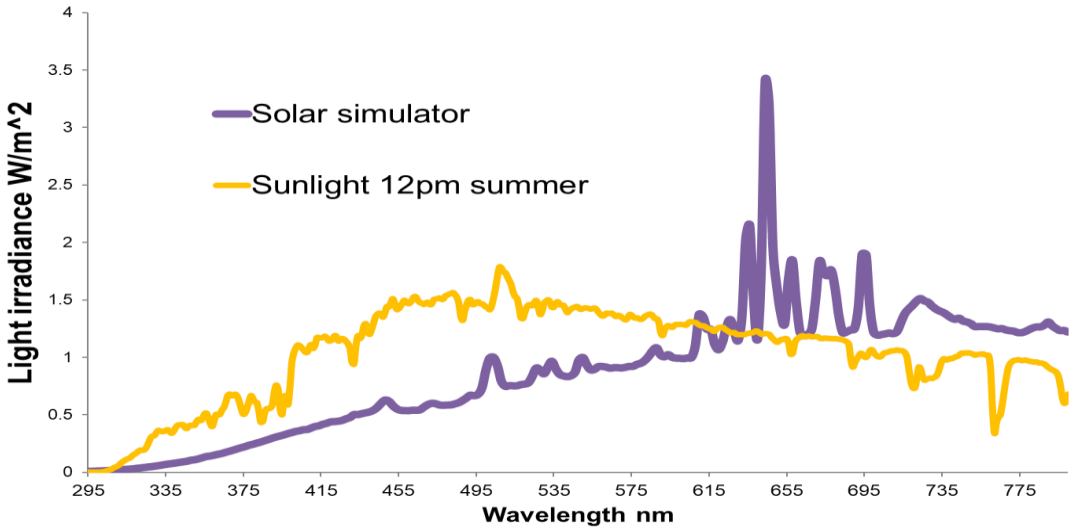
For the less persistent and photoreactive compounds this effect was not observed. Enrofloxacin showed a rapid response to the natural sunlight and photodegraded in 30 minutes compared to the indoor test where it shows a half life of 3.5 hours. In this work, borosilicate 3.3 flasks were used which were different than the quartz vessels used in the indoor set up. The difference in the observed results, point out the relevance of the experimental set up in the photooxidation kinetics. (Matamoros *et al.*, 2009)

However, for the biotic systems (flasks containing filtered and unfiltered surface water) all compounds showed higher half-lives values compared to the indoor set up. One possible explanation why there are these differences in the degradation rates in lake surface water between the solar simulator and the sunlight is that the main sources of the free radicals generated in humic acid during irradiation originate from the aromatic structures that absorb in the range above 280 nm to 340 nm. (**Figure 4-4**) The solar simulator system shows a lower light intensity in those regions. This means that more ROS are produced during the exposure at the sunlight to those in the solar simulator system. (Leal *et al.*, 2015) Humic acid has a large specific surface area and more surface reaction sites, and has good adsorption performance, which will adsorb some pollutants and lead to a decrease in the photodegradation rate of pollutants. An additional explanation for the differences between the irradiation setups in transformation kinetics could be that other chromophores are excited at 254 nm than at longer wavelengths. (Willach *et al.*, 2018)

The variation in the intensity of solar light should also be reflected in the photodegradation rate of antibiotics, however most photodegradation studies usually simulate a solar intensity at noon which could largely overestimate the degradation rate of the chemicals (Guo *et al.*, 2023) Sulfamethoxazole appear to be more affected by seasonal variety due to the only tail overlapped with irradiation spectrum. Daily averaged half-lives of sulfamethoxazole were found to vary from about 300 to 750 hours

depending on seasonal change during irradiation under natural sunlight at 22°N. (Wei *et al.*, 2019) As natural water contains dissolved organic matter or particulate organic matter and other sensitizers, it is believed that the environmental half-life of sulfadiazine under field conditions will be far less than in pure water, as demonstrated also in this study. (Sukul *et al.*, 2008b) For oxytetracycline and enrofloxacin the average half-lives are quite short and appeared less dependent on seasonality, solar irradiance and more depended on the water type tested. (Xuan *et al.*, 2010b) (Sturini *et al.*, 2010) To better understand how field conditions eg. overcast skies, limited photic zone, decreasing angular height of the sun or other aqueous constituents affect the photochemical behaviour of compounds further and more extensive studies are required (Sukul *et al.*, 2008b) (Bodrato and Vione, 2014).

Figure 4-4: Comparison of simulated vs natural sunlight

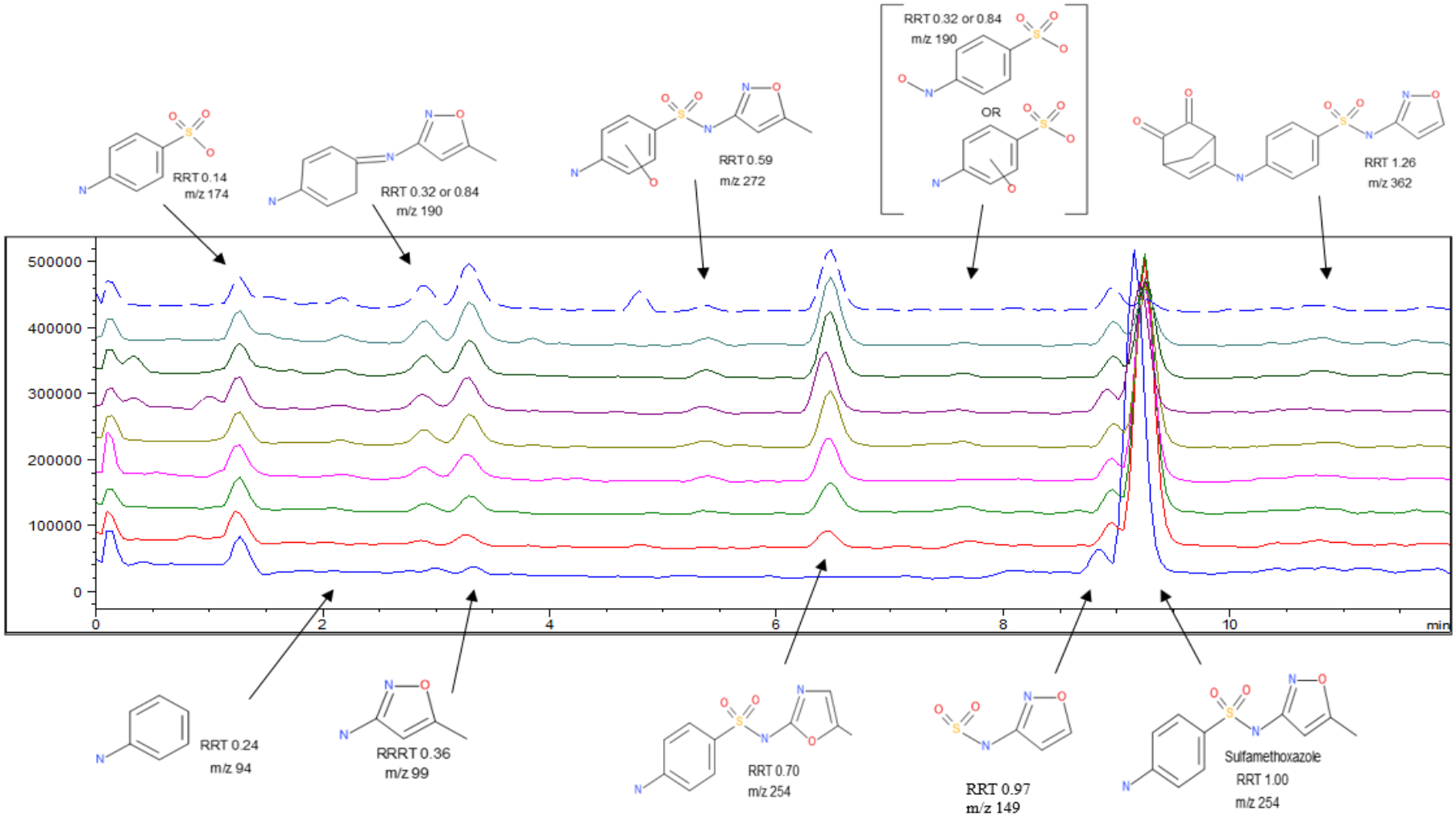


4.2.4 Transformation products

The phototransformation products of oxytetracycline, enrofloxacin, sulfadiazine and sulfamethoxazole were studied in different media including pH 4 and 8 buffer solutions, low and high nitrate enriched solutions and in the presence of low and high concentration of fulvic acid. Representative example chromatograms are located in Appendix 7. A list of observed masses along with the relative retention times can be found in **Figure 4-5**, **Figure 4-9**, **Figure 4-13** and **Figure 4-17** for sulfamethoxazole, sulfadiazine, oxytetracycline and enrofloxacin respectively. . Unfortunately, due to instrument limitations an attempt to identify them further was not made and possible structures for the transformation products has been appointed by available literature. The chromatographic conditions developed in this study have the capacity to separate the relevant metabolites with base line resolution however most of the time even though a single mass could be obtained further fractionation/confirmation was not possible.

The evolution and decline of the phototransformation products expressed as % of applied concentration is plotted against time and presented in **Figure 4-6** to **Figure 4-8** for sulfamethoxazole, **Figure 4-10** to **Figure 4-12** for sulfadiazine, **Figure 4-14** to **Figure 4-16** for oxytetracycline and **Figure 4-18** to **Figure 4-20** for enrofloxacin. To its transformation product a name was assigned based on the relevant retention time in comparison to the parent which has a relative retention time (RRT) of 1.00.

Figure 4-5: Transformation products of Sulfamethoxazole during photolysis and their RRT



Relevant masses identified in the literature (Gmurek *et al.*, 2015)(Gao *et al.*, 2021)

Figure 4-6: Degradation of Sulfamethoxazole in buffered solutions at pH 4 and ph 8 and formation of transformation products

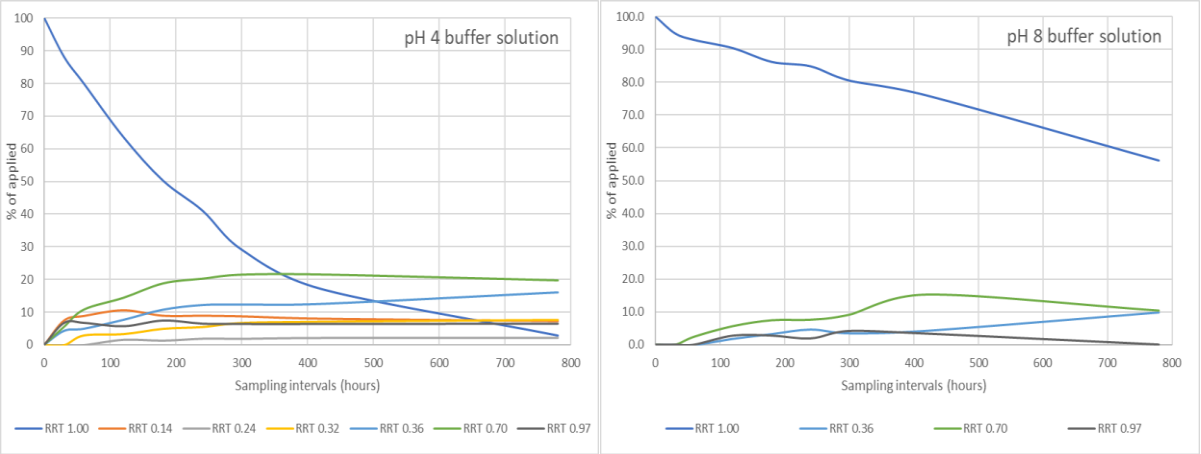


Figure 4-7: Degradation of Sulfamethoxazole in fulvic acid enriched solutions and formation of transformation products

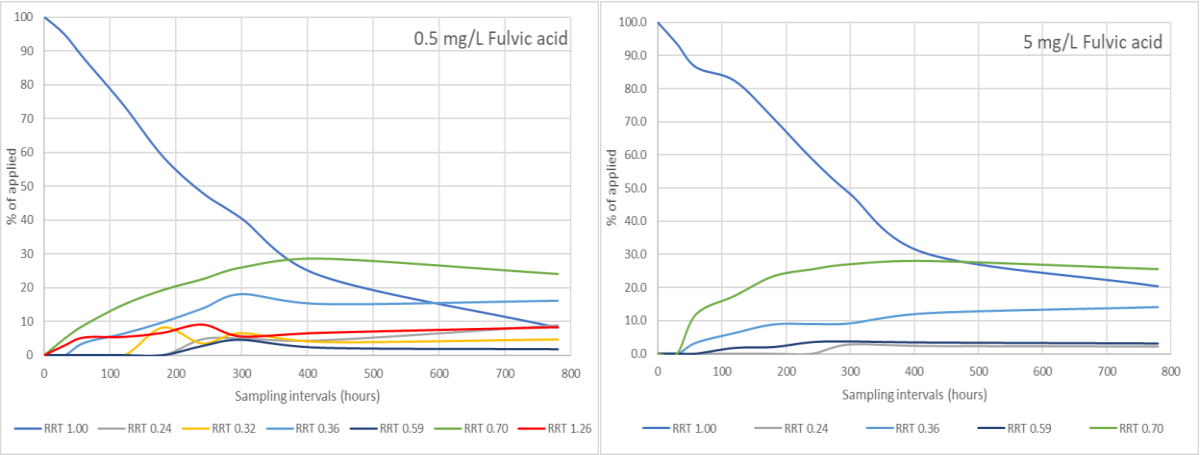
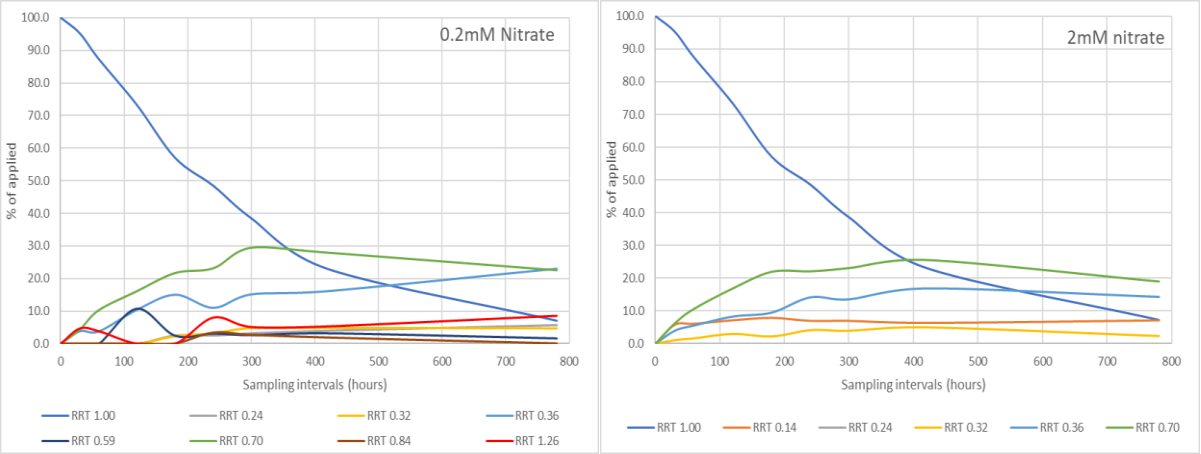


Figure 4-8: Degradation of Sulfamethoxazole in nitrate enriched solutions and formation of transformation products



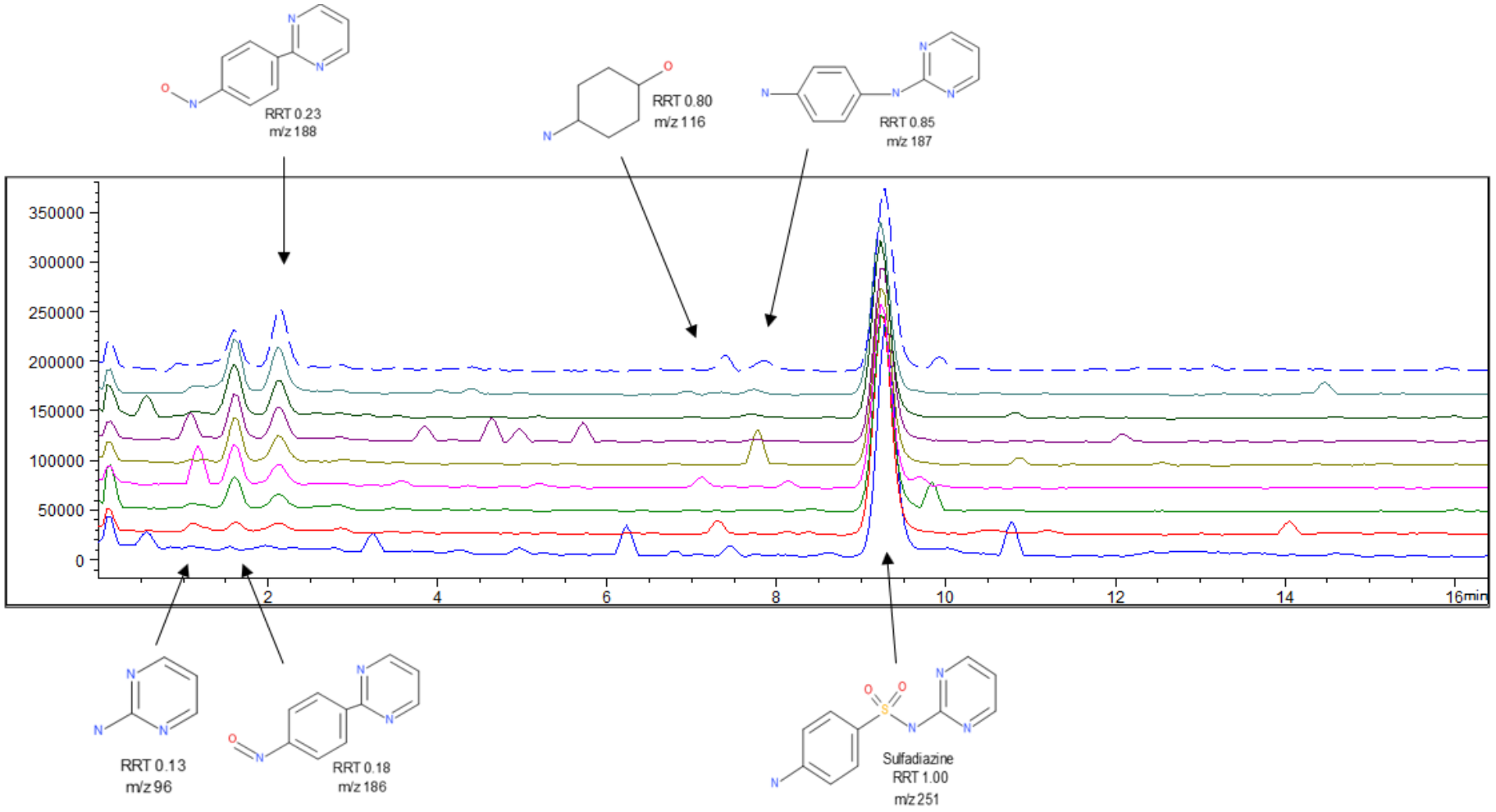
After 12 hours of irradiation under simulated sunlight sulfamethoxazole was still present in solution along with some of its transformation products. Most of the transformation products appear to be reaching a plateau at 12 hours with some of them even start to disappear. Two of them with RRT 0.36 and 0.70 appeared consistently in all matrices and showed high concentations. The literature mainly reports the transformation reactions occurring to sulfomethoxazole, and these involve acetylation, various types of single or multiple hydroxylation (N-hydroxylation, hydroxylation at aromatic ring or R-moiety, ipso-hydroxylation), nitrosation, nitration, deamination, desulfonation, formylation. (Montone *et al.*, 2024)

A product with RRT 0.70 shows *m*/*z* of 254, which is the same *m*/*z* value as sulfamethoxazole itself but with less retention on the reversed-phase chromatographic column. This finding is in agreement with other studies and represent a rearrangement of the isoxazole ring which is induced by irradiation, leading to the oxazole isomer. (Trovó *et al.*, 2009) This product was present in all the matrices tested and showed the highest observed concentration.

The second most observed transformation product showed an RRT of 0.36 and a m/z of 99 and is formed due to S−N bond fracture.

Sulfamethoxazole show high photochemical persistence but relatively low toxicity with the inhibition rate only slightly decreasing after 70 hours of irradiation for the three tested Gram-negative bacteria (Escherichia coli, Vibrio sp. and Aeromonas sp.) (Leal *et al.*, 2017) However it was observed that the eco-toxicity of transformation products when tested on fish, daphnid and green algae even though it decreases with the degradation of sulfamethoxazole there are some products that still maintain harmful levels to organisms, especially to daphnid. (Yang *et al.*, 2020)

Figure 4-9: Transformation products of Sulfadiazine during photolysis and their RRT



Relevant masses identified in the literature (Baena-Nogueras *et al.*, 2017)

Figure 4-10: Degradation of Sulfadiazine in buffered solutions at pH 4 and ph 8 and formation of transformation products

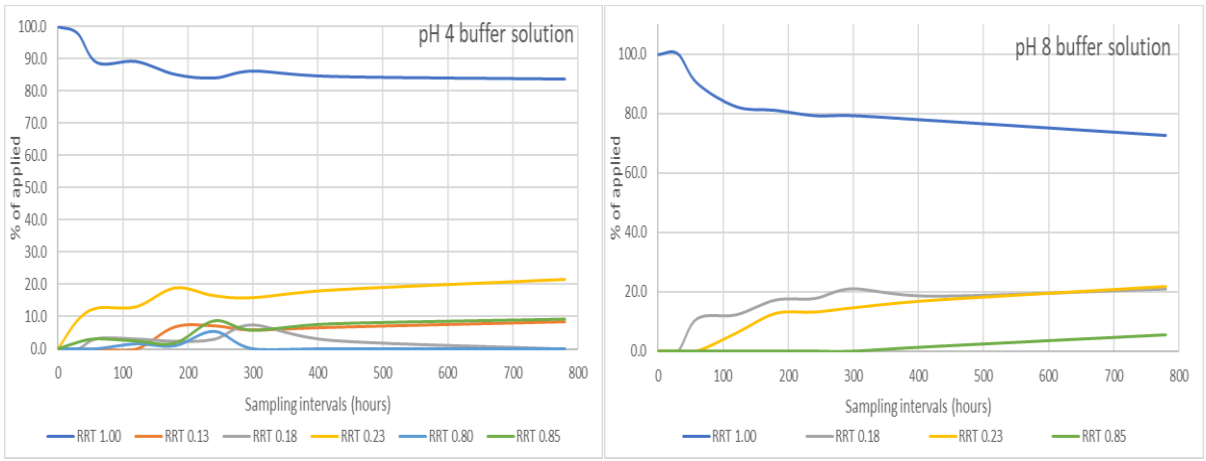


Figure 4-11: Degradation of Sulfadiazine in fulvic acid enriched solutions and formation of transformation products

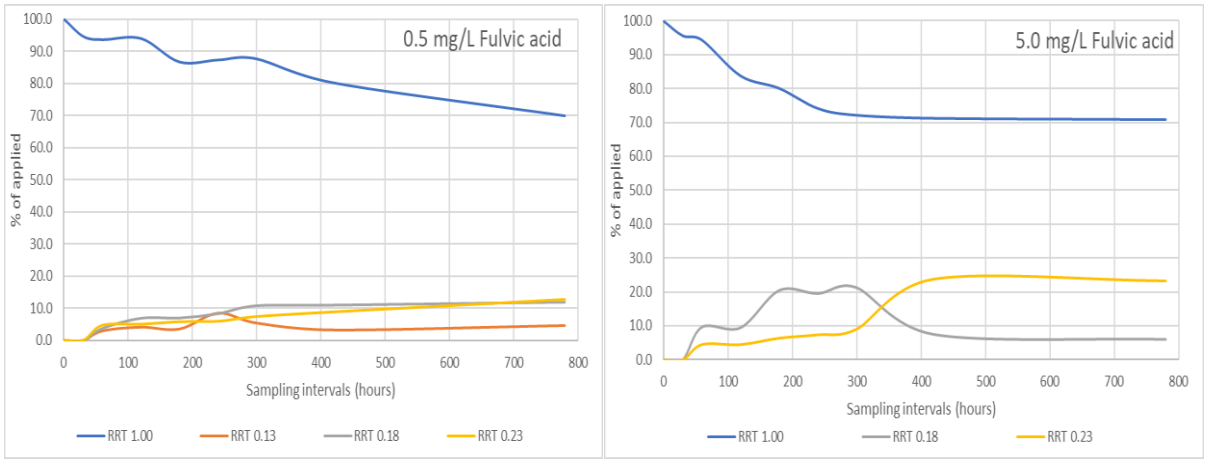
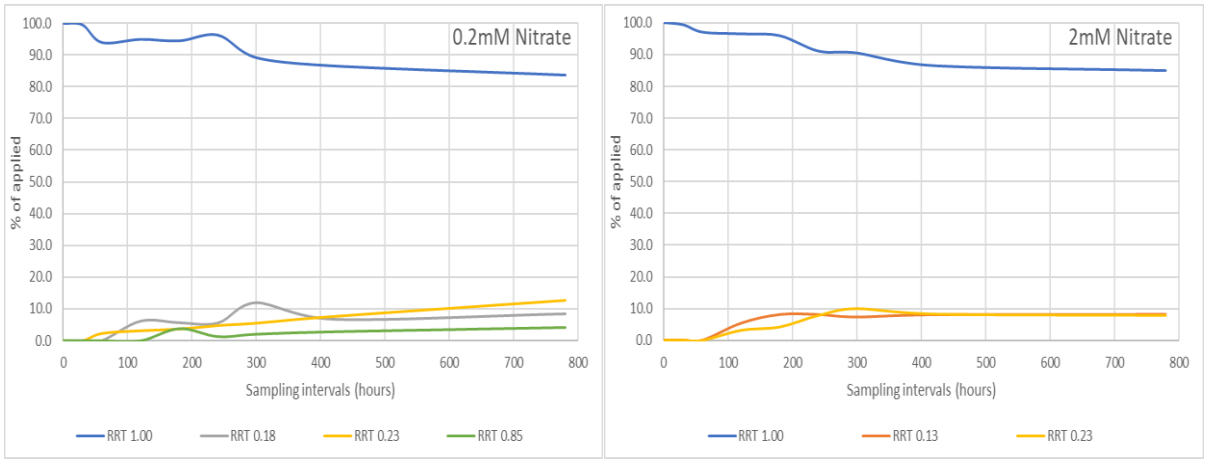


Figure 4-12: Degradation of Sulfadiazine in nitrate enriched solutions and formation of transformation products



The concentration profiles of sulfadiazine photodegradation and its transformation products versus time in different matrices are shown on **Figure 4-10** to **Figure 4-12**. Sulfadiazine appeared relatively resistant to photolysis within the timeframe of this study. The highest number of transformation products was observed in the pH 4 buffer solution. Most of the transformation products appear to be increasing slowly over the duration of the experiment (12 hours).

Four transformation products with RRT 0.13 (m/z 96), RRT 0.18 (m/z 186), RRT 0.23 (m/z 188) and RRT 0.85 (m/z 187) were present at higher concentrations at the end of experiments. Transformation product RRT 0.13 detected with a *m/z* of 96 and appeared to be corresponding to the aminopyrimidine ring. (Pfeifer *et al.*, 2005)

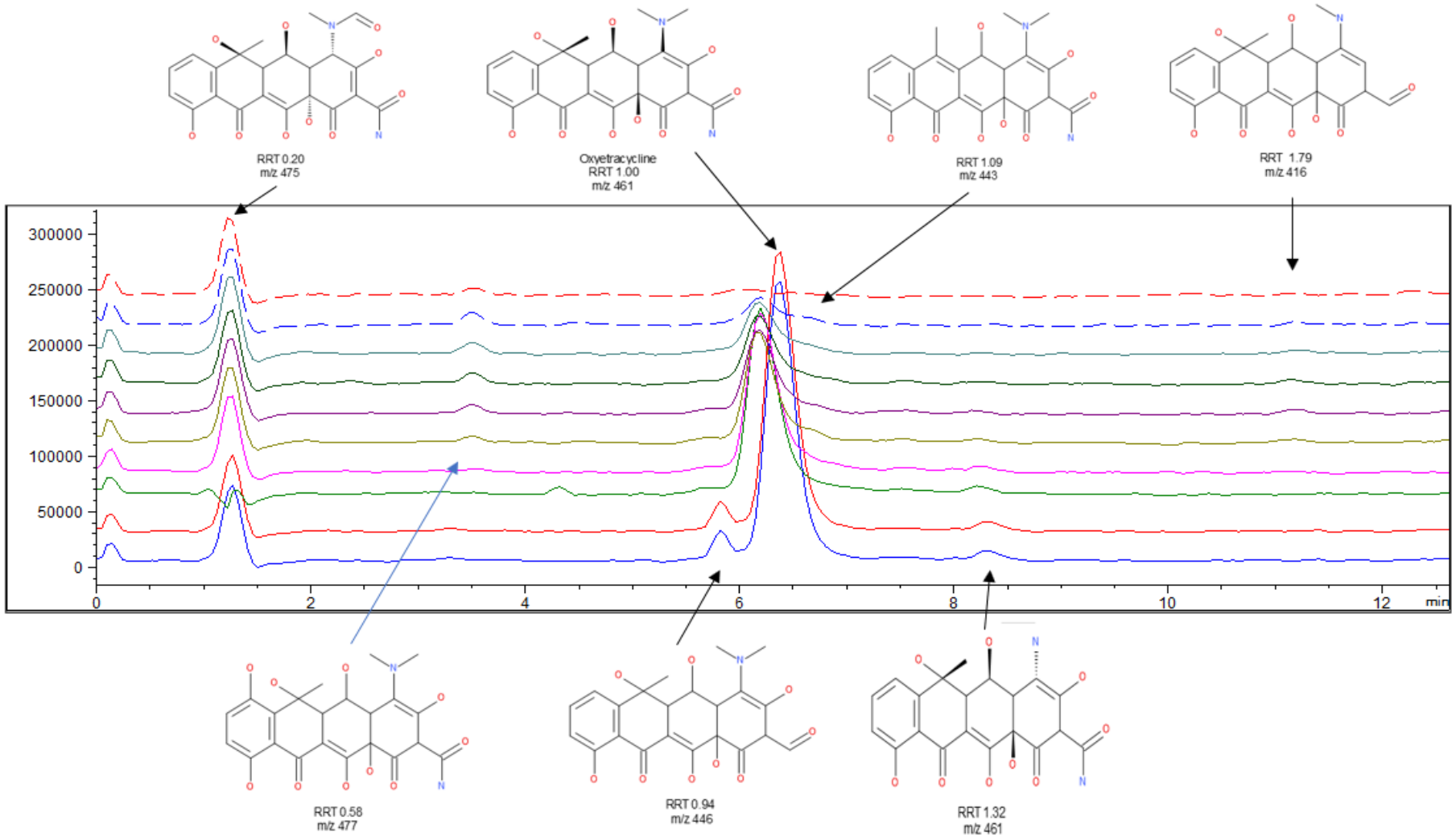
The product with RRT 0.18 showed the most abundant mass as m/z 186 corresponding to . It was observed in all matrices apart from the one containing the high nitrate concentration.

The product with RRT 0.23 showed the most abundant mass as m/z 188 and is a result of the oxidation following the desulfonation and denitrification of sulfadiazine. The product with RRT 0.18 showed the most abundant mass as m/z 186 corresponding to reduction of RRT 0.23. (Baena-Nogueras *et al.*, 2017) It was observed in all matrices apart from the one containing the high nitrate concentration.

The product with RRT 0.85 showed the most abundant mass as m/z 187 corresponding to SO_2 extrusion. It was present in both pH testing solutions and in low nitrate concentrations. (Sukul *et al.*, 2008b)

Sulfadiazine and in particular its phototransformation products seemed to be of lower phototoxicity and did not show any adverse effect to species tested (Pseudomonas fluorescens and Bacillus subtilis) (Voigt *et al.*, 2017)

Figure 4-13: Transformation products of Oxytertacycline during photolysis and their RRT



Relevant masses identified in the literature (Jiao *et al.*, 2008c)

Figure 4-14: Degradation of Oxytetracycline in buffered solutions at pH 4 and ph 8 and formation of transformation products

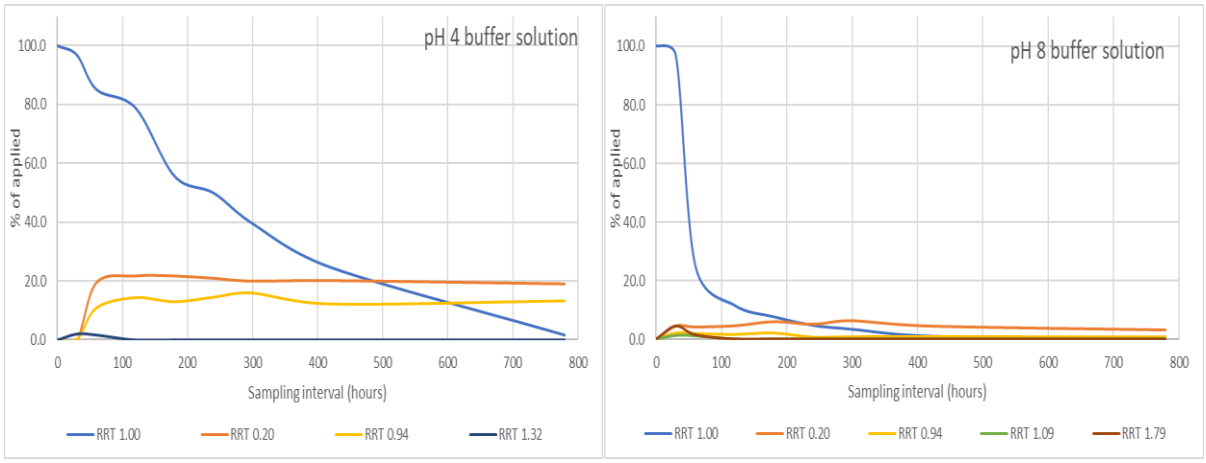


Figure 4-15: Degradation of Oxytetracycline in fulvic acid enriched solutions and formation of transformation products

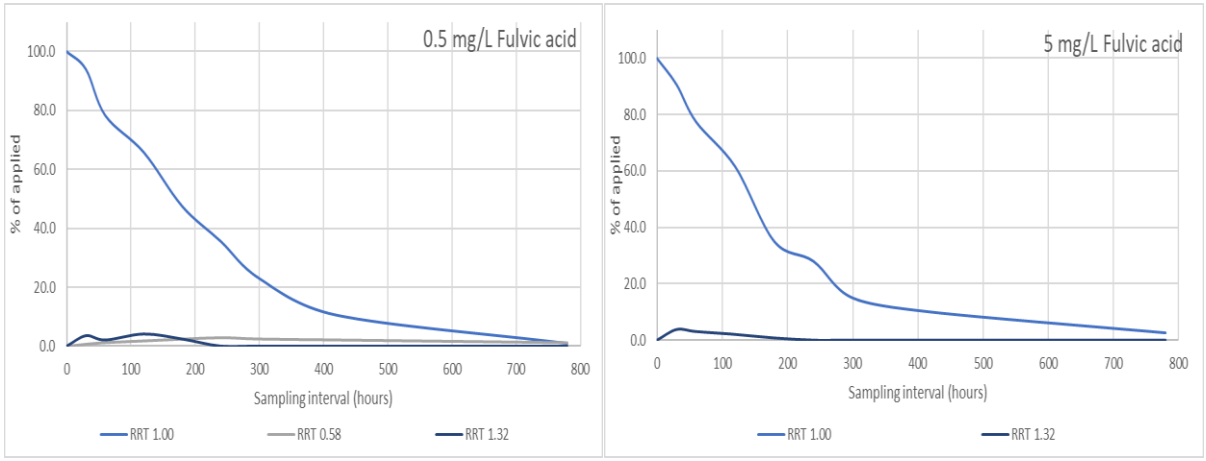
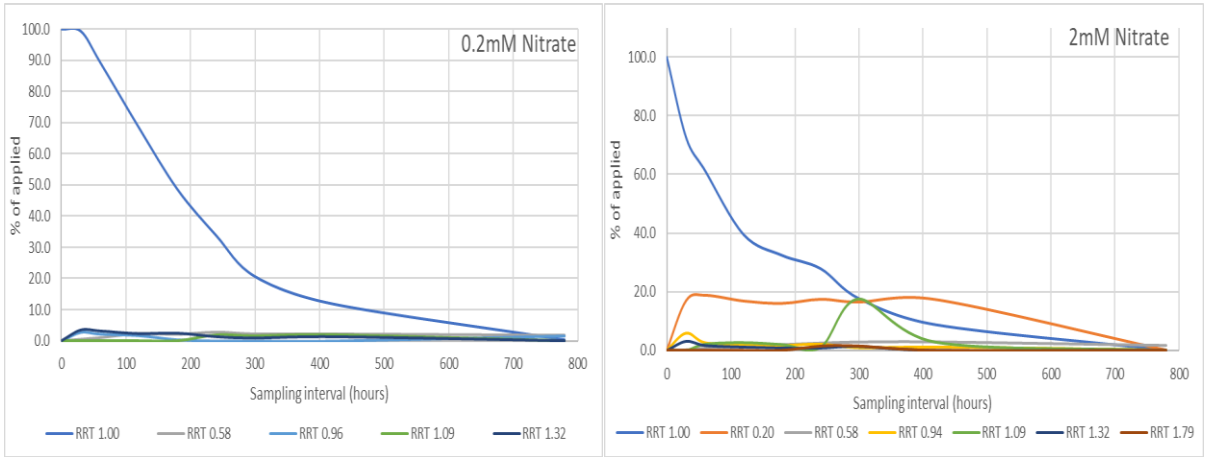


Figure 4-16: Degradation of Oxytetracycline in nitrate enriched solutions and formation of transformation products



The concentration profiles of oxytetracycline photodegradation and its transformation products versus time in different matrices are shown on **Figure 4-14** to **Figure 4-16**. The highest number of transformation products was observed in the nitrate enriched water. Most of the transformation products reached their maximum concentration in the first 100 min of irradiation, dropping down slowly or disappearing over the duration of the experiment (12 hours).

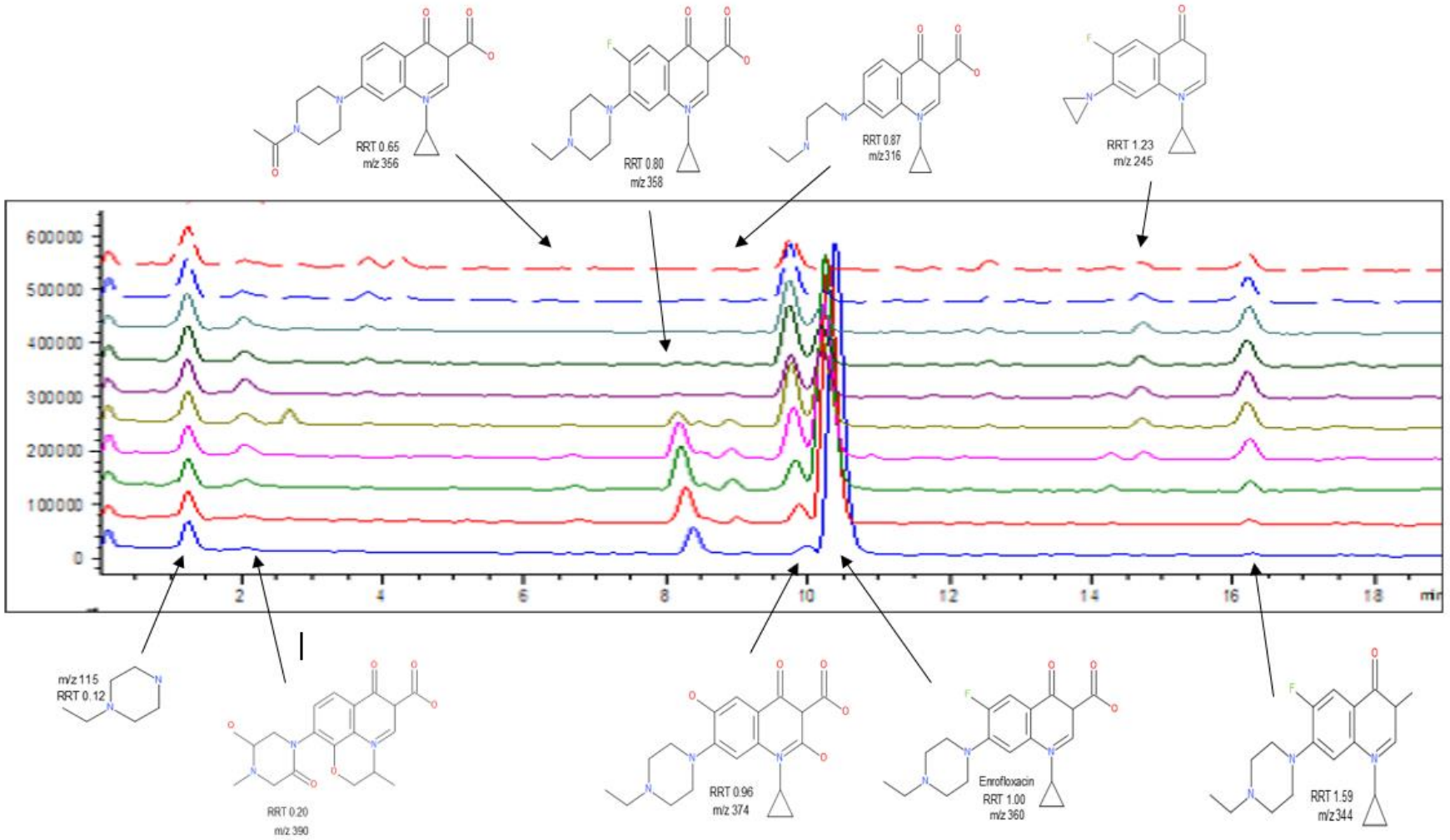
Three transformation products with RRT 0.20 (m/z 475), RRT 0.94 (m/z 446) and RRT 1.09 (m/z 443) were present at higher concentration at the end of experiments. Transformation product RRT 0.20 detected with a *m/z* of 475 was a common photodegradation path under most tested conditions and seems to be formed as a result of subsequent hydroxylation.

The product with RRT 0.94 showed the most abundant mass as m/z 446 and is a result of dihydroxylation followed by oxidation to the amide group.(He *et al.*, 2021) It was observed in all matrices and reached the highest concentration in the pH 4 buffer solutions.

The product with RRT 1.09 showed the most abundant mass as m/z 443 and is a result of the oxytetracycline molecule oxidation through a loss of water molecule. (Jin *et al.*, 2017) The photolysis products appeared the same in different conditions tested but showed the highest concentration in the 2mM nitrate enriched water.

After irradiation the resulted photoproducts showed an increased inhibition rate to P. phosphoreum, (Jiao *et al.*, 2008c) and three Gram-negative bacteria (Escherichia coli, Vibrio sp. and Aeromonas sp.) (Leal *et al.*, 2017) The results revealed a clear effect of simulated sunlight, resulting on the decrease or elimination of the antibacterial activity for all strains and in all aqueous matrices due to oxytetracycline photodegradation. (Leal *et al.*, 2017)

Figure 4-17: Transformation products of Enrofloxacin during photolysis and their RRT



Relevant masses identified in the literature (Baena-Nogueras *et al.*, 2017)(Babić *et al.*, 2013b)

Figure 4-18: Degradation of Enrofloxacin in buffered solutions at pH 4 and ph 8 and formation of transformation products

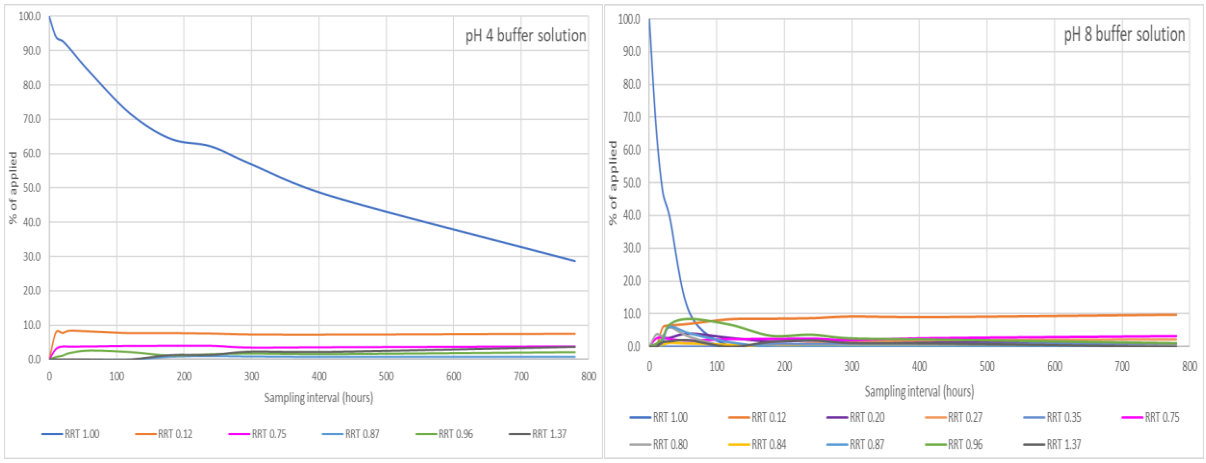


Figure 4-19: Degradation of Enrofloxacin in fulvic acid enriched solutions and formation of transformation products

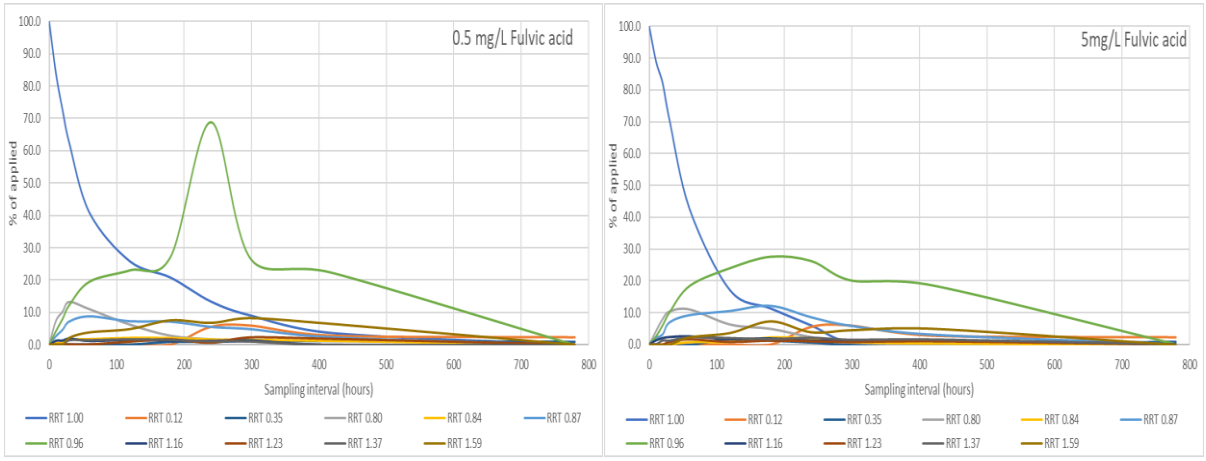
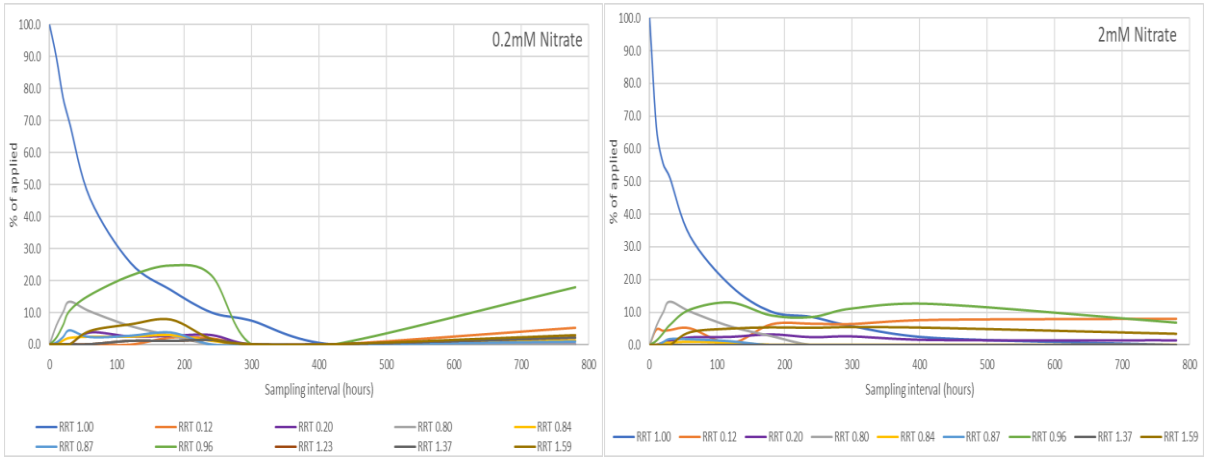


Figure 4-20: Degradation of Enrofloxacin in nitrate enriched solutions and formation of transformation products



It is identified in the literature that primarily three processes occur during the photodegradation of fluroquinolones. These are (i) oxidative degradation of the ethyl-piperazine side chain;(ii) fluorine solvolysis; and (iii) reductive defluorination. (Sturini *et al.*, 2010)

The concentration profiles of enrofloxacin photodegradation and its transformation products versus time in different matrices are shown on **Figure 4-18** to **Figure 4-20**. The highest number of transformation products was observed in the fulvic acid enriched water. Most of the transformation products reached their maximum concentration in the first 300 min of irradiation, dropping down slowly or disappearing over the duration of the experiment (12 hours).

Ciprofloxacin which is a primary degradation product of enrofloxacin (Yahya *et al.*, 2014), was also detected during the photolysis of enrofloxacin but only in pH 4 buffer solutions. Two transformation products with RRT 0.12 (m/z 115) and RRT 0.96 (m/z 374) were present at higher concentration at the end of experiments. Transformation product RRT 0.12 detected with a *m/z* of 115 was a common photodegradation path under most tested conditions and is representing the disconnected *N*-ethyl-piperazine ring that was created through the N-C bond cleavage between the quinolone and the piperazine rings. (Babić *et al.*, 2013b)

The product with RRT 0.96 showed the most abundant mass as m/z 374. It was observed in all matrices and reached the highest concentration in the solutions containing fulvic acid. Based on the molecular mass and literature search a possible structure was suggested resulting from reductive defluorination followed by solvolysis.

Enrofloxacin photoproducts exhibit an extremely significant effect towards the two bacterial strains (Escherichia coli and Staphylococcus) tested with the effect being more noticeable with the photoproducts formed under alkaline conditions. (Klementová *et al.*, 2022) (Li *et al.*, 2011b) also demonstrated that the generation of enrofloxacin's photoproducts showed an inhibition rate to Vibrio fischeri of up to 67.2%, however as the experiment was progressing the inhibition rate was decreasing indicating that the intermediates were degrading further to less toxic products.

5 ALGAE AS A REMEDIATION TECHNIQUE FOR ELIMINATING ANTIBIOTICS

5.1 Introduction

Large-scale production of wastewater is an inevitable consequence of all contemporary societies. Among all the chemicals that have drawn attention in the recent years pharmaceuticals and its related products have raised significant concerns due to their persistence and potential risk to human health and the environment. (Kumar *et al.*, 2022) Low levels of antibiotics enter and accumulate in natural environments, inducing the development of antibiotic resistant bacteria and genes.(Koch *et al.*, 2021)

The conventional remediation approaches for wastewater management employed either by pharmaceutical industries or municipal wastewater treatment plants appear to be unable to remove the antibiotics completely. (Phoon *et al.*, 2020) Concentrated animals feeding operations (CAFOs) also pose a great risk for the receiving environment primarily due to the uncontrolled spreading of manure into soils that releases high volumes of organic matter and nutrients into water bodies. (González *et al.*, 2008)

Untreated wastewaters pose a risk to human populations and the receiving fauna, so some form of treatment is always required before they are released into streams, lakes, seas, and land surfaces. (Ullah Bhat and Qayoom, 2022) When not properly managed, the high organic matter, nitrogen and phosphorous concentrations present in wastewaters can cause severe environmental problems such as eutrophication of water bodies (Carvalho *et al.*, 2013) These high levels of organics can facilitate the growth of algae, especially in areas where the water appears stagnant.

Studies have demonstrated the ability of micro-algae to treat livestock and municipal wastewater effluents to reduce the nutrients presence (Daneshvar *et al.*, 2018; Nagarajan *et al.*, 2022) In addition, wastewater remediation by microalgae is a promising eco-friendly process with no secondary pollution as long as the biomass produced is reused and utilized to produce value-added products of industrial significance. (Rawat *et al.*, 2011) Utilisation of biomass can take place in various industrial applications such as cosmetics, pigments, food, nutraceuticals, pharmaceuticals, animal feed,

and the biofertilizer industry (Barsanti and Gualtieri, 2018).The advantage is that while the microalgae will be removing excess nutrients, organic contaminants and pathogens in the wastewater, there will be also accumulation of biomass for downstream processing which might result in accomplishment of making profit from agricultural wastewater. (Alavianghavanini *et al.*, 2024)

However, there are several drawbacks and safety risks to this approach including (1) toxic effects of antibiotics and unfavorable growth conditions could inhibit photosynthetic metabolism of microalgae, leading to a low photosensitizer yield (2) microalgae growth is susceptible to wastewater compositions and environmental conditions which make the regulation of the microalgae-based antibiotics treatment performance more complex; (3) antibiotic resistance genes may transfer and accumulate after the microalgal treatment of antibiotics (Wei et al., 2021).

Micro algal cultivation is either phototrophic, mixotrophic or heterotrophic depending on the algal strains' adaptation with their environment. Micro algae have immense potential for adapting with the fluctuating environmental conditions. (Ummalyma and Sukumaran, 2014) Algae is an effective photosensitizer that can generate reactive oxygen species (ROS) under irradiation. (Nicodemus *et al.*, 2020) Algae-induced photodegradation of residual antibiotics involves many substances in the algae solution that can induce the generation of reactive oxygen species such as chlorophyll, enzymes, and extracellular organic matters. (Wei *et al.*, 2021a)

Recently there was an increase in studies where they present the removal of antibiotics utilizing different algal species. (Xiong *et al.*, 2020; Fayaz *et al.*, 2024; Wei *et al.*, 2021b) (Xiong *et al.*, 2017) studied the removal of enrofloxacin from five different algal species (Scenedesmus obliquus, Chlamydomonas mexicana, Chlorella vulgaris, Ourococcus multisporus, Micractinium resseri). (Bai and Acharya, 2017) investigated the removal of trimethoprim, sulfamethoxazole, ciprofloxacin by green alga Nannochloris sp. (Chen *et al.*, 2020) These studies have provided evidence that microalgae-based techniques might be a potential alternate strategy as an additional treatment to improve the wastewater quality.

Most of the studies are testing the effect of removal of antibiotics by using laboratory strains and performing the experiment in a clean media or under controlled environment. Only (Bai and Acharya, 2017) have tested the removal of antibiotics by cultivating freshwater algae originated from a lake. In nature, most microalgae is found in association with cyanobacteria and other aerobic or anaerobic microorganisms that can also contribute to the removal of pollutants from aquatic environments. (Subashchandrabose *et al.*, 2011) During cyanobacterial and algal photosynthesis oxygen is the key provides oxygen, a key electron acceptor to the pollutant-degrading heterotrophic bacteria. In turn, bacteria support photoautotrophic growth of the partners by providing carbon dioxide and other stimulatory means. The objective of this study is to evaluate the ability of algal and cyanobacterial consortium to remove antibiotics from aqueous solutions and check whether the high concentrations present inhibit the algal growth. Also, an attempt was made to correlate the production

of reactive oxygen species with the enhanced degradation of the antibiotics. The chemicals were chosen based on the persistence they showed during the photolysis experiments in Chapter 4. Sulfadiazine and sulfamethoxazole have high solubility and low Koc and that makes them quite mobile and diffusible in aquatic environment. (Hu *et al.*, 2022) They are frequently detected in wastewater effluents and surface waters at concentrations up to several hundreds of μg/L. (Felis *et al.*, 2020)

5.2 Experimental

5.2.1 Materials and methods

5.2.1.1 Reagents and chemicals

Standards of sulfamethoxazole and sulfadiazine were of PESTANAL® grade and purchased from Sigma-Aldrich (UK). Standard stock solutions (500 μg mL^{-1}) of all the compounds were prepared in methanol and stored in the dark at nominally -20ºC.

Treatment solutions of mix standards and mix calibration standards were prepared by dilution of the stock solutions in ultrapure water:methanol (50:50 v/v). Ultrapure water was produced in the laboratory using a Milli-RO plus 30® and a Milli-Q purifiers from Millipore (UK) Methanol (Optima grade or equivalent), formic acid and Whatman™ nylon membranes filters were obtained from Fisher Scientific (UK) and PES Captiva syringe filters were purchased from Agilent (UK).

5.2.1.2 Analytical methodology

An Agilent MSD single quadrupole mass spectrometer MS equipped with an electrospray ionization source (ESI) and connected to an Agilent 1100 HPLC system (Agilent, UK) was used for sample analysis. Separations were achieved on a Poroshell 120 EC-C18 column (2.1x100 mm, 2.7 μm particle size) equipped with a safe guard column, both purchased from Agilent. The pump was operated at a flow rate of 0.2 mL/min. The column oven temperature was 30 °C, and the injection volume was 20 μL.

The separation was performed using a simple binary gradient mobile phase consisting of 0.1% formic acid in water (A) and methanol (B). Gradient conditions were used for analysis starting at 5%B and slowly increased to 80% B over 15 minutes before returning to the initial conditions. The LC-MS was operated in positive ion mode. The drying gas temperature was 300 °C, and the capillary voltage was 4.0 kV. Nitrogen was used as a carrier gas at a flow rate of 9 L/min nebuliser pressure was set at 40psig. Analysis was performed at a full scan range of 200-800 m/z.

5.2.2 Algal culture

Algal species were cultivated for natural water sources by maintaining optimal growth conditions and a high nutrient environment. Water was collected from a lake with low anthropogenic input and filtered through a GF/A filter and transferred to a 5 L borosilicate glass bottle. In the beginning

of the cultivation period in order to create a nutrient rich media a mixture of potassium nitrate, monosodium phosphate, magnesium sulfate and calcium chloride was added to the water and placed in a growth chamber (Snijder Scientific Microclima 1750) at 20°C, ambient CO_2, 70% relative humidity and 150 μmol photons $m^{-2} s^{-1}$ in 12 : 12 h light : dark. The solution was mixed and aerated at frequent intervals. Once a week the growing algae was allowed to precipitate to the bottom of the flask and the surface water was replenished with fresh natural water from a lake with low anthropogenic input o increase the natural microorganism abundance.

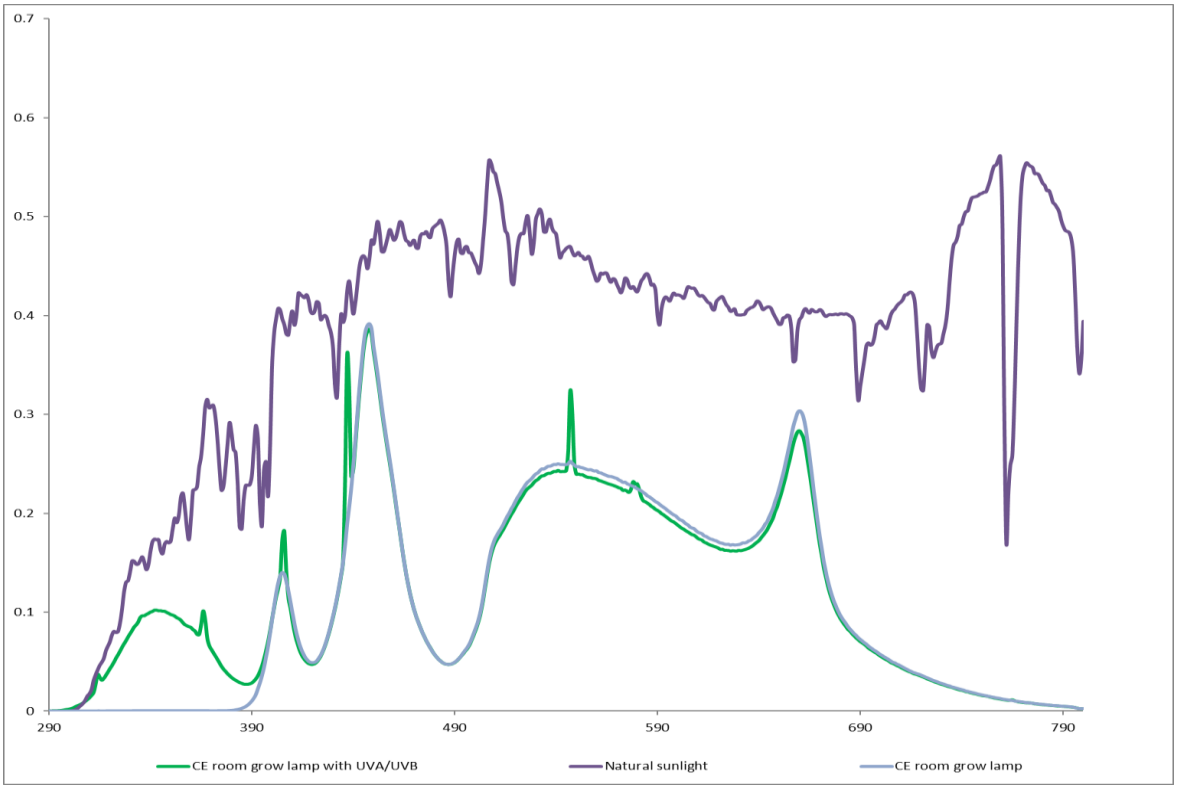
Bacterial abundance was determined by staining with SYBR gold (Molecular Probes, Invitrogen, Paisley, UK) after 10-mL aliquots were filtered onto 0.2-μm black polycarbonate filters. Enumeration was carried out under epifluorescence microscopy using a blue filter at ×1000. Bacterial cell volumes were determined from measurements on 50 cells prior to experiment initiation. For the algae determination 40-mL aliquots were stained with 4′6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Co. Ltd, Gillingham, UK) and filtered onto 0.2 μm polycarbonate filters. Analyses for abundances were undertaken using epifluorescence microscopy (×1000) using both the UV and blue excitation.

The initial concentration of algae was calculated as 3.48 x 10^7 cells/mL. The absorbance of the culture was monitored at 680 nm using a UV/Vis spectrophotometer. The concentration factor was then evaluated based on the measured absorbance against the counted cell numbers according to the linear regression model. This “calibration line” was later used to evaluate the growth during the experiment. Identification of predominant species were done using 1000x magnification, under oil immersion, with a Zeiss Axioskop microscope. The most abandoned species identified were Chlamydomonas, a type of green algae that consists of unicellular flagellates and Synechococcus a unicellular cyanobacterium. (Appendix 8)

5.2.2.1 Study set up

The study was conducted in a 3.4 m × 4.15 m walk-in Controlled Environment room (CE room) at the Lancaster Environment Centre (LEC, Lancaster University, UK). Illumination was provided by 12 400 W metal halide lamps (HQI-T 400N; Osram, St Helens, UK) for a 12 hour photoperiod (06.00 h to 20.00 h). Room air temperature ranged between 16 and 18 °C and relative humidity ranged from 60% to 80%. Room temperature and humidity were recorded by an Ektron II C sensor (HortiMaX B.V., Pijnacker, the Netherlands). The lamps were only intended for plant growth so in order to obtain lower wavelengths into the UV region to resemble natural sunlight, 3 UVA and 3 UVB lamps (Philips) were also used. Irradiation was performed under an acetate film that was used to filter any wavelength below 290nm. Macam Q203 Quantum radiometer (Macam Photometrics LTD, Livingstone, UK) was used to measure light intensity. The figure below shows the irradiance spectra for the CE room and compares the results with the light sources used for plant growth and natural sunlight.

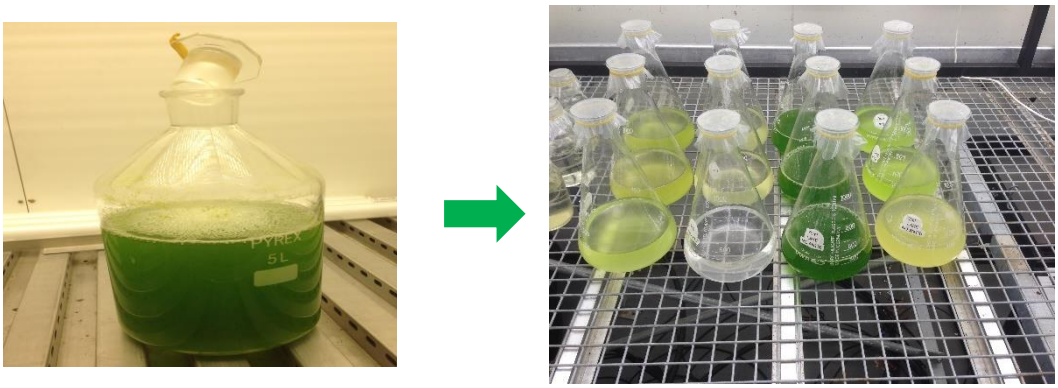
Figure 5-1: Light irradiation reading for CE room



5.2.2.2 Sample preparation

Flasks for irradiation were prepared by adding algal cells from the stock culture (7 or 70mL) to 1L Erlenmeyer flasks containing lake water. Two algal concentrations were tested with initial density of approximately 2.5×10^5 cells/ml and 2.5×10^6 cells/ml. 2 mL of the standard mix was added to each flask to achieve a final concentration of 1 mg L^{-1}. 1 mL of methanol was then added to the control flask to determine the effect of the added organic might have on the algal growth. For reference flasks were prepared with MilliQ and lake water and fortified with the standard solution as described above. Four additional flasks per test system were prepared, fortified and covered in foil. See set up in figure below. The flasks were covered with plastic to eliminate loss by evaporation.

Figure 5-2: Study set up



5.2.2.3 Sampling

Samples (1 mL) of each flask were withdrawn at regular intervals of cultivation at 0, 1, 3, 5, 7, 9, 12 hours and 1, 1.5, 2, 2.5, 3, 3.5, 4 and 5 days. The samples were then centrifuged at 15,000 rpm for 10 minutes. The supernatant was filtered through a 0.20 mm PES syringe filter (Agilent, UK) and used for determination of the residual concentrations of sulfadiazine and sulfamethoxazole in the aqueous medium using high-pressure liquid chromatography.

All of the experiments were conducted in four replicates. An aliquot was also removed to measure the algal growth in the solution by UV/vis. Measurements of pH, conductivity and dissolved oxygen were measured at each sampling interval. Dissolved oxygen values were measured close to 9 mg/L in the flasks containing algae indicating oxygen saturation in the solutions.

5.2.2.4 Reactive oxygen species measurement

To detect the production of reactive oxygen species 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) purchased from Sigma-Aldrich (UK). DCFH-DA is a non-fluorescent dye which when in contact with ROS it hydrolyses to is polar and highly fluorescent form 2',7'-dichlorofluorescein (DCF). (Rastogi *et al.*, 2010) Standard stock solution (2mM) was prepared in ethanol and stored in the dark at nominally -20ºC until further use. A 200 μL aliquot was removed at 0, 1, 2, 3, 4 and 5 days and was dispensed into a 96 well plate, the redox sensitive dye DCFH-DA was added to a final concentration of 5 μM and plates incubated in the dark for 1 hour. Fluorescence of DCF was measured by a microplate reader (FLUOstar Omega) using an excitation wavelength of 485 nm and an emission band at 500-600 nm. Although DCFH-DA is widely used for the detection of ROS, it should be noted, however, that the dye cannot be used as an indicator for a specific form of ROS.(Rajneesh *et al.*, 2017)

5.3 Results and discussion

5.3.1 Kinetic parameters and removal rates

Computer Assisted Kinetic Evaluation (CAKE) v3.3, by Tessella was used to generate degradation kinetics. The degradation followed the single first order (SFO) kinetic. Results were calculated using Single First-Order Rate Model (SFO)

$C_t = C_0 e^{-kt}$

Where, C_t = concentration at time t
C_0 = initial concentration or percent applied radioactivity
e = base e
k = rate constant of decline 1/days
t = time

The results were evaluated based on the error (chi2) observed which was <15% for all matrices tested and the t-test or confidence intervals which were <0.05. r^2 values were slightly lower than the

ones observed when biphasic models First Order Multi Compartment (FOMC) or Double First-Order in Parallel (DFOP) were used however the other parameters calculated exceed the acceptable limits.

Table 5-1: Kinetics statistics and DT_{50} calculations for sulfamethoxazole

Parameters	MilliQ water	Surface water	Low algal concentration	High algal concentration
DT_{50} (days)	1.87	14.8	3.3	3.52
DT_{90} (days)	6.22	49	30.2	11.7
chi2	9.64	2.21	3.3	3.63
r^2	0.8941	0.7551	0.7675	0.9413
t-test	6.06e-022	6.30e-014	9.92e-018	1.096e-031
k (day^{-1})	0.3701	0.04696	0.07625	0.1969

Figure 5-3: Removal percentages for sulfadiazine in irradiated and dark control samples

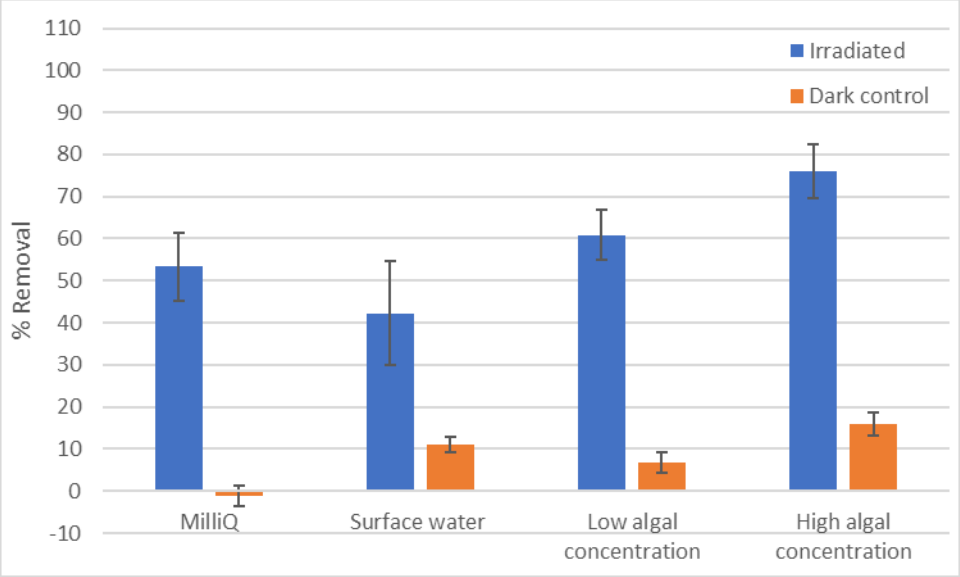
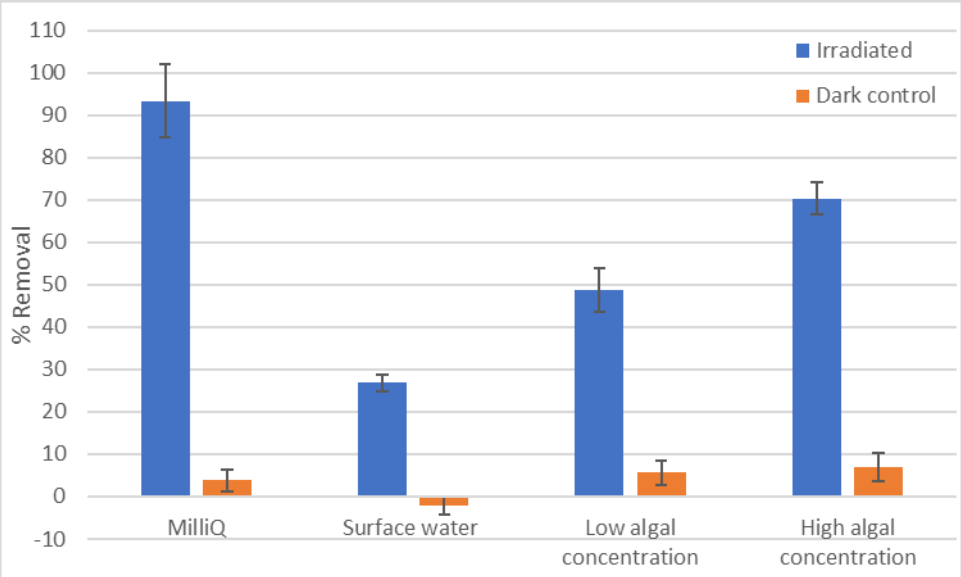


Table 5-2: Kinetics statistics and DT_{50} calculations for sulfadiazine

Parameters	MilliQ water	Surface water	Low algal concentration	High algal concentration
DT_{50} (days)	4.29	5.56	3.24	2.74
DT_{90} (days)	14.2	18.5	10.8	9.09
chi2	7.51	3.61	8.06	6.25
r^2	0.7906	0.8259	0.8087	0.8154
t-test	2.26e-019	1.10e-016	1.04e-019	5.55e-018
k (day^{-1})	0.1618	0.1247	0.2137	0.2500

Figure 5-4: Removal percentages for sulfamethoxazole in irradiated and dark control samples



The degradation of sulfadiazine attributed to direct photolysis in a clean media (MilliQ water) and surface water was 60 and 56% (mean value over 4 replicates) respectively. For sulfomethoxazole the corresponding values were 93% and 26%. A much shorter half life for MilliQ water was observed in this set up when compared to the results obtained in Chapter 3. The irradiation spectra of the light source shows an increased intensity in the UVA and UVB region compared to the one obtained from xenon lamp and in that region sulfadiazine and sulfamethoxazole show a higher overlap with this light setting. (Batchu *et al.*, 2013) also found that when sulfamethoxazole is irradiated under 350 nm it degrades much faster than under simulated sunlight. Also the lower % removal observed in the surface water is because sulfamethoxazole at environmental relevant pHs (pH 7-8) appears to be less susceptible to photolysis because it exists primarily in its anionic form. (Boreen *et al.*, 2004)

For sulfadiazine in the presence of the algal/cyanobacteria consortium the removal was enhanced and reached 79%. The hydrolysis observed in the dark control containing lake water was up to 11%. This value for the dark control containing high algal concentration was 16%. This indicated that there was a minimal degree of bioaccumulation into the algal species and degradation was primarily due to algae mediated photolysis via indirect photodegradation.

Sulfomethoxazole exhibited a similar pattern even though the overall removal attributed to algal species was 68%, it showed that algae enhanced the removal by 42% when compared to the surface water alone. Accumulation into the cells was minimal and reached a maximum of 6% in the dark control when compared to the lake water. (Xie *et al.*, 2020) showed that the biosorption of sulfadiazine to microalga Chlamydomonas sp. Tai-03 was also minimal.

It has been noted that the removal of certain antibiotics can be enhanced through indirect photodegradation when algae present in the system with the presence of reactive oxygen species such as hydroxyl radicals. (Xiong *et al.*, 2016) It is difficult to find comparable results in the literature because most studies use a single laboratory grown strain where in this instance the algal species were a mixture of different native species to the lake water. (Xiong *et al.*, 2017) tested the removal of enrofloxacin in five different algal species and their consortium. This study demonstrated that each culture behaves differently and when present in a mixture it was more sensitive to enrofloxacin. Overall, the removal capacity of the consortium was 26% which was comparable to the removal capacity observed in the individual strains and was in the range of 18 to 26%. However this study highlighted the need for further evaluation of competing mechanisms when there is a mixture of algal cultures in order to understand their action mechanisms.

(Bai and Acharya, 2016) studied the removal of sulfomethoxazole by Nannochloris sp and showed low removal (30%) compared to 74% (average for Haematococcus pluvialis, Selenastrum capricornutum, Scenedesmus quadricauda and Chlorella vulgaris) (Kiki *et al.*, 2020) For sulfadiazine (Chen *et al.*, 2020) showed a removal rate using Chlorella vulgaris and a cyanobacterium (Chrysosporum ovalisporum) of 5.97% to 15.11%, respectively. These results are comparable to the values find in this study as the algal driven degradation over photodegradation was 23%.

It has been identified in the literature that the main pathways of antibiotic removal include bioadsorption, biodegradation, bioaccumulation, photodegradation, volatilization, and hydrolysis. (Li *et al.*, 2022) However as to what degree these pathways and mechanisms will take place is completely dependent on the specific physicochemical properties of the individual compound and also the species used. (Hena *et al.*, 2021)

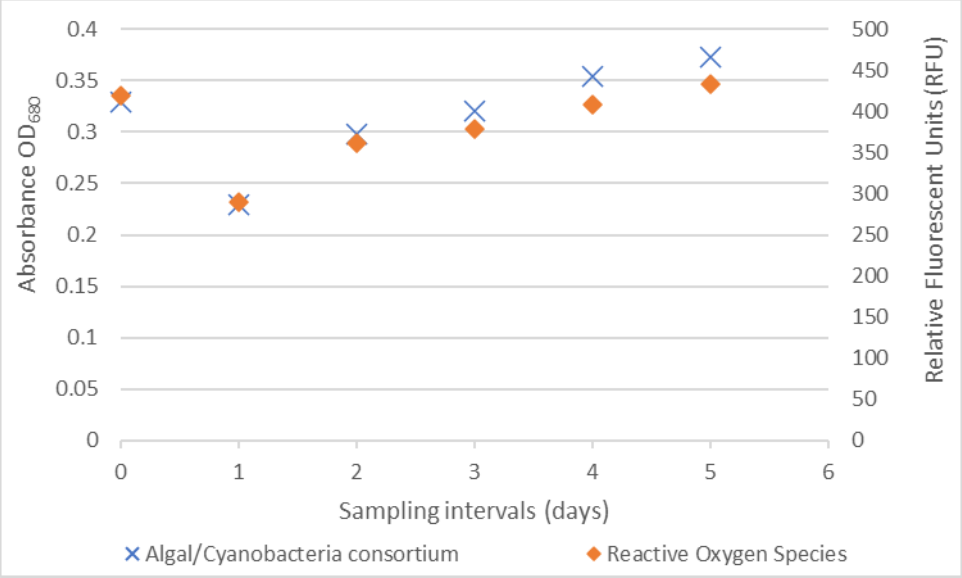
5.3.2 Growth capacity and ROS production

In general, there are a lot of studies that showcase the toxicity and negative response of antibiotics against species such as algae (Chen and Guo, 2012). Algal species are routinely used as indicator organisms in risk assessment studies for human and veterinary pharmaceuticals due to the fact that they show quick response times and high sensitivity. (EMEA, 2006) When exposed to antibiotics, algae initiate stress response mechanisms that degrade toxic antibiotics and assist algal survival. (Zhang *et al.*, 2021) These stress responses usually contain variations of growth patterns, photosynthetic activity, intracellular enzyme and biochemical components and which will affect the antibiotic removal. (Wan *et al.*, 2021) (Tian *et al.*, 2019) has demonstrated that the algal induced photodegradation of

ciprofloxacin is attributed to the presence of $^{3}EOM*$ that are generated by algae under irradiation but also to the small amount of other ROS, such as $^{1}O_2$, ·OH. Contrary to that (Hsiao *et al.*, 2021) indicated that the presence primarily of $^{3}IOM*$, $^{3}DOM*$ and ·OH were the dominant reactive species responsible for the degradation of acetaminophen. In this study the presence of reactive oxygen species was verified by using DCFH-DA however even though the dye can be used as an indicator further identification of the exact ROS can not be made.

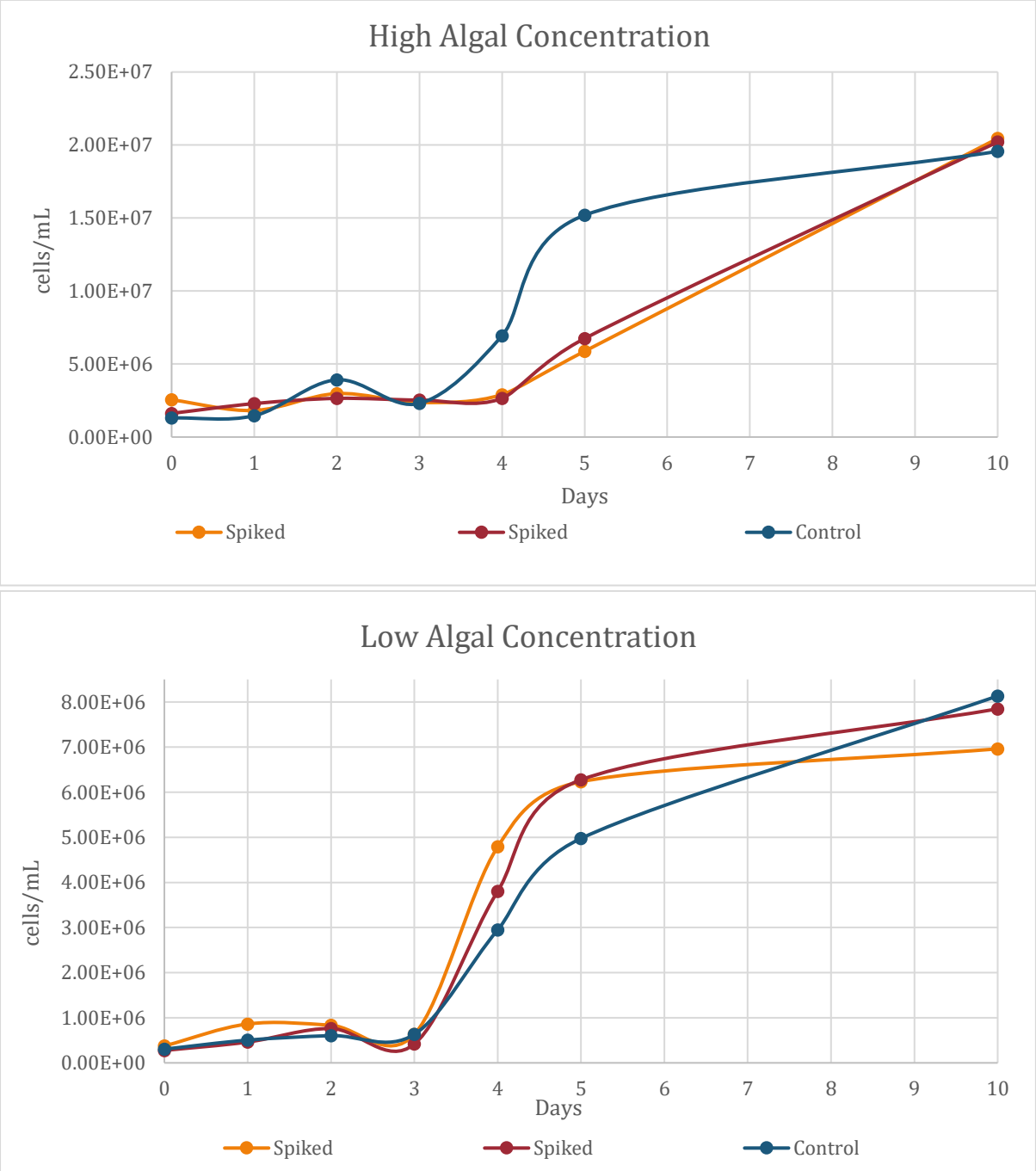
Figure 5-5 shows how the consortium recovers from the toxicity induced by high concentrations of antibiotics by producing reactive oxygen species as a mechanism against oxidative damage.

Figure 5-5: Correlation of growth patterns of algal/cyanobacterial consortium under antibiotic stress with production of Reactive Oxygen Species (ROS)



For the first two days the growth has slowed down but as this was also observed in the control samples it might be attributed to the presence of methanol. Optical density at 680nm for the high algal concentration solutions spiked with antibiotics decreased from 0.189 at day 0 to 0.138 at day 1 before starting to rise again to 0.219 by day 2. The same pattern was followed also in the control solution with the absorbance values being 0.113, 0.102 and 0.285, respectively. After the initial shock the samples that were spiked with antibiotics showed the same exponential growth with the control indicating that the species were resistant to the antibiotic presence. (Kurade *et al.*, 2016) after showing that microalgae needed a 2 day adaptation period to synthesize enzymes that are required for the metabolism of organic contaminants also showed a significant enhancement of the microalgal growth in the exponential growth phase suggesting less/non-toxic nature of the photoproducts.

Figure 5-6: Growth patterns of algal organic matter under antibiotic stress



Under mixotrophic cultivation, microalgae can simultaneously utilize light and organics, which facilitates higher biomass accumulation and growth rate in a specific cycle (Gao et al., 2022) This have also been observed by (Cao *et al.*, 2018) where solutions of Chlamydamonas spiked with cefradine concentrations within the range 0.5–10 mg/L promoted the algal growth instead of inhibiting it. Also (Xiong *et al.*, 2016) showed that five different microalgae species (Scenedesmus obliquus, Chlamydomonas mexicana, Chlorella vulgaris, Ourococcus multisporus and Micractinium resseri) was negligibly influenced when dosed with enrofloxacin concentration <1 mg L^{-1} and could recover from

the toxicity induced by high concentrations. The growth might have also been promoted by the removal of most of the antibiotics by day 10. These responses can be interpreted as an internal defence mechanism and might allow us to establish a better understanding on how we can utilise microalgae strains or cyanobacterial/algal/bacterial consortiums for the treatment of aquatic environments contaminated with emerging pollutants.

6 CONCLUSIONS

A fast and simple method for the analysis of 15 commonly used antibiotics in water samples deriving from a catchment area was developed. The method combines online solid phase extraction using a reusable online trapping column combined with analytical separation on a C18 analytical column and detection by a single quadrupole mass spectrometer. The method was fully validated for detection and quantification limits as well as linearity, repeatability, and matrix effects. The method gave an excellent linear response ($r^2 > 0.99$) and detection limits for all compounds (1–50 ng L^{-1}). These levels are comparable with other method that employed more sensitive instruments for analysis. This work has showed that online SPE using just and additional pump and a clean up cartridge is a quick and cheap alternative on sample pre-treatment without the need of extra expensive instrumentation. The method was used to monitor diffuse pollution from farm and WwTWs in a rural area.

Out of the 15 antibiotics monitored thirteen of the antibiotics were detected in at least one sample. The only ones that were not detected were two cephalosporins, cefquinome and ceftiofur. Highest concentrations were observed for sulfamethoxazole, marbofloxacin, oxytetracycline, penicillin G, anhydroerythromycin and cefalonium and reached 1659, 1009, 931, 816, 544 and 293 ng/L respectively. Other antibiotics were also detected but concentrations were mostly below 50ng/L. The highest measured concentrations were associated with a discharge point of WWTP. Sampling during March and April showcased that wet weather conditions facilitate the steady release of antibiotics in rural areas around the farms. However, the one source that was impacting the most the aquatic ecosystem in Eden catchments was the effluent discharge of the wastewater plant. These findings highlight the requirement of a prominent and coordinated upgrade of WWTP treatments in order to be able to successfully remove antibiotics during the process. However, environmental quality standards or other recognised limits for antibiotics do not exist, meaning their release into the environment is not currently regulated. This is partly due to the lack of a standardised experimental method that can be used routinely to generate data on the observed concentrations of antibiotic in the environment. Experimental monitoring method should not only focus of monitoring the water quality, but it needs to include soil and sediment. This will allow a better understanding on the exact distribution of these antibiotics in the environment.

Once released into the aquatic environment, pharmaceuticals may undergo different degradation processes. Photodegradation is an important route of elimination for light-sensitive pharmaceuticals, such as antibiotics. In this study, the fate of two sulphonamides, one tetracycline and

one fluoroquinolone were investigated in different matrices to establish possible degradation patterns. A comparison between laboratory acquired photolysis data and field data was made that identified an increase need for more routine field work. Degradation under natural sunlight for more photosensitive compounds such as oxytetracycline and enrofloxacin, appeared in line with the laboratory results however there wear big discrepancies between the laboratory obtained values and the ones derived from the outdoor experiment for the more persistent compounds sulfamethoxazole and sulfadiazine. An attempt was made to identify the major degradation products and gradient were developed that successfully separates them for easier identification. Unfortunately, the exact mass and possible metabolite elucidation was not achieved due to instrument limitations. Future work should include the positive identification of these metabolites. This will increase the knowledge on the presence of antibiotics and relevant metabolites in waters from Eden catchment. Also, it could enable the expansion of the current online SPE method to allow for a more efficient follow-up monitoring in the near future for evaluation of water quality and perform risk assessment studies for contaminants of emerging concern in surface waters.

Recently, microalgae-based technology has been explored as a potential alternative for the treatment of wastewater containing antibiotics by adsorption, accumulation, biodegradation, photodegradation, and hydrolysis. In this role a primitive study was conducted to evaluate the removal rates and degradation of two sulphonamides in the presence of naturally grown algae.

Both of compounds exhibited faster degradation in the presence of algal species when combined with irradiation. Removal of sulfadiazine and sulfamethoxazole in the presence of algae was enhanced by 23 and 42% respectively when compared to the degradation rate achieved only by direct photolysis. The hydrolysis in the dark control for sulfadiazine and sulfamethoxazole was 11% and 13% respectively in the lake sample compared to 16 and 19% in the high concentration algal solution. This indicate that the uptake of the antibiotics in the algal cells was minimal.

Further work is required to understand under which mechanism algae is promoting degradation of antibiotics but nevertheless it appears that the utilisation of natural grown cultures in settling ponds might be the way forward to tackle diffuse pollution deriving from farm run off. Further work might include studying the interactions between different microalgae species during the degradation of organic pollutants or investigating the competitive and synergetic effects of reactive oxygen species and which contribute more on the photodegradation of pollutants in the presence of natural organic matter in surface waters.

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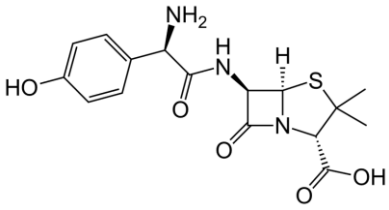
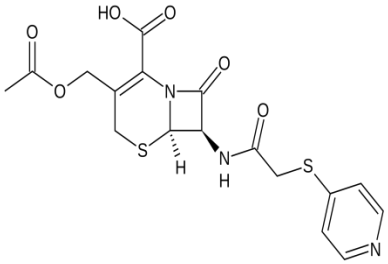
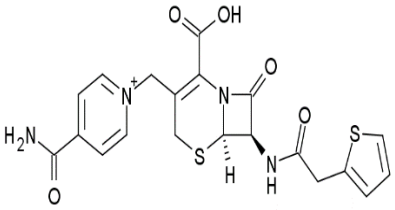
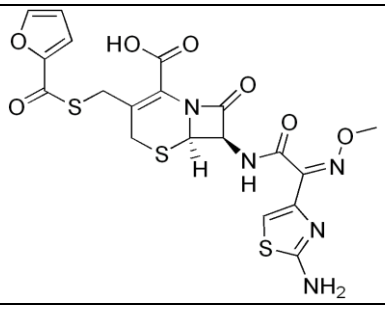
8 APPENDICES

Appendix 1 Physical-chemical properties and molecular structures of major antibiotics

Name	**Amoxicillin (AMX)** [1]	**Cephapirin (CFP)** [2]
CAS Number	26787-78-0	21593-23-7
Structure		
Molecular formula	$C_{16}H_{19}N_3O_5S$	$C_{17}H_{17}N_3O_6S_2$
Molecular weight	365.4	423.5
pKa	2.63, 7.16, 9.55	2.74, 5.13
Solubility in water (g/L)	4.0	1.03
logKow	0.87	-1.15
Koc	108.4	44.8
Excretion rate	48-75%	48%
Name	Cefalonium (CFL) [3]	Ceftiofur (CFT) [4]
CAS Number	5575-21-3	80370-57-6
Structure		
Molecular formula	$C_{20}H_{18}N_4O_5S_2$	$C_{19}H_{17}N_5O_7S_3$
Molecular weight	458.51	523.5
pKa	3.3	2.68
Solubility in water (g/L)	0.0798	0.023
logKow	-2.63	0.54
Koc	182.2	3700
Excretion rate	7-13%	30-55%

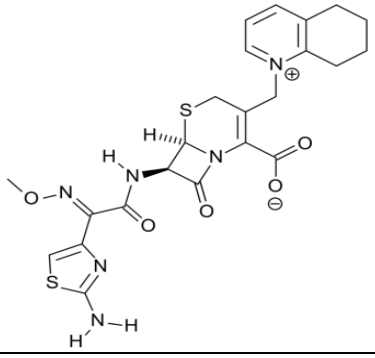
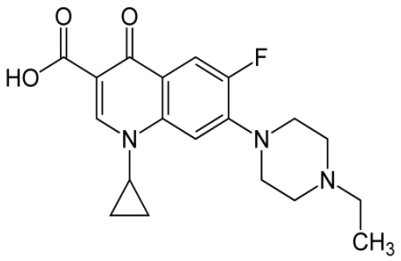
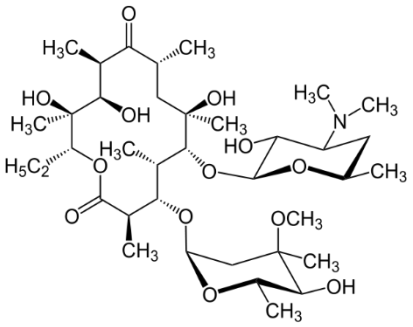
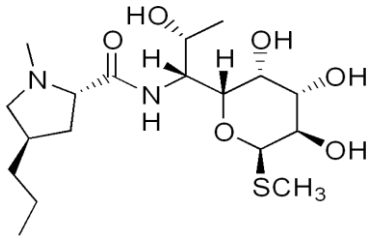
[1] https://echa.europa.eu/registration-dossier/-/registered-dossier/12616/4/22 (accessed on 05 April 2020)

[2,3] EPI Suite v4.11 for Koc, [2] https://sitem.herts.ac.uk/aeru/vsdb/Reports/1845.htm (accessed on 31 March 2020) , [2,4] (Ribeiro and Schmidt, 2017)

[3] (Anon, 1999)

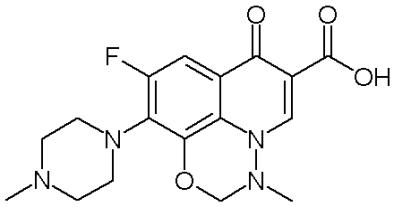
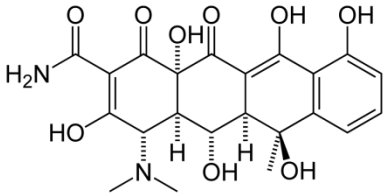
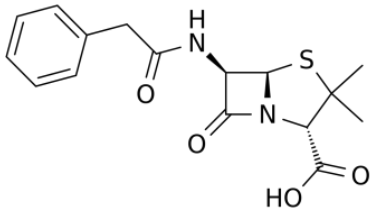
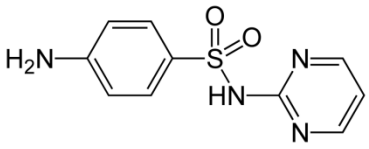
[4] https://sitem.herts.ac.uk/aeru/vsdb/Reports/1822.htm (accessed on 31 March 2020)

Appendix 1 Physical-chemical properties and molecular structures of major antibiotics (continued)

Name	**Cefquinome (CFQ)** [5]	**Enrofloxacin (ENR)** [6]
CAS Number	84957-30-2	93106-60-6
Structure		
Molecular formula	$C_{23}H_{24}N_6O_5S_2$	$C_{19}H_{22}FN_3O_3$
Molecular weight	528.60	359.4
pKa	2.75, 3.75, 7.54	6.21
Solubility in water (g/L)	9.0	130
logKow	-1.49	4.7
Koc	-	16506-768740
Excretion rate	33-49%	17%
Name	Erythromycin (ERY) [7]	Lincomycin (LIN) [8]
CAS Number	114-07-8	154-21-2
Structure		
Molecular formula	$C_{37}H_{67}NO_{13}$	$C_{18}H_{34}N_2O_6S$
Molecular weight	733.9	406.5
pKa	8.88	7.6
Solubility in water (g/L)	2.0	0.9
logKow	3.06	0.29
Koc	570	59
Excretion rate	27.2-36.1%	60-85%

[5](Dołhań *et al.*, 2017), (EMEA/MRL/405/98-Final, 1998), https://sitem.herts.ac.uk/aeru/vsdb/Reports/1797.htm (accessed on 31 March 2020)
[6](EMEA/MRL/388/98-Final, 1998), https://sitem.herts.ac.uk/aeru/vsdb/Reports/1762.htm (accessed on 31 March 2020)
[7]https://sitem.herts.ac.uk/aeru/vsdb/Reports/1850.htm (accessed on 31 March 2020),
(EMEA/MRL/720/99-Final, 2000)
[8]https://sitem.herts.ac.uk/aeru/vsdb/Reports/1886.htm(accessed on 31 March 2020), (EMEA/V/A/123, 2008)

Appendix 1 Physical-chemical properties and molecular structures of major antibiotics (continued)

Name	**Marbofloxacin (MAR)** [9]	**Oxytetracycline (OTC)** [10]
CAS Number	84957-30-2	79-57-2
Structure		
Molecular formula	$C_{17}H_{19}FN_4O_4$	$C_{22}H_{24}N_2O_9$
Molecular weight	362.5	460.4
pKa	5.69, 8.02	3.57, 7.49, 9.88
Solubility in water (g/L)	1000	1000
logKow	-2.92	-1.22
Koc	33.39	27792-93317
Excretion rate	51.6-56.7 %	60-75%
Name	Penicillin G (PEN) [11]	Sulfadiazine (SDZ) [12]
CAS Number	61-33-6	68-35-9
Structure		
Molecular formula	$C_{16}H_{18}N_2O_4S$	$C_{10}H_{10}N_4O_2S$
Molecular weight	334.4	250.2
pKa	2.74	2.49, 6.48
Solubility in water (g/L)	0.1	0.13
logKow	1.5	0.09
Koc	2.68	59-1625
Excretion rate	50-70%	44%

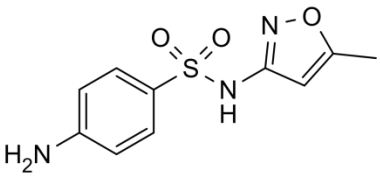
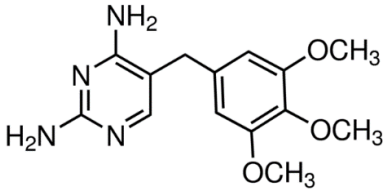
[9] (Schneider *et al.*, 2014), EPI Suite v4.11 for Koc

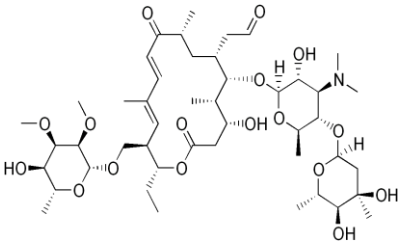
[10] https://sitem.herts.ac.uk/aeru/bpdb/Reports/3144.htm, http://www.inchem.org/documents/jecfa/jecmono/v27je06.htm (accessed on 31 March 2020)

[11] (Hirsch *et al.*, 1999) [12] https://sitem.herts.ac.uk/aeru/vsdb/Reports/1740.htm (accessed on 31 March 2020)

[12] (Veterinary Medicines Directorate, 2018), (Lamshöft *et al.*, 2007)

Appendix 1 Physical-chemical properties and molecular structures of major antibiotics (continued)

Name	**Sulfamethoxazole (SXM)** [13]	**Trimethoprim (TMP)** [14]
CAS Number	723-46-6	738-70-5
Structure		
Molecular formula	$C_{10}H_{11}N_3O_3S$	$C_{14}H_{18}N_4O_3$
Molecular weight	253.2	290.32
pKa	5.9	7.12
Solubility in water (g/L)	0.610	0.4
logKow	0.890	0.91
Koc	1.2-94.9	606-1650
Excretion rate	15%	60%

Name	Tylosin (TYL) [15]
CAS Number	1401-69-0
Structure	
Molecular formula	$C_{46}H_{77}NO_{17}$
Molecular weight	916.10
pKa	7.73
Solubility in water (g/L)	5.0
logKow	1.63
Koc	110-95532
Excretion rate	6-33%

[13,15] (Cycoń *et al.*, 2019)

[14] (Veterinary Medicines Directorate, 2018)

[15] http://www.inchem.org/documents/jecfa/jecmono/v29je08.htm (accessed on 31 March 2020)

Appendix 2 Antiobiotic concentrations detected in receiving environmental matrices

Table A2-1: Antibiotics concentrations in wastewater effluents and receiving environmental matrices in different geographical regions

Target analytes	Country	Matrices analysed	Antibiotics most frequently present	Highest environmental concentrations (ng/L)	Comments	Reference
70 pharmaceutical EPA priority pharmaceuticals	United States	WWTP effluent SW (river)	Clarithromycin Erythromycin Sulfamethoxazole Trimethoprim	WWTP effluent: 172 / SW: 580 WWTP effluent: 1200 / SW: 52 WWTP effluent: 53 / SW: 210 WWTP effluent: 429 / SW: 105	Method developed to facilitate routine multi residue monitoring of pharmaceuticals in drinking water and wastewater samples	(Ferrer *et al.*, 2010)
25 pharmaceuticals (12 antibiotics)	United States	WWTP effluent SW1 (discharge point) SW2 (downstream) SW3 (far downstream)	Lincomycin Monensin Sulfamethazine Sulfadimethoxine Sulfamethoxazole	WWTP effluent: 3.6 / SW1: 2.0 / SW2: 0.9 / SW3: 5.7 WWTP effluent: 1.2 / SW1: 0.8 / SW2: 0.6 / SW3: 0.8 WWTP effluent: 6.8 / SW1: 5.5 / SW2: 3.5 / SW3: 4.1 WWTP effluent: 1.2 / SW1: 0.8 / SW2: 0.6 / SW3: 0.8 WWTP effluent: 49.9 / SW1: 50.4 / SW2: 37.4 / SW3: 41.2	Detection of antibiotics 1500m away from the discharge point showed that effluent residues can be transported significant distances in receiving streams. Higher concentrations were observed in the less advanced WWTP.	(Brown *et al.*, 2015)
20 pharmaceuticals (12 antibiotics)	Colombia	WWTP influent WWTP effluent	Azithromycin Ciprofloxacin Sulfamethoxazole Trimethoprim	WWTP effluent: 4570 WWTP effluent: 1070 WWTP effluent: 831 WWTP effluent: 456	Incomplete removal of antibiotics observed. Higher concentrations derived from the WWTP that used only physicochemical processes in contrast with the other that used also biological processes	(Botero-Coy *et al.*, 2018)

Appendix 2 Antibiotic concentrations detected in receiving environmental matrices (continued)

Table A2-1: Antibiotics concentrations in wastewater effluents and receiving environmental matrices in different geographical regions (continued)

Target analytes	Country	Matrices analysed	Antibiotics most frequently present	Highest environmental concentrations (ng/L)	Comments	Reference
18 antibiotics	China	WWTP influent WWTP effluent	Ofloxacin Sulfamethoxazole Sulfadiazine Erythromycin Roxithromycin	WWTP effluent: 1200 WWTP effluent: 460 WWTP effluent: 560 WWTP effluent: 300 WWTP effluent: 360	Insufficiently removal ranges from -34 to 72% The concentrations of antibiotics in winter were higher than in spring and autumn.	(Gao *et al.*, 2012)
12 antibiotics	China	WWTP influent WWTP effluent SW (receiving WWTP effluent)	Sulfadiazine Sulfamethoxazole Sulfamethazine Amoxicillin Ofloxacin Roxithromycin	WWTP effluent: 14.14 / SW: 53.91 WWTP effluent: 70.60 / SW: 25.97 WWTP effluent: 14.20 / SW: 10.08 WWTP effluent: 3.48 / SW: 2.29 WWTP effluent: 1308.0 / SW: 16.14 WWTP effluent: 33.37 / SW: 2.01	Elimination of antibiotics varied from -442.8 to 100% showcasing that the removal of antibiotics through the STPs might be incomplete.	(Wu *et al.*, 2016)
43 antibiotics	China	SW (heavily industrialised-populated area)	Sulfamethoxazole Sulfadoxine Lincomycin Florfenicol Erythromycin Doxycycline	SW: 234 SW: 210 SW: 53.8 SW: 63.5 SW:4.66 SW:947	Presence of veterinary antibiotics showed that livestock also contribute to the antibiotic loading of Lake Taihu along with domestic sources.	(Zhou *et al.*, 2016)
14 antibiotics	China	Sediment, SW (receiving WWTP effluent)	Sulfamethoxazole Sulfadiazine Trimethoprim Roxithromycin Erythromycin Cefalozin Cefotaxime Tetracycline Oxyteracycline Chlortetracycline	SW: 464 SW: 34.8 SW: 106 SW: 223 SW: 300 SW: 11.6 SW: 33.2 SW: 854 SW: 164 SW: 272	Tetracyclines were also present in sediment samples (max.2080 μg/kg) Concentrations were higher in summer months. Redundancy analysis concluded that anthropogenic activities contribute significantly to the persistence of antibiotics pollution.	(Jia *et al.*, 2018)

Appendix 2 Antibiotic concentrations detected in receiving environmental matrices (continued)

Table A2-1: Antibiotics concentrations in wastewater effluents and receiving environmental matrices in different geographical regions (continued)

Target analytes	Country	Matrices analysed	Antibiotics most frequently present	Highest environmental concentrations (ng/L)	Comments	Reference
26 antibiotics	China	WWTP effluent SW (receiving WWTP effluent)	Sulfamethoxazole Norfloxacin Oflaxocin Anhydroerythromycin Lincomycin Clarithromycin	WWTP effluent: 29.67 / SW: 11.68 WWTP effluent: 37.62/ SW:15.47 WWTP effluent: 94.47/ SW: 9.08 WWTP effluent: 52.35 / SW: 6.37 WWTP effluent: 19.31 / SW: 1.56 WWTP effluent: 27.02 / SW: 3.89	Quinolines most detected antibiotics. Up to 22 antibiotics detected 4 km downstream of WWTP	(Zheng *et al.*, 2022)
55 pharmaceuticals (22 antibiotics)	Japan	WWTP influent WWTP effluent SW (river)	Azithromycin Clarithromycin Roxythromycin Ofloxacin Sulfamethoxazole Sulfapyridine Trimethoprim	WWTP effluent: 251 / SW: n.d WWTP effluent: 812 / SW: 46 WWTP effluent: 84 / SW: 12 WWTP effluent: 558 / SW: 7 WWTP effluent: 142 / SW: 38 WWTP effluent: 255 / SW: 38 WWTP effluent: 88 / SW: 6	The presence of small amount of lincomycin and sulfamonomethoxine in rivers showed a positive correlation with swine population in the catchment area.	(Hanamoto *et al.*, 2018)
13 antibiotics	South Korea	SW (receiving WWTP effluent)	Oxytetracycline Tetracycline Chlortetracycline Trimethoprim Sulfamethazine Sulfamethoxazole Sulfadimethoxime Enrofloxacin Florfenicol	SW: 1236 SW: 2093 SW: 793 SW: 587 SW: 67 SW: 270 SW: 80 SW: 113 SW: 340	Four WWTP's with capacities of > 1 million m^3/day Higher concentrations observed during dry season.	(Kim *et al.*, 2016)
12 antibiotics	Japan	WWTP influent WWTP effluent SW (river)	Azithromycin Clarithromycin Erythromycin Sulfamethoxazole Sulfapyridine Sulfamethazine Trimethoprim	WWTP effluent: 622 / SW: 329 WWTP effluent: 233 / SW: 714 WWTP effluent: 209 / SW: 138 WWTP effluent: 74 / SW: 33.9 WWTP effluent: 260 / SW: 151 WWTP effluent: n.d / SW: 62.9 WWTP effluent: 54 / SW: 13.6	Elevated residues were measured in urban rivers than in rural rivers and were correlated with sewage markers (crotamiton and carbamazepine).	(Murata *et al.*, 2011)

Appendix 2 Antibiotic concentrations detected in receiving environmental matrices (continued)

Table A2-1: Antibiotics concentrations in wastewater effluents and receiving environmental matrices in different geographical regions (continued)

Target analytes	Country	Matrices analysed	Antibiotics most frequently present	Highest environmental concentrations (ng/L)	Comments	Reference
14 antibiotics	Italy	WWTP influent WWTP effluent SW (river)	Ofloxacin Clarithromycin Erythromycin Lincomycin Spiramycin Sulfamethoxazole	WWTP effluent: 133 / SW: 18.1 WWTP effluent: 580 / SW: 44.8 WWTP effluent: 39.3 / SW: 8.1 WWTP effluent: 8.5/ SW: 10.9 WWTP effluent: 209 / SW: 17.9 WWTP effluent: 35.6 / SW: 11.4	Macrolides and quinolones showed the lower removal rates. Calculated that STPs contribute 5 kg/day, or about 1.8 tons/year of antibiotics in River Po.	(Zuccato *et al.*, 2010)
12 antibiotics	Italy	WWTP influent WWTP effluent SW (river)	Azithromycin Ciprofloxacin Clarithromycin Trimethoprim	WWTP effluent: 176 / SW: 7 WWTP effluent: 979 / SW: 25 WWTP effluent: 304 / SW: 6 WWTP effluent: 47 / SW: 2	The sampling was conducted during a dry period, to avoid dilution effects due to rainfall.	(Verlicchi *et al.*, 2014)
23 antibiotics	France	WWTP effluent SW1 (upstream) SW2 (downstream) SW3 (far downstream)	Erythromycin Trimethoprim Sulfamethoxazole Norfloxacin Ciprofloxacin Ofloxacin	WWTP effluent: 1492 / SW2: 913 SW3: 572 WWTP effluent: 5316/ SW2: 1573 SW3: 1364 WWTP effluent: 12848 / SW2: 3066 SW3: 2708 WWTP effluent: 9347 / SW2: 1261 SW3: 181 WWTP effluent: 3403 / SW2: 1523 SW3: 414 WWTP effluent: 8637/ SW2: 2888 SW3: 855	Upstream only fluoroquinolones were measured at ≤10 ng L^{-1} indicating the possible transfer from agricultural fields. WWTP built in 1979 showed higher effluent values compared to the one built in 2009.	(Dinh *et al.*, 2017)
43 pharmaceuticals (9 antibiotics)	Spain	WWTP effluent SW (river)	Roxithromycin Clarithromycin Erythromycin Tylosin Trimethoprim Metronidazole	WWTP effluent: 63.8 / SW: - WWTP effluent: 212 / SW: 27.9 WWTP effluent: 24.5 / SW: 42.4 WWTP effluent: 266.9/ SW: 0.77 WWTP effluent: 249 / SW: 29.1 WWTP effluent: 111 / SW: 17.4	Residues were interlinked with the river flow and dilution factor, resulting in higher concentrations in small tributary rivers than in the Ebro river.	(Silva *et al.*, 2011)

Appendix 2 Antibiotic concentrations detected in receiving environmental matrices (continued)

Table A2-1: Antibiotics concentrations in wastewater effluents and receiving environmental matrices in different geographical regions (continued)

Target analytes	Country	Matrices analysed	Antibiotics most frequently present	Highest environmental concentrations (ng/L)	Comments	Reference
77 pharmaceuticals (20 antibiotics)	Spain	SW (river receiving WWTP effluent)	Azithromycin Clarithromycin Sulfadiazine Sulfamethazine Trimethoprim Ofloxacin	33.4 141 50.9 53.1 59.9 79.9	Sulfadiazine and Clarithromycin detected in all the samples. Concentrations observed along the river remained unaffected by the dilution effect due the continuous release of WWTP effluents.	(López-Serna et al., 2012)
81 pharmaceuticals (7 antibiotics)	Spain	WWTP effluent SW1 (upstream) SW2 (discharge point) SW3 (downstream)	Ciprofloxacin Azithromycin Ofloxacin Clarithromycin Sulfamethoxazole Trimethoprim	WWTP effluent: 370 / SW1: 6 SW2: 36 WWTP effluent: 287/ SW1: 29 SW2: 43 / SW3: 27 WWTP effluent: 157 / SW1: 19 SW2: 33 / SW3: 25 WWTP effluent: 238 / SW1: 19 SW2: 17 / SW3: 2 WWTP effluent: 19 / SW1: 8 SW2: 9 / SW3: 8 WWTP effluent: 11/ SW1: 8 SW2: 9 / SW3: 8	Pharmaceuticals also detected before the WWTP discharge point, indicating a either diffuse contamination or other possible sources.	(Collado *et al.*, 2014)
21 pharmaceuticals (9 antibiotics)	Spain	SW (receiving runoff from agricultural areas and WWTP effluent)	Trimethoprim Sulfamethazine Sulfadiazine Enrofloxacin Salinomycin	73.4 63.8 55.6 118.4 58.3	Out of the 51% positive samples, 13 % originated from sites downstream of a WWTP and 38 % of the samples near the collection point of a DWTP were positive.	(Iglesias *et al.*, 2014)

Appendix 2 Antibiotic concentrations detected in receiving environmental matrices (continued)

Table A2-1: Antibiotics concentrations in wastewater effluents and receiving environmental matrices in different geographical regions (continued)

Target analytes	Country	Matrices analysed	Antibiotics most frequently present	Highest environmental concentrations (ng/L)	Comments	Reference
17 antibiotics	Kenya	SW (river receiving WWTP effluent) WWTP effluent Hospital effluent	Sulfamethoxazole Spiramycin Trimethoprim Spectinomycin Ampicillin Oxacillin	WWTP effluent: 1280 / SW: 18.1 WWTP effluent: 380 / SW: 300 WWTP effluent: 740 / SW: 3160 WWTP effluent: 200 / SW: 260 WWTP effluent: 200 / SW: 240 WWTP effluent: 130 / SW: 210	Antibiotics concentrations in hospital wastewater were 3–10 times higher than the ones detected in WWTP and surface water.	(Ngigi *et al.*, 2020)

Appendix 2 Antibiotic concentrations detected in receiving environmental matrices (continued)

Table A2-2: Antibiotics concentrations in wastewater effluents and receiving environmental matrices in different geographical regions

Target analytes	Country	Matrices analysed	Antibiotics most frequently present	Highest environmental concentrations (ng/L)	Comments	Reference
19 pharmaceuticals (16 antibiotics)	United States	SW river in agricultural region	Erythromycin Sulfamethoxazole Sulfamerazine Meclocycline Chlortetracycline	180 80 60 110 40	Sediment concentration observed higher than in the river aqueous phase indicating higher dissipation tendencies of the antibiotics.	(Kim and Carlson, 2007)
13 antibiotics	United States	SW watershed in a heavily agricultural region	Lincomycin Monensin Sulfamerazine	68 49 13	High frequency of detected antibiotics observed (40-94.5%). Mean values reported.	(Jaimes-Correa *et al.*, 2015)
17 veterinary antibiotics, (7 transformation products)	Canada	Swine manure Soil Drainage water (after manure application	Lincomycin Tetracycline Demeclocycline 4-epimeclocycline Chlortetracycline 4-epichlortetracycline Isochlortetracycline 4-epi-isochlortetracycline	79 9.2 21 1159 29 29 3256 3290	High tetracyclines concentrations were observed in swine manure slurry (max. 663µg/L) but also in the soil (max 1020 ng/g). Degradation products appeared in concentrations higher than the parent in some cases.	(Solliec *et al.*, 2016)
13 antibiotics	China	(summer / winter) Swine WW influent Swine WW effluent SW (Lake water) GW (Ground water)	Enrofloxacin Ofloxacin Norfloxacin Ciprofloxacin Oxytetracycline Doxycycline Sulfamerazine	8.5–21,692.7 / 32.8-11276.6 7.9-1172.3 / 5.8-409.5 5.7-11.6 / 6.7-11.7 1.6-8.6 / 2.0-7.3	Reported as ranges of concentrations based on matrix. Detection frequency increased during winter sampling.	(Tong *et al.*, 2009)

Appendix 2 Antibiotic concentrations detected in receiving environmental matrices (continued)

Table A2-2: Antibiotics concentrations in wastewater effluents and receiving environmental matrices in different geographical regions

Target analytes	Country	Matrices analysed	Antibiotics most frequently present	Highest environmental concentrations (ng/L)	Comments	Reference
10 antibiotics	China	Animal WW Animal/farm effluent SW (receiving river) PW (Pond water)	Sulfadiazine Sulfamethazine Sulfamethoxazole Sulfadoxine Chlortetracycline Tetracycline Oxytetracycline	WW: 550 / EF: 890 SW:1000/ PW:290 WW:21100/ EF:16900 SW:4660 /PW:460 WW: 6360 / EF: 570 SW: 560 / PW: 190 WW: 630 / EF: 100 SW: 460/ PW: 290 WW: 1100 / EF: 3670 SW: 2420/ PW: 570 WW: 10300 / EF: 6440 SW: 810/ PW: 930 WW: 72900 / EF: 11100 SW: 220 / PW: 6870	There was an evident correlation between the detected antibiotic's concentrations and the animal species in each farming location.	(Wei *et al.*, 2011)
12 antibiotics	China	SW rural area with intensive farming activities	Sulfadiazine Sulfamethoxazole Sulfameter Sulfachinoxallin Oflaxacin Enrofloxacin Ciprofloxacin Oxytetracyline	385.7 230.0 387.0 13.95 102.0 13.41 24.8 100.0	Increasing antibiotics concentrations were observed from up- to mid- and downstream in the two tributaries. No clear relationship was observed in the antibiotic resistance frequency with corresponding antibiotic concentration.	(Zhang *et al.*, 2014b)
14 antibiotics	China	SW / GW (highly affected by agriculture and aquacuculture)	Erythromycin Norfloxacin Enrofloxacin Chlortetracycline Ciprofloxacin Sulfamethoxazole Tetracycline	SW: 2910 / GW: 28.9 SW: 277 / GW:17.4 SW:136 SW: 109 / GW: 15.1 SW: 96 SW: 30.2 SW:122 / GW: 25.2	The total concentrations of target compounds in the water samples were higher in spring than those in summer and winter.	(Yao *et al.*, 2017)

Appendix 2 Antibiotic concentrations detected in receiving environmental matrices (continued)

Table A2-2: Antibiotics concentrations in wastewater effluents and receiving environmental matrices in different geographical regions

Target analytes	Country	Matrices analysed	Antibiotics most frequently present	Highest environmental concentrations (ng/L)	Comments	Reference
12 antibiotics	Japan	WW (Treated Livestock wastewater) SW (river)	Azithromycin Clarithromycin Erythromycin Sulfamethoxazole Sulfapyridine Sulfamethazine Trimethoprim	WW: 1.09 / SW: 20.72 WW: 0.57 / SW: 27.34 WW: n.d / SW: 12.28 WW: 378 / SW: 70.23 WW: 0.98 / SW: 15.61 WW: 1.98 / SW: 26.40 WW: 6999 / SW: 9.91	Sulfonamides were mostly detected in rural rivers with intensive farming. However. in some areas the distinction between human and veterinary origin was hindered	(Murata *et al.*, 2011)
13 antibiotics	South Korea	SW (receiving runoff from agricultural areas and livestock facilities)	Oxytetracycline Tetracycline Chlortetracycline Trimethoprim Sulfathiazole Sulfamethazine Sulfamethoxazole Enrofloxacin	SW: 30 SW: 37 SW: 50 SW: 27 SW: 123 SW: 123 SW: 147 SW: 333	Higher concentrations observed during wet season.	(Kim *et al.*, 2016)
20 pharmaceuticals (16 antibiotics)	France	SW (agricultural watershed)	Sulfamethoxazole Sulfadiazine Trimethorpim Lincomycin Oxytetracycline	181 35 23 71 144	Animal-specific pharmaceuticals were detected mainly during runoff events and periods of manure spreading. LIN – 373 at the WWTP	(Jaffrézic *et al.*, 2017)
38 veterinary products (21 antibiotics)	France	SW (intensive husbandry area) DW (drinking water)	Florfenicol Lincomycin Neospiramycin Oxytetracycline Tylosin Sulfadiazine Sulfamethazine Trimethoprim	930 6 25 325 9 2946 66 468	Florfenicol, Tylosin and Sulfadiazine were also present in tap water at maximum concentrations of 211, 5 and 7 ng/L respectively	(Charuaud *et al.*, 2019a)

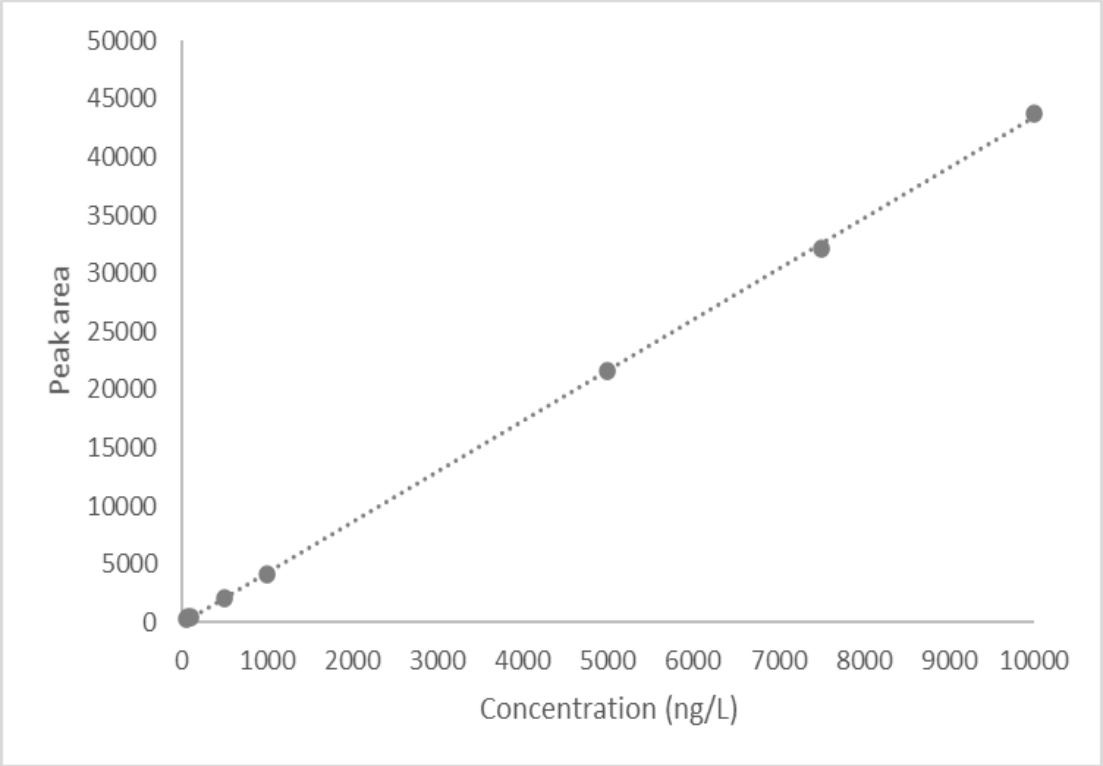
Appendix 2 Antibiotic concentrations detected in receiving environmental matrices (continued)

Table A2-2: Antibiotics concentrations in wastewater effluents and receiving environmental matrices in different geographical regions

Target analytes	Country	Matrices analysed	Antibiotics most frequently present	Highest environmental concentrations (ng/L)	Comments	Reference
38 veterinary products (11 antibiotics)	Spain	SW (river in rural area)	Monensin Salinomycin Sulfadiazine Sulfamethazine Sulfamethoxypyridazine Sulfapyridine Sulfaquinoxaline Trimethoprim	16.7 17.4 2978.6 33.5 148.8 177.8 40.8 126.1	Highest Sulfadiazine concentration observed near a swine farm (>100 animals)	(Iglesias *et al.*, 2012)
21 pharmaceuticals (9 antibiotics)	Spain	SW (receiving agricultural and WWTP effluent)	Trimethoprim Sulfamethoxypyridazine Sulfadiazine Monensin	85.4 11.6 38 30	Trimethoprim, Sulfamethoxypyridazine and Sulfadiazine detected close a DWTP	(Iglesias *et al.*, 2014)
19 veterinary drugs (9 antibiotics)	Spain	DW (from milking facilities)	Enrofloxacin Monensin Salinomycin Sulfachloropyridazine Sulfadiazine Sulfamethazine Sulfamethoxypyridazine Sulfapyridine Sulfaquinoxaline Trimethoprim	28 56 23 3826 3941 180 905 400 337 552	The samples were from private water wells (52% of the samples), from distribution systems (17%), from both sources as the farm has access to both water systems (4%), and unknown origin (27%). 57% of the collected samples contained at list one antibiotic.	(Veiga-Gómez *et al.*, 2017)
45 veterinary drugs (44 antibiotics)	Poland	DW (water supply on farms)	Enrofloxacin Doxycycline Sulfamethoxazole Trimethoprim Lincomycin	1670000 1650000 587000 17800 304000	52% of samples collected were tested positive for antibiotics. Residues might be due to the lack of a cleaning system, accumulations of residues or overdosing.	(Gbylik-Sikorska *et al.*, 2015)

Appendix 3 Calibration curves for analytes – direct injection

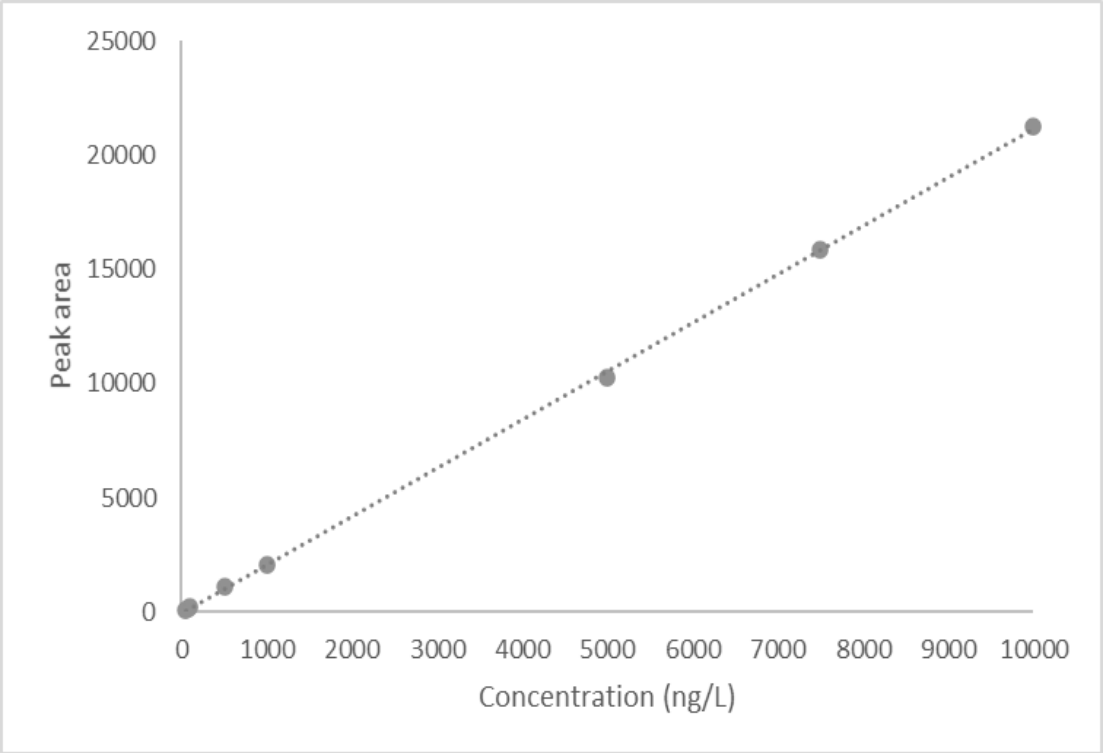
Figure A3-1: Linearity Graph for Sulfadiazine



Standard Concentration (ng/L)	pg on column based on a direct 9µL injection	Peak Area
50	0.5	255
75	0.7	397
100	0.9	445
500	4.5	2008
1000	9.0	4109
5000	45.0	21580
7500	67.5	32127
10000	90.0	43745
Intercept	-81	
Slope	4.347	
Coefficient of Determination (r^2)	0.9998	

Appendix 3 Calibration curves for analytes – direct injection (continued)

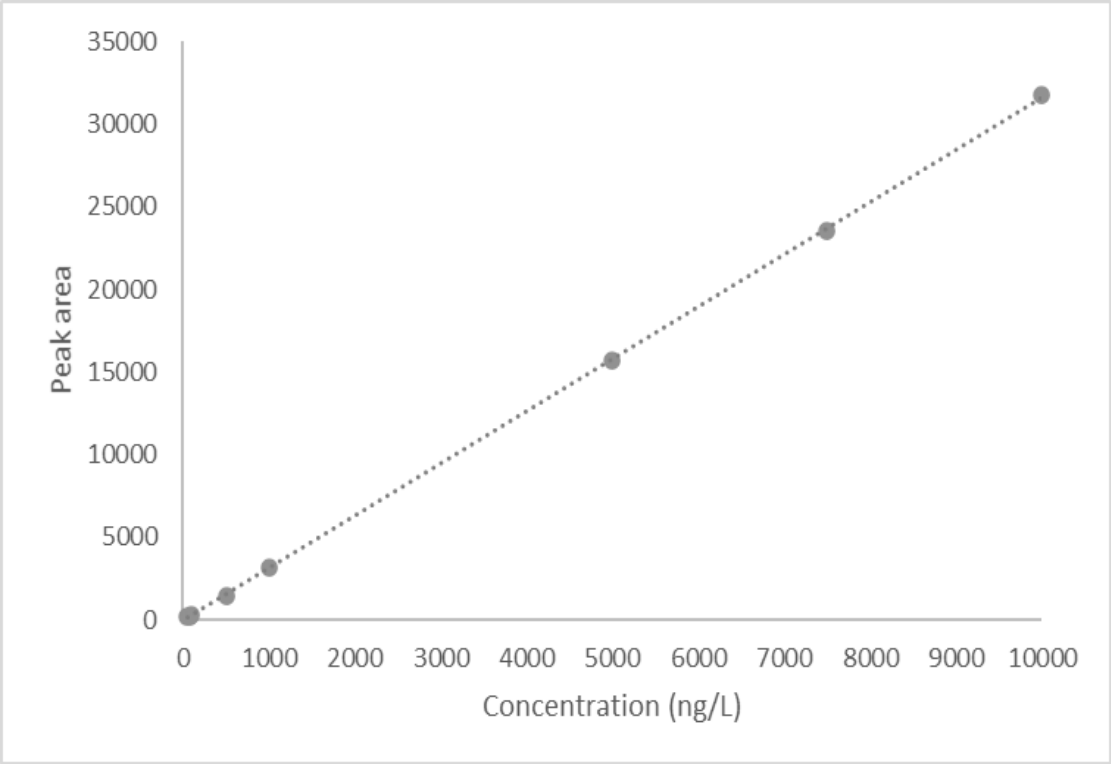
Figure A3-2: Linearity Graph for Amoxicillin



Standard Concentration (ng/L)	pg on column based on a direct 9µL injection	Peak Area
50	0.5	80
75	0.7	145
100	0.9	190
500	4.5	1130
1000	9.0	2079
5000	45.0	10287
7500	67.5	15873
10000	90.0	21235
Intercept	-31	
Slope	2.116	
Coefficient of Determination (r^2)	0.9998	

Appendix 3 Calibration curves for analytes – direct injection (continued)

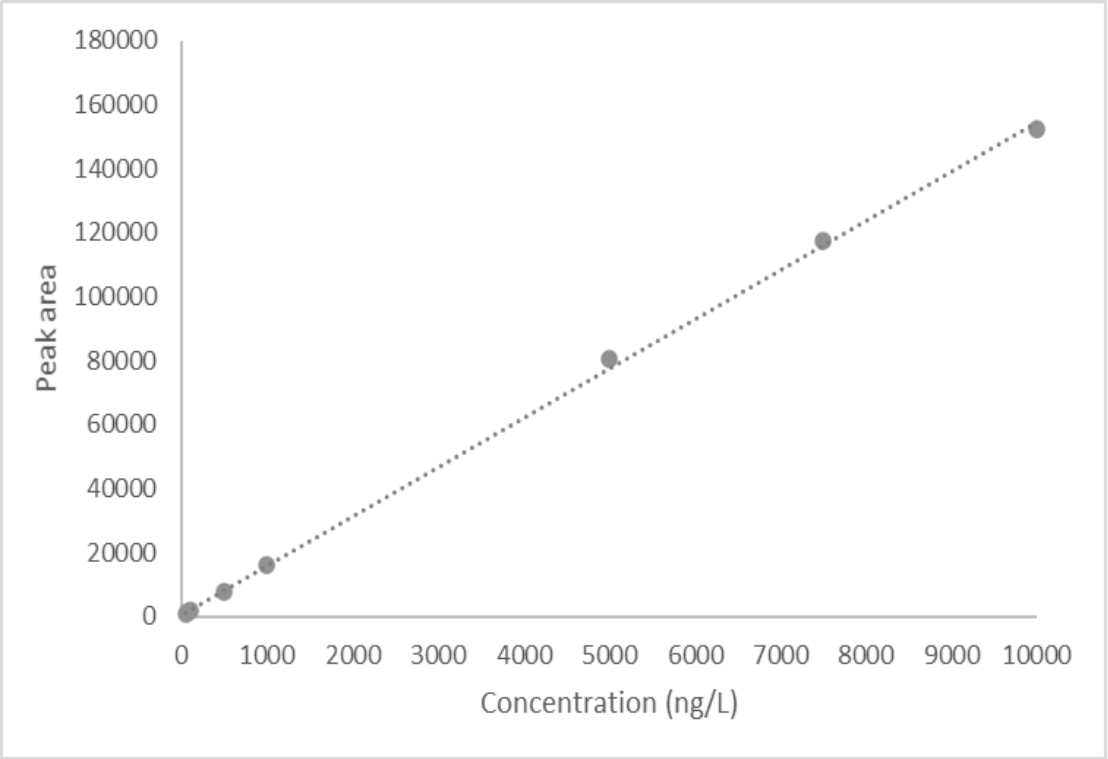
Figure A3-3: Linearity Graph for Cephapirin



Standard Concentration (ng/L)	pg on column based on a direct 9µL injection	Peak Area
50	0.5	161
75	0.7	228
100	0.9	294
500	4.5	1483
1000	9.0	3152
5000	45.0	15744
7500	67.5	23522
10000	90.0	31749
Intercept	-40	
Slope	3.164	
Coefficient of Determination (r^2)	0.9999	

Appendix 3 Calibration curves for analytes – direct injection (continued)

Figure A3-4: Linearity Graph for Lincomycin

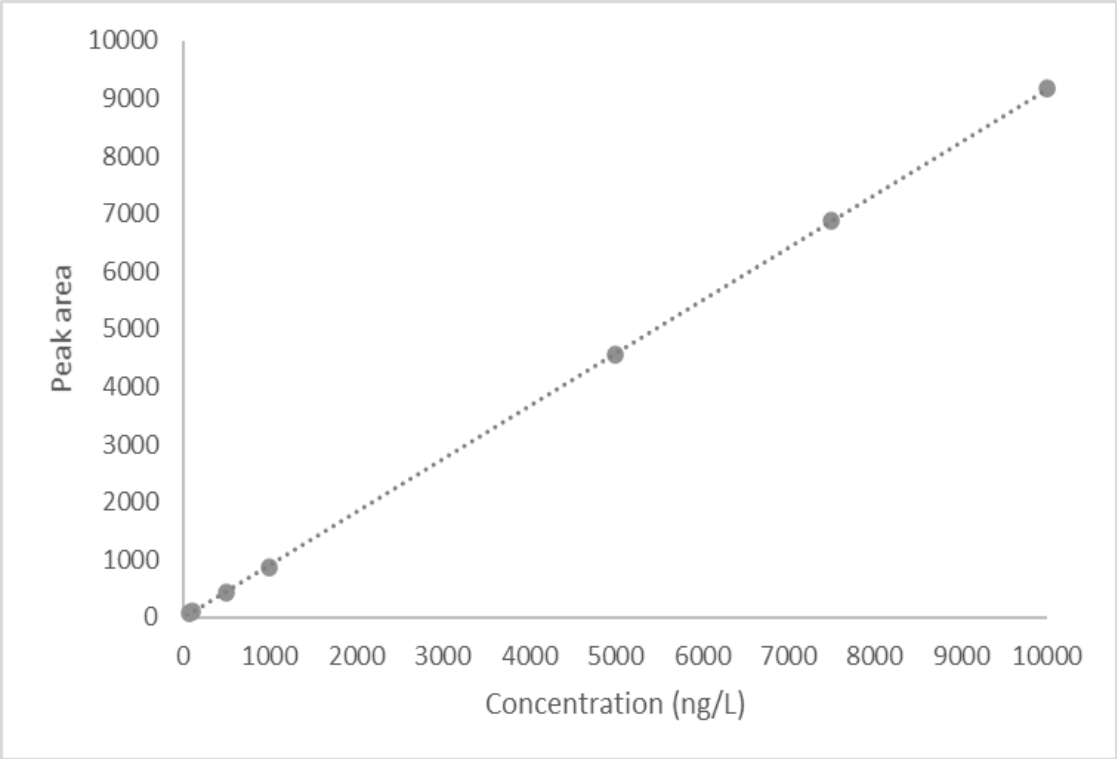


Standard Concentration (ng/L)	pg on column based on a direct 9µL injection	Peak Area
25	0.25	416
50	0.5	780
75	0.7	1171
100	0.9	1599
500	4.5	7936
1000	9.0	16062
5000	45.0	80585
7500	67.5	117255
10000	90.0	152496

Intercept	539
Slope	15.420
Coefficient of Determination (r^2)	0.9994

Appendix 3 Calibration curves for analytes – direct injection (continued)

Figure A3-5: Linearity Graph for Cefalonium

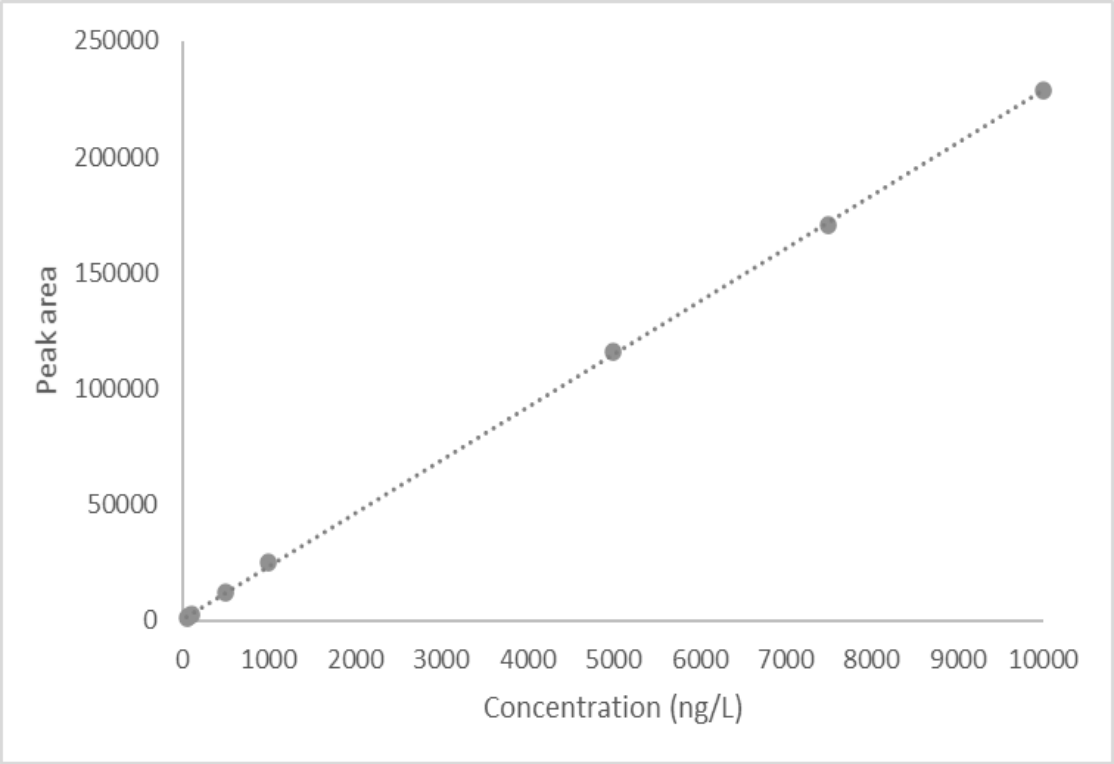


Standard Concentration (ng/L)	pg on column based on a direct 9µL injection	Peak Area
100	0.9	108
500	4.5	425
1000	9.0	871
5000	45.0	4555
7500	67.5	6871
10000	90.0	9169

Intercept	-14
Slope	9.17
Coefficient of Determination (r^2)	1.0000

Appendix 3 Calibration curves for analytes – direct injection (continued)

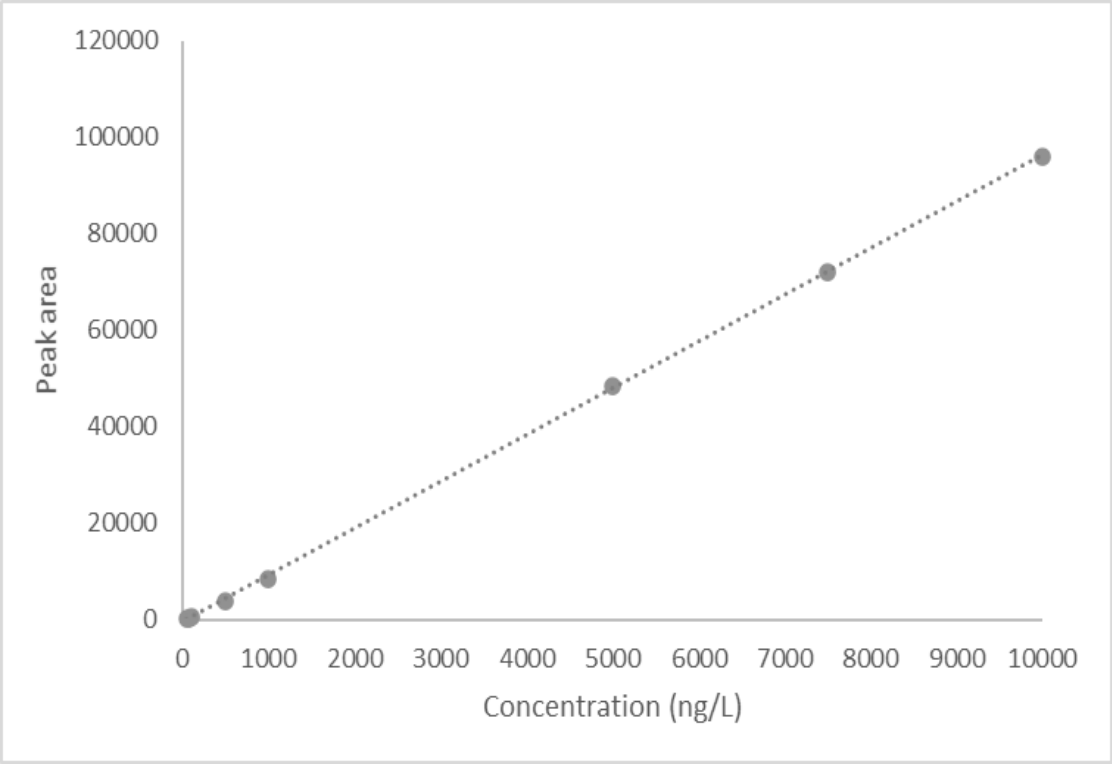
Figure A3-6: Linearity Graph for Trimethoprim



Standard Concentration (ng/L)	pg on column based on a direct 9µL injection	Peak Area
25	0.25	515
50	0.5	1093
75	0.7	1686
100	0.9	2321
500	4.5	12258
1000	9.0	24995
5000	45.0	116157
7500	67.5	170964
10000	90.0	228823
Intercept	654	
Slope	22.830	
Coefficient of Determination (r^2)	0.9999	

Appendix 3 Calibration curves for analytes – direct injection (continued)

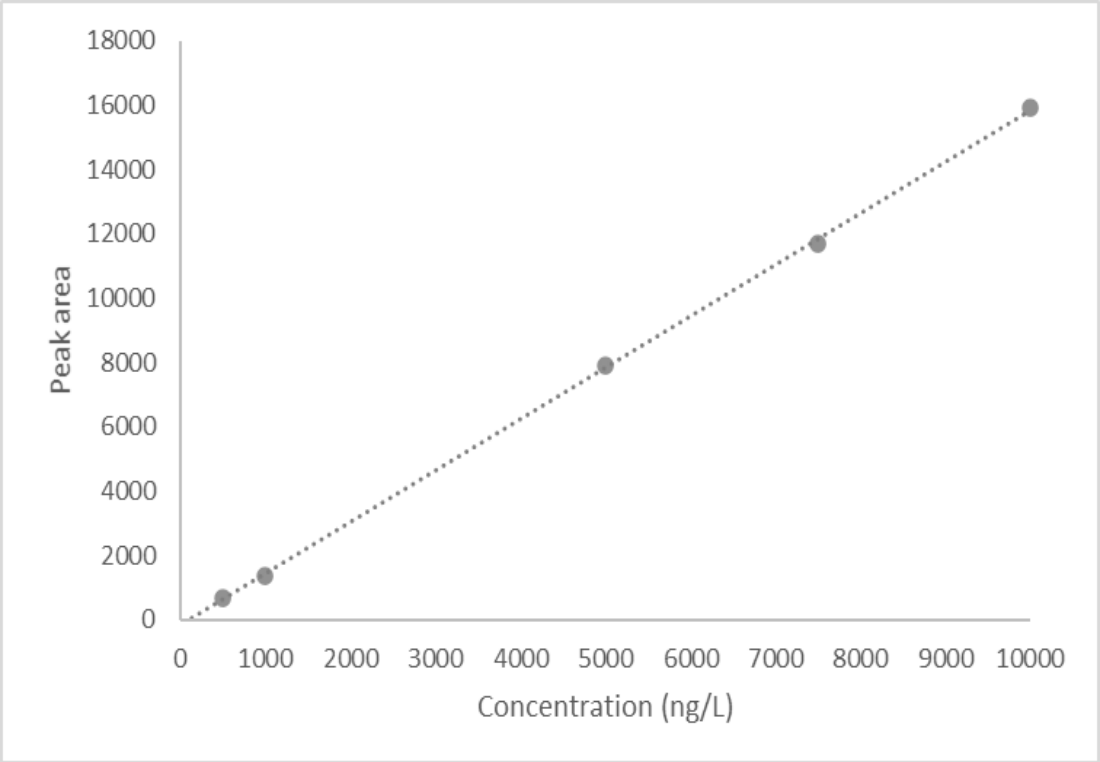
Figure A3-7: Linearity Graph for Marbofloxacin



Standard Concentration (ng/L)	pg on column based on a direct 9µL injection	Peak Area
50	0.5	204
75	0.7	361
100	0.9	585
500	4.5	3931
1000	9.0	8421
5000	45.0	48503
7500	67.5	72205
10000	90.0	96073
Intercept	-561	
Slope	9.691	
Coefficient of Determination (r^2)	0.9999	

Appendix 3 Calibration curves for analytes – direct injection (continued)

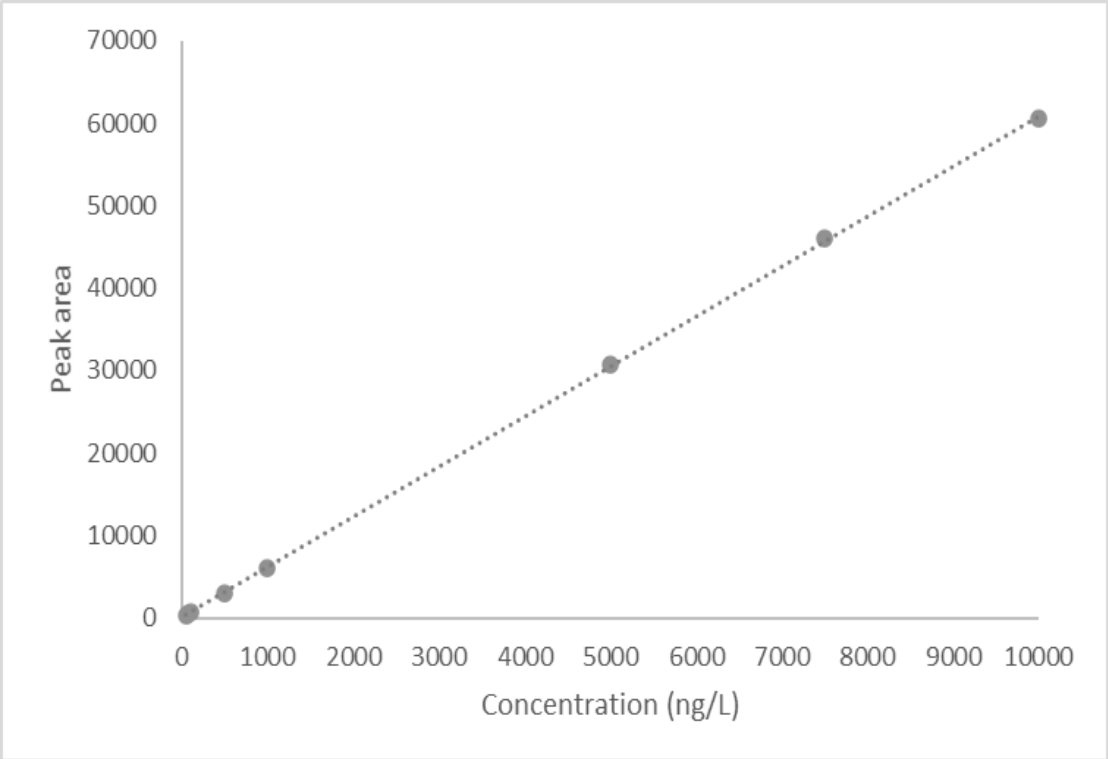
Figure A3-8: Linearity Graph for Oxytetracycline



Standard Concentration (ng/L)	pg on column based on a direct 9µL injection	Peak Area
250	2.25	353
500	4.5	651
1000	9.0	1372
5000	45.0	7925
7500	67.5	11699
10000	90.0	15951
Intercept	-190	
Slope	1.606	
Coefficient of Determination (r^2)	0.9998	

Appendix 3 Calibration curves for analytes – direct injection (continued)

Figure A3-9: Linearity Graph for Sulfamethoxazole

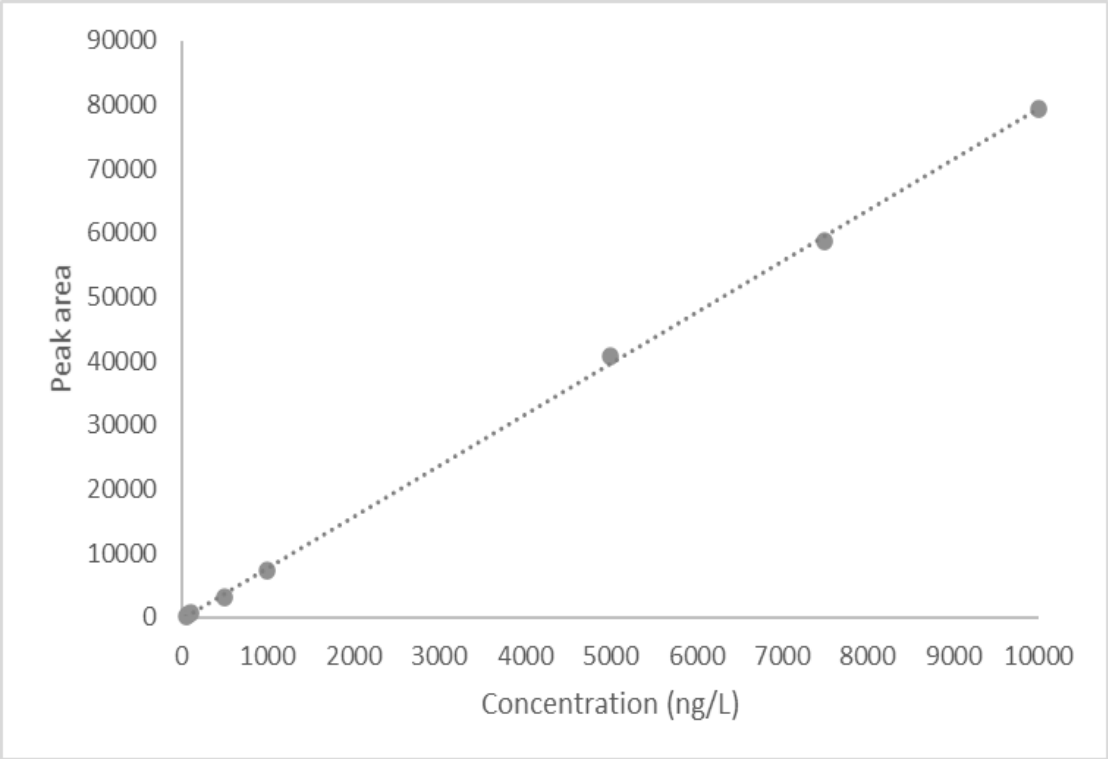


Standard Concentration (ng/L)	pg on column based on a direct 9µL injection	Peak Area
50	0.5	359
75	0.7	511
100	0.9	700
500	4.5	3081
1000	9.0	6083
5000	45.0	30816
7500	67.5	46150
10000	90.0	60558

Intercept	96
Slope	6.088
Coefficient of Determination (r^2)	0.9999

Appendix 3 Calibration curves for analytes – direct injection (continued)

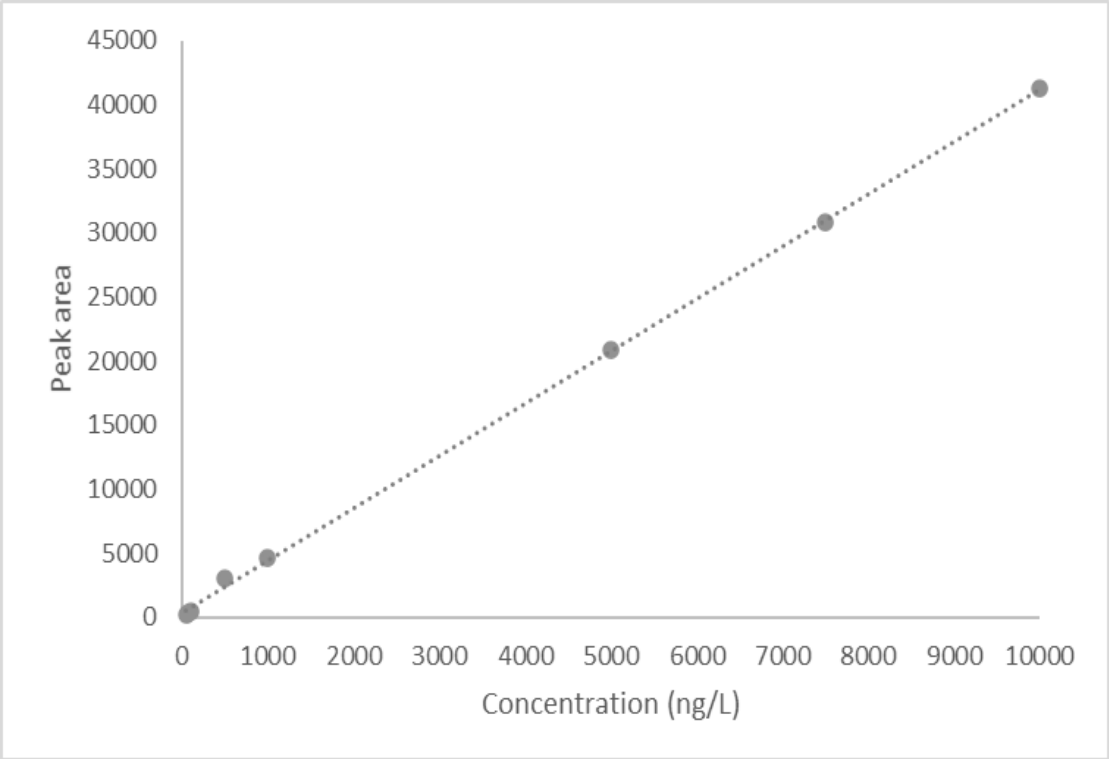
Figure 3-10: Linearity Graph for Enrofloxacin



Standard Concentration (ng/L)	pg on column based on a direct 9µL injection	Peak Area
50	0.5	273
75	0.7	343
100	0.9	586
500	4.5	3132
1000	9.0	7206
5000	45.0	40689
7500	67.5	58724
10000	90.0	79453
Intercept	-352	
Slope	7.976	
Coefficient of Determination (r^2)	0.9997	

Appendix 3 Calibration curves for analytes – direct injection (continued)

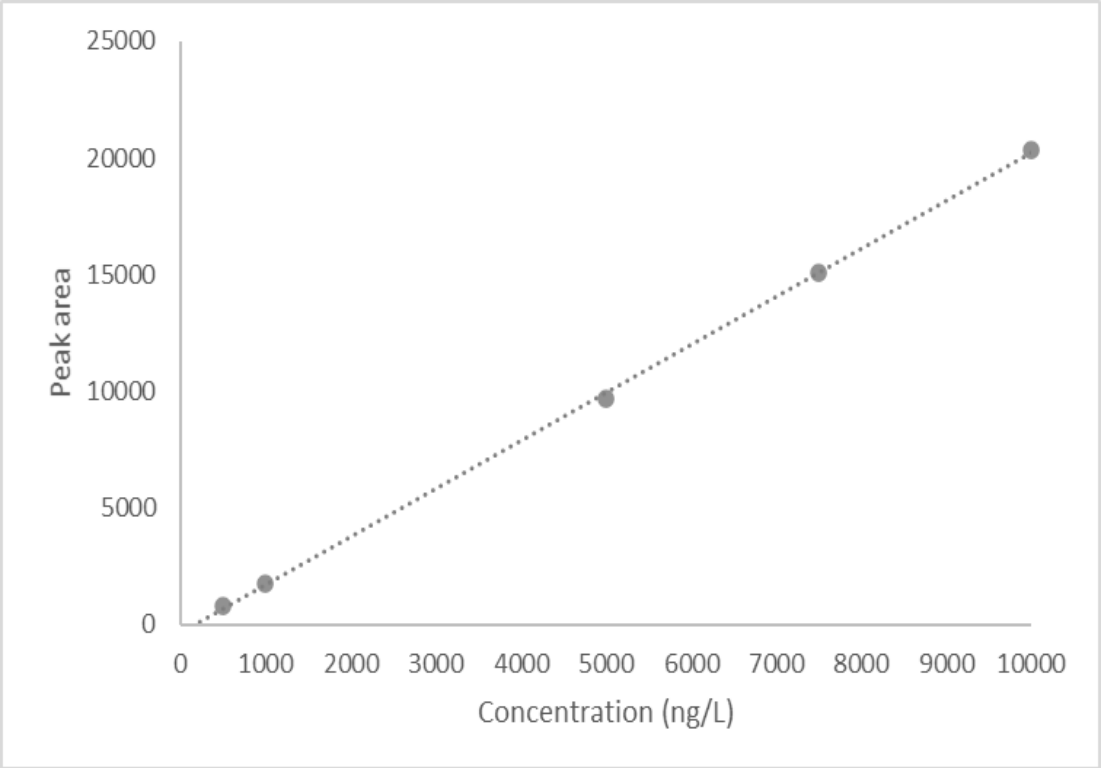
Figure 3-11: Linearity Graph for Ceftiofur



Standard Concentration (ng/L)	pg on column based on a direct 9µL injection	Peak Area
50	0.5	225
75	0.7	319
100	0.9	452
500	4.5	2994
1000	9.0	4641
5000	45.0	20887
7500	67.5	30889
10000	90.0	41270
Intercept	306	
Slope	4.096	
Coefficient of Determination (r^2)	0.9996	

Appendix 3 Calibration curves for analytes – direct injection (continued)

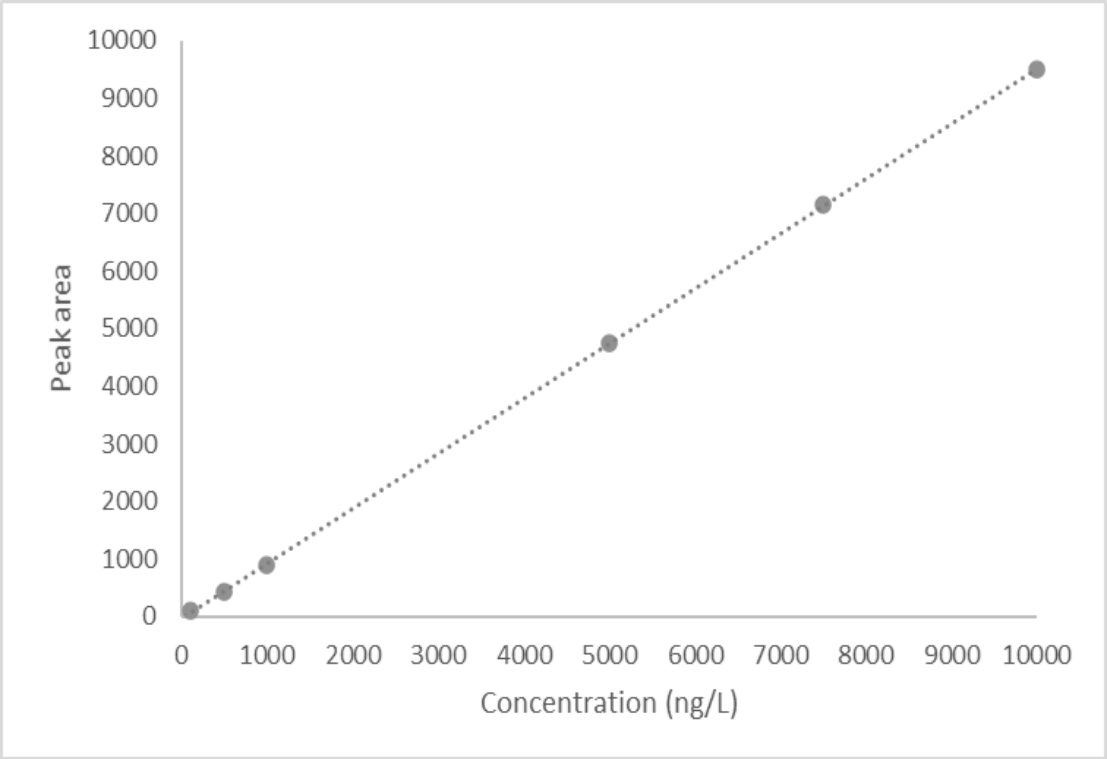
Figure A3-12: Linearity Graph for Penicillin



Standard Concentration (ng/L)	pg on column based on a direct 9µL injection	Peak Area
250	2.25	368
500	4.5	776
1000	9.0	1725
5000	45.0	9659
7500	67.5	15061
10000	90.0	20346
Intercept	-365	
Slope	2.058	
Coefficient of Determination (r^2)	0.9996	

Appendix 3 Calibration curves for analytes – direct injection (continued)

Figure A3-13: Linearity Graph for Tylosin

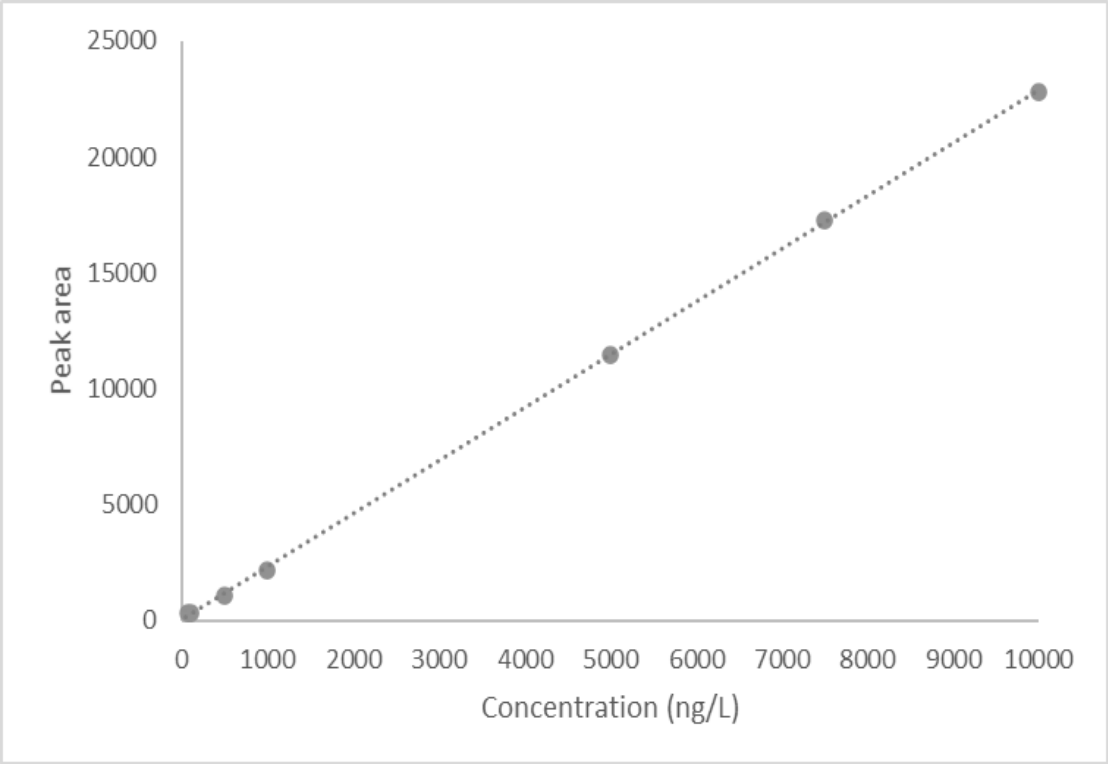


Standard Concentration (ng/L)	pg on column based on a direct 9µL injection	Peak Area
250	2.25	249
500	4.5	426
1000	9.0	888
5000	45.0	4740
7500	67.5	7146
10000	90.0	9502

Intercept	38
Slope	0.955
Coefficient of Determination (r^2)	1.0000

Appendix 3 Calibration curves for analytes – direct injection (continued)

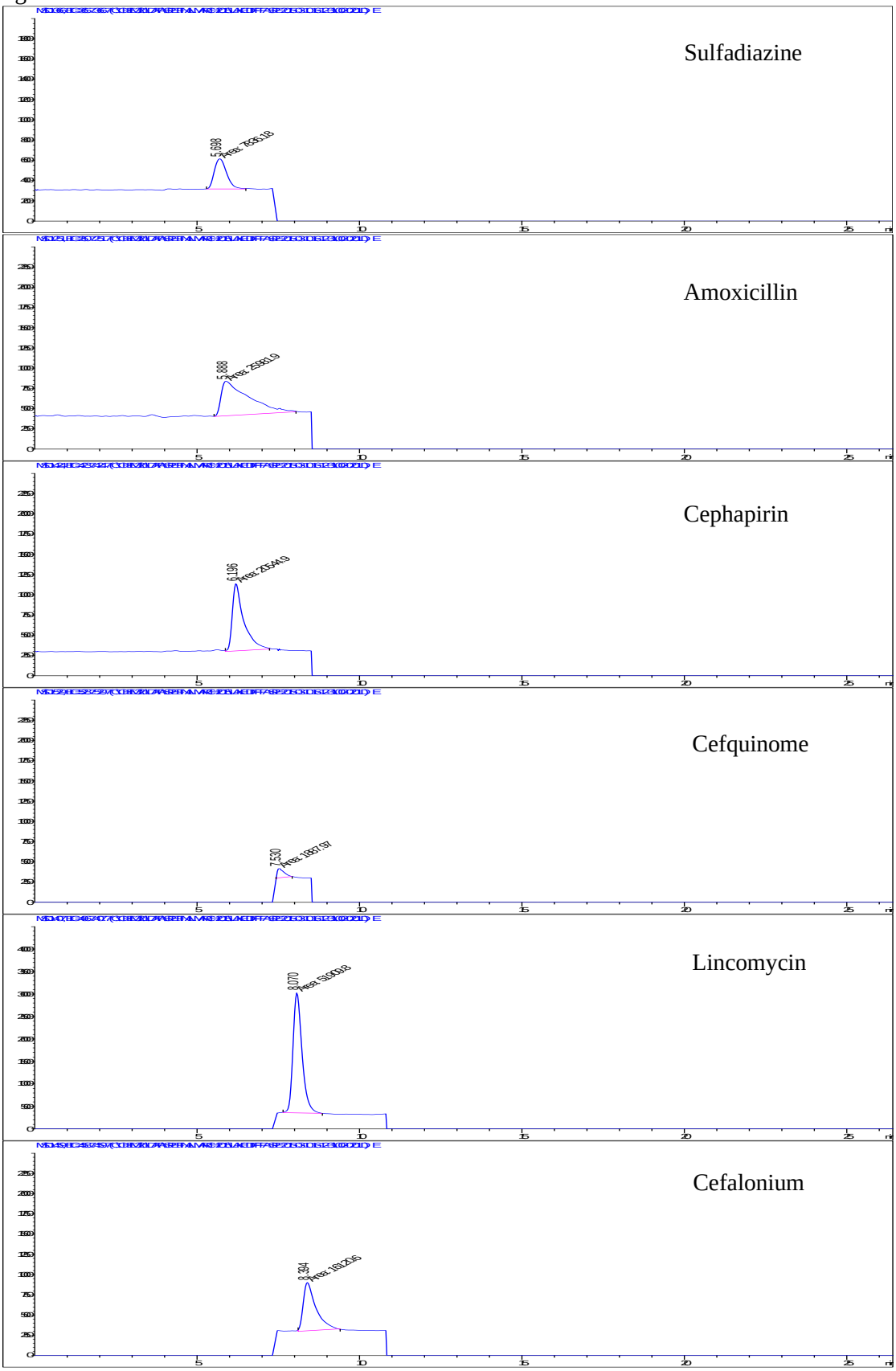
Figure A3-14: Linearity Graph for Erythromycin



Standard Concentration (ng/L)	pg on column based on a direct 9µL injection	Peak Area
100	0.9	355
500	4.5	1106
1000	9.0	2135
5000	45.0	11490
7500	67.5	17301
10000	90.0	22838
Intercept	33	
Slope	2.287	
Coefficient of Determination (r^2)	0.9999	

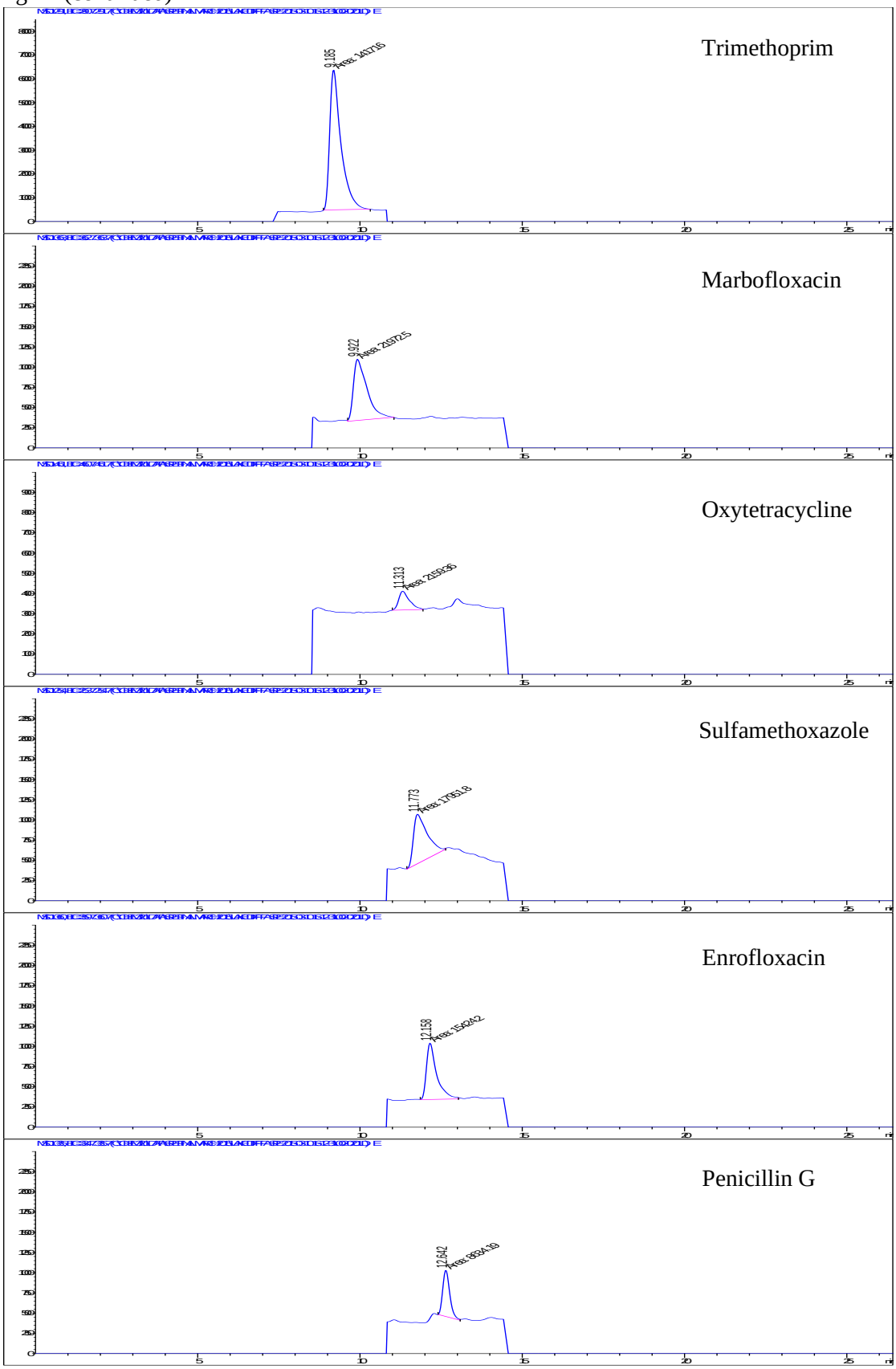
Appendix 4 Example chromatography

Figure A4-1: Representative chromatograms during online SPE extraction at LOQ level, 100 ng L^{-1}



Appendix 4 Example chromatography (continued)

Figure A4-1: Representative chromatograms during online SPE extraction at LOQ level, 100 ng L^{-1} (continued)



Appendix 4 Example chromatography (continued)

Figure A4-1: Representative chromatograms during online SPE extraction at LOQ level, 100 ng L^{-1} (continued)

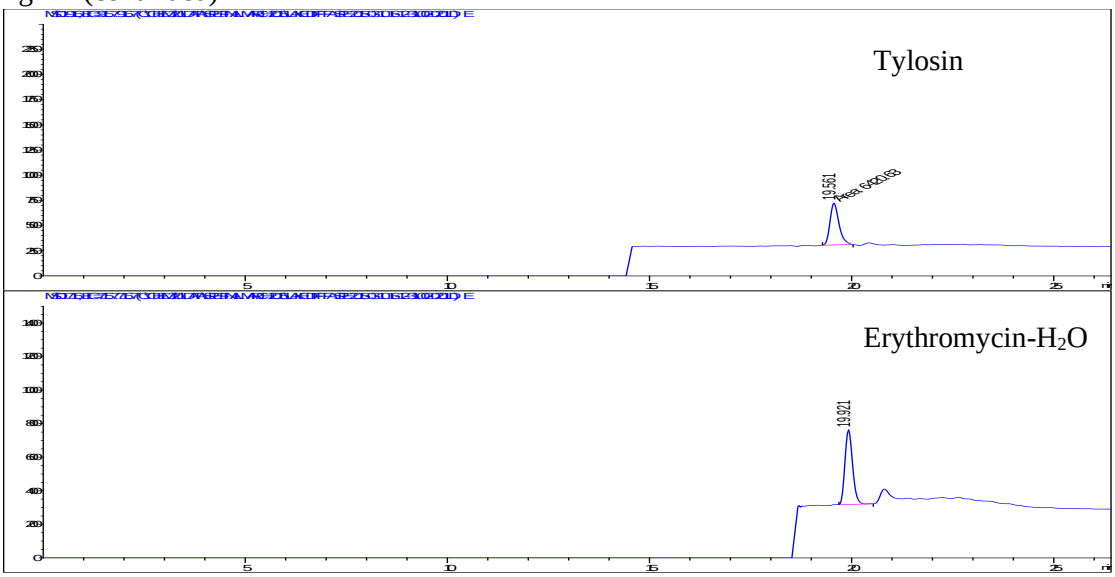
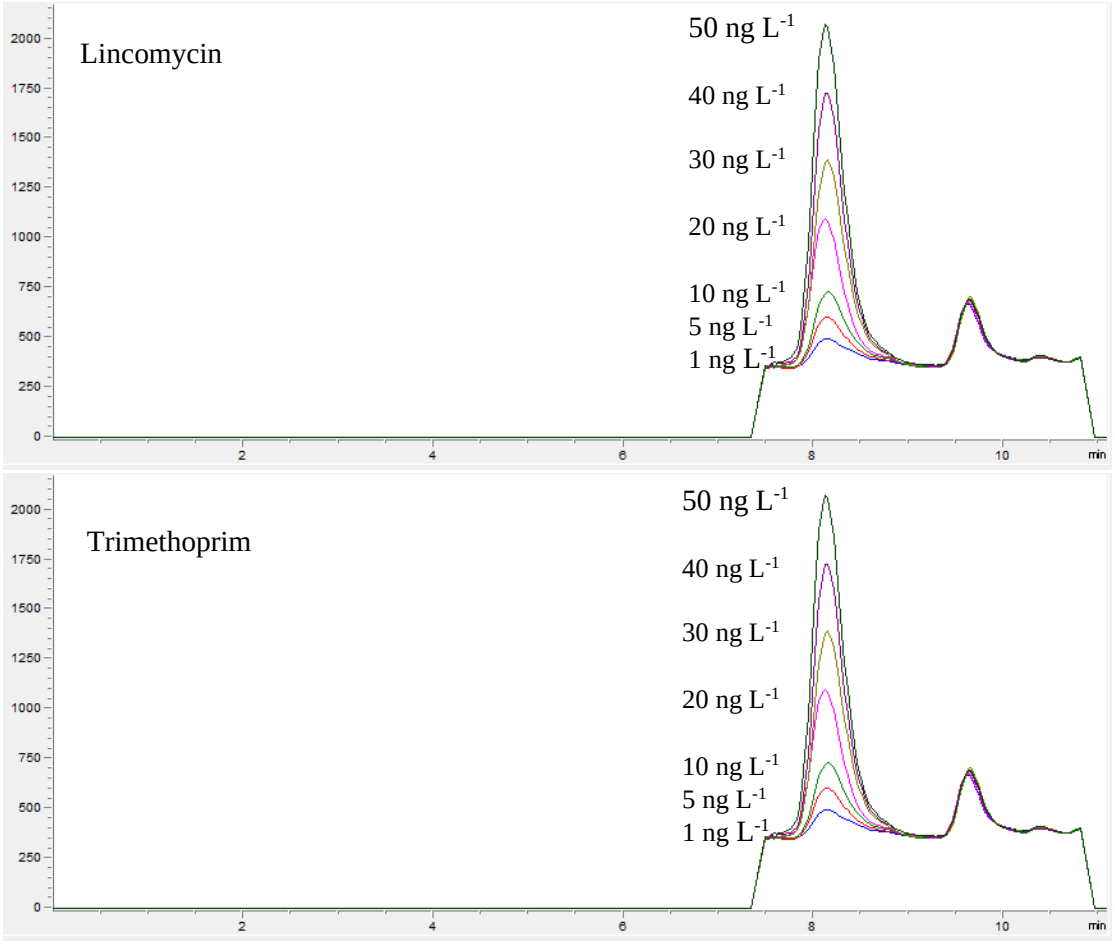
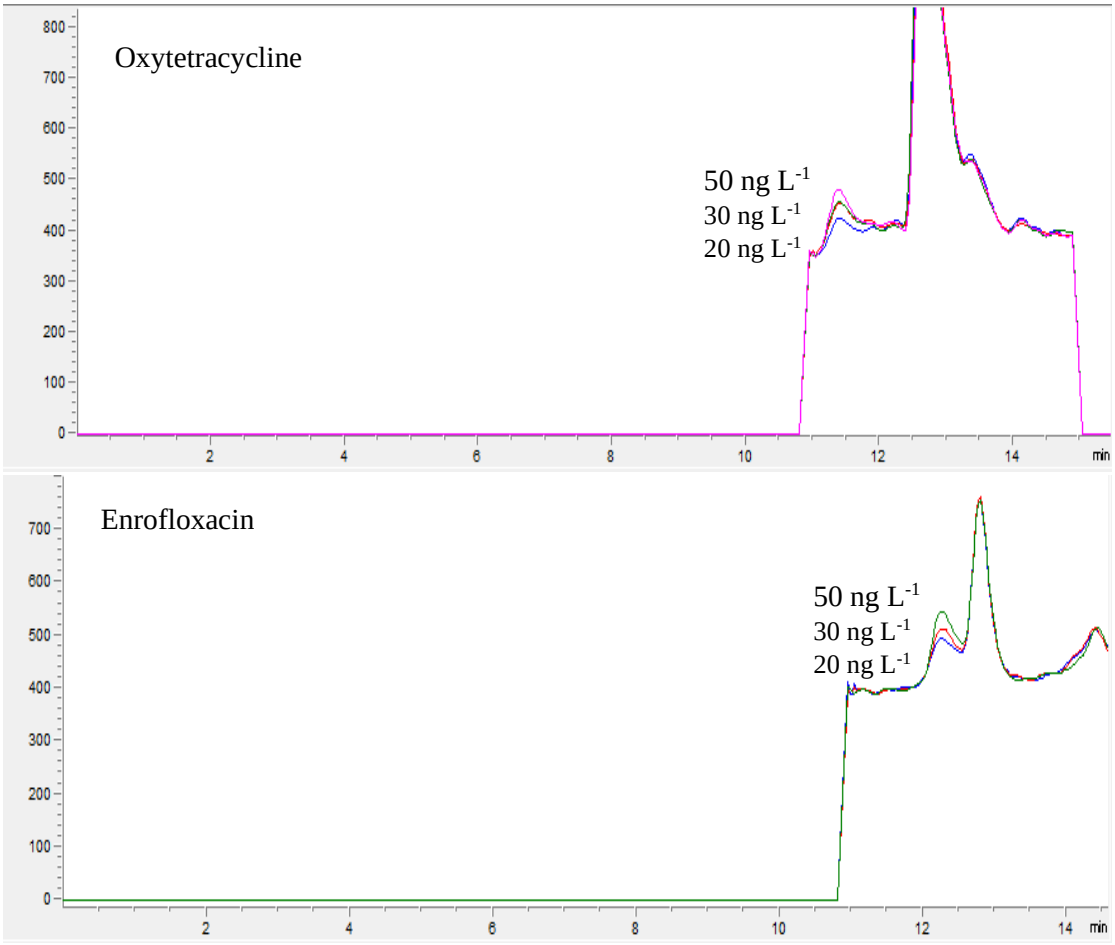


Figure A4-2: Example chromatograms of the least and most sensitive compounds at during LOD testing



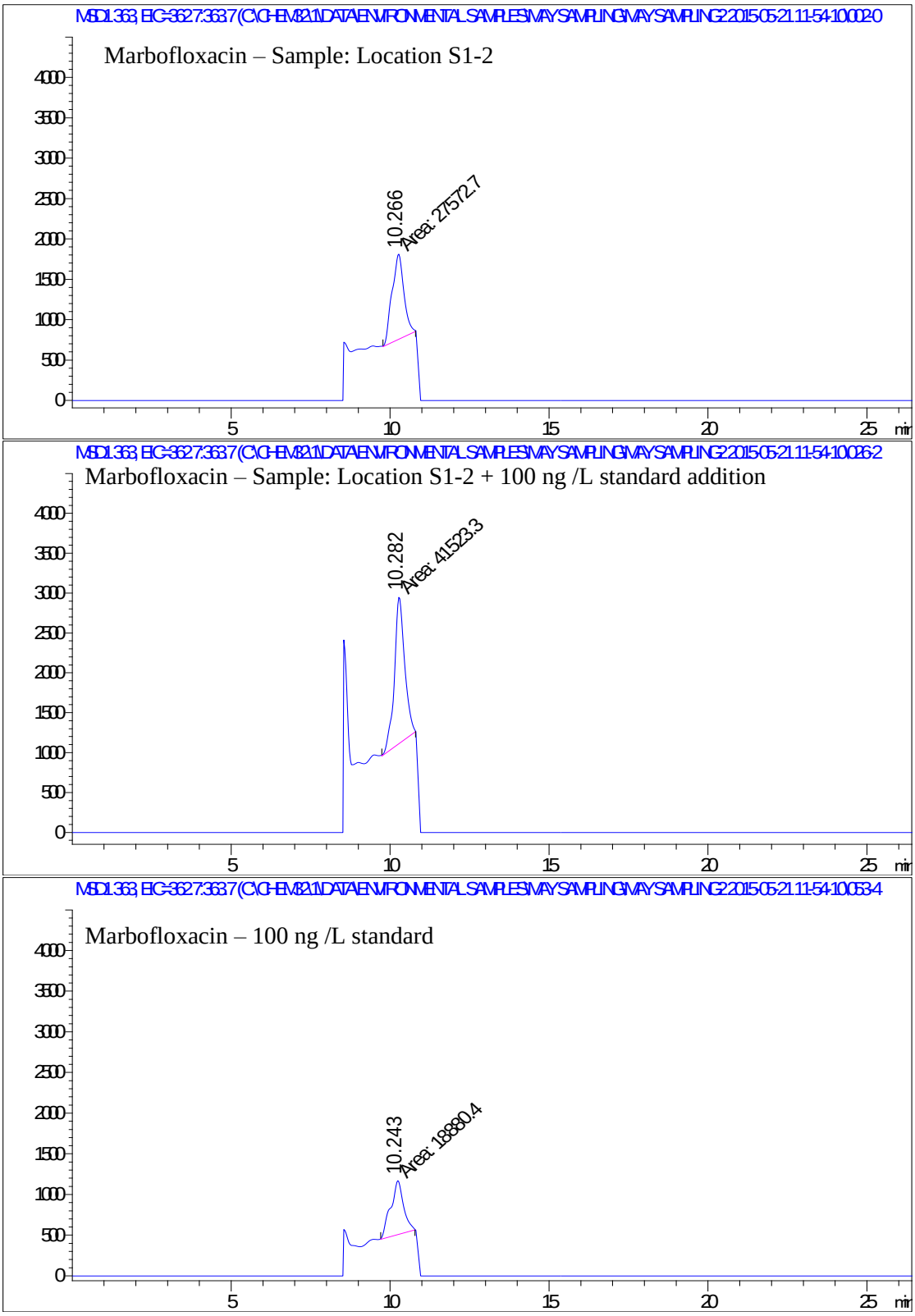
Appendix 4 Example chromatography (continued)

Figure A4-2: Example chromatograms of the least and most sensitive compounds at during LOD testing (continued)



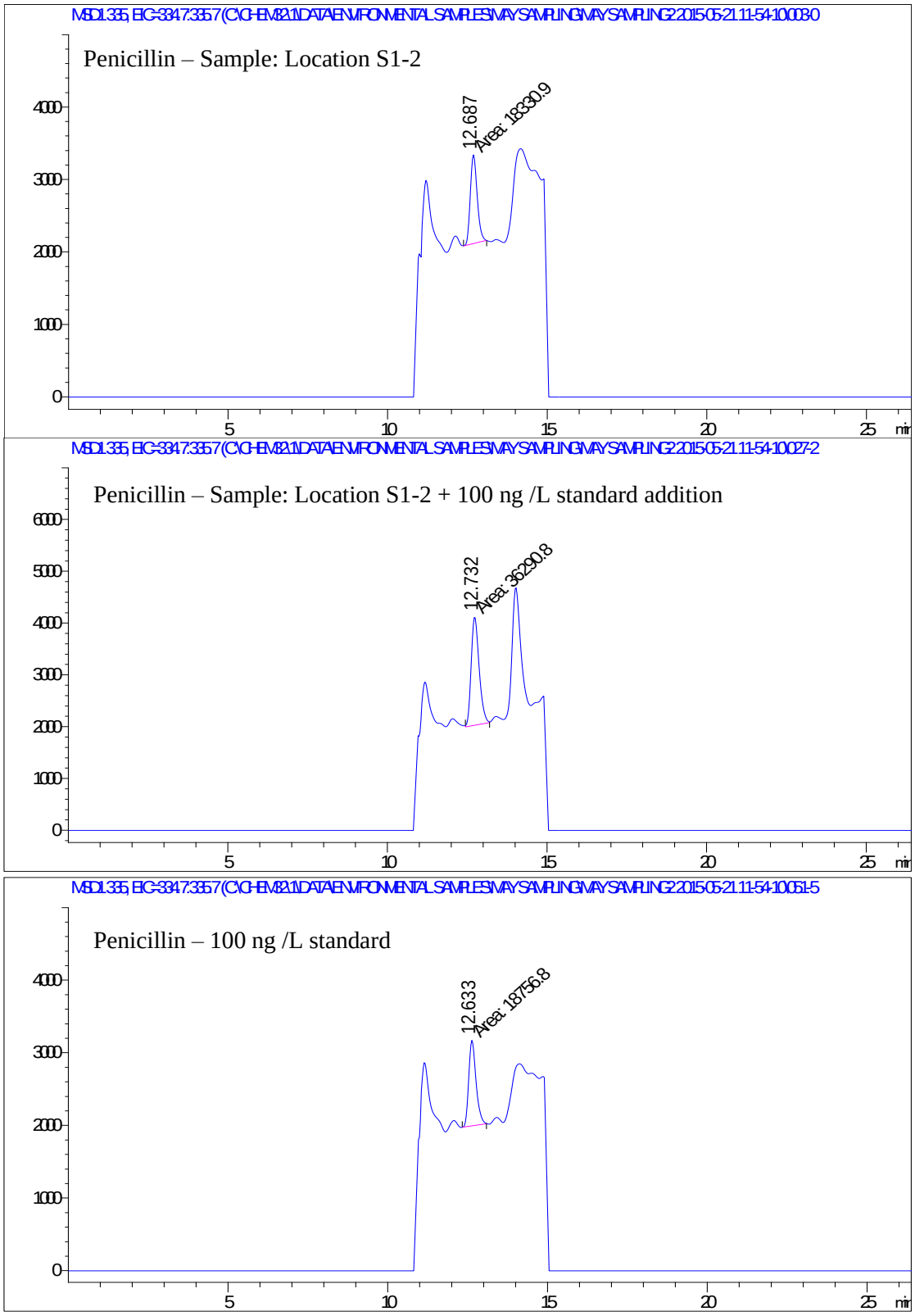
Appendix 4 Example chromatography (continued)

Figure A4-3: Example chromatograms of environmental samples – Marbofloxacin



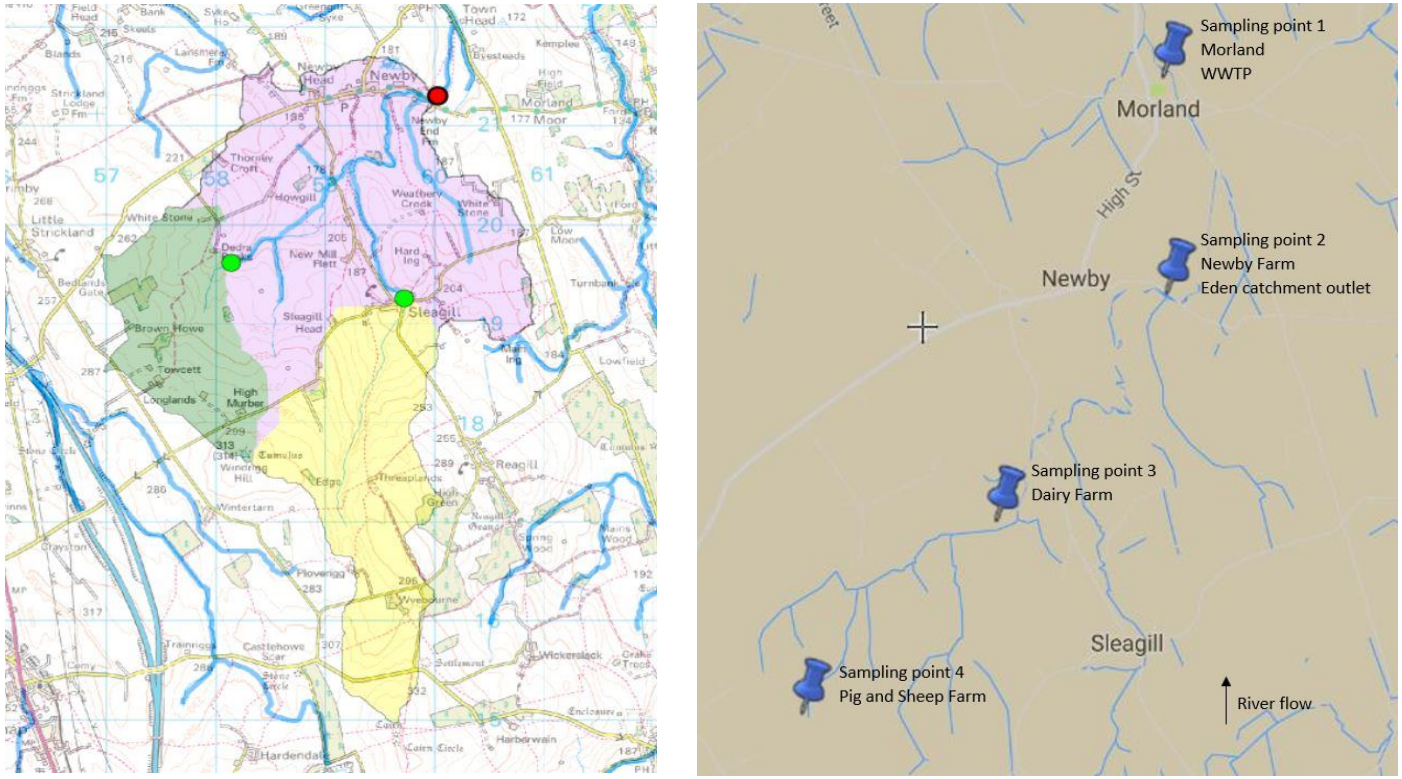
Appendix 4 Example chromatography (continued)

Figure A4-4: Example chromatograms of environmental samples – Penicillin



Appendix 5 Concentrations of target antibiotic residues in environmental water samples per sampling location

Figure A5-1: Sampling locations – Catchment area



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Appendix 5 Concentrations of target antibiotic residues in environmental water samples (continued)

Table A5-1: Concentrations of target antibiotic residues detected in samples taken from sources around catchment area

Analyte	March Sampling per location															
	S1-1	**S1-2**	**S1-3**	**S2-1**	**S3-1**	**S3-2**	**S3-3**	**S3-4**	**S3-5**	**S3-6**	**S4-1**	**S4-2**	**S4-3**	**S4-4**	**S4-5**	**S4-6**	**S4-7**
Sulfadiazine				-	-	-	-	-			-				-	(9.9)	-
Amoxicillin				-	-	-	-	-			-				-	-	(34.0)
Cephapirin				-	-	-	-	-			-				-	(17.8)	(12.8)
Cefquinone				-	-	-	-	-			-				-	-	-
Lincomycin				-	-	-	-	-			-				-	-	-
Cefalonium				-	-	-	-	-			-				-	182.1	293.3
Trimethoprim				-	-	-	-	-			-				-	-	-
Marbofloxacin				-	-	-	-	(38.5)			-				-	-	-
Oxytetracycline				-	-	-	-	-			-				-	(48.8)	(40.9)
Sulfamethoxazole				-	-	-	-	-			-				-	-	-
Enrofloxacin				-	-	-	-	-			-				-	-	-
Ceftiofur				-	-	-	-	-			-				-	-	-
Penicillin G				-	-	-	-	(16.8)			-				-	-	-
Tylosin				-	-	-	-	-			-				-	-	-
Erythromycin-H_20				-	-	-	-	-			-				-	-	-
	April Sampling per location																
Sulfadiazine				-	-	-	-	-		(13.3)	-	-	-	-	-	-	
Amoxicillin				-	-	-	-	-		-	-	-	-	-	-	-	
Cefapirin				-	-	-	-	(6.8)		-	-	-	-	-	-	-	
Cefquinone				-	-	-	-	-		-	-	-	-	-	-	-	
Lincomycin				-	-	-	-	-		-	-	-	-	-	-	-	
Cefalonium				-	-	-	-	-		-	-	-	-	-	-	-	
Trimethoprim				-	-	-	-	-		-	-	-	-	-	(11.5)	(6.2)	
Marbofloxacin				-	-	-	-	-		-	-	-	-	-	-	-	
Oxytetracycline				-	-	-	-	-		-	-	(25.5)	-	143.4	-	-	
Sulfamethoxazole				-	-	-	-	-		-	-	-	-	-	-	-	
Enrofloxacin				-	-	-	-	-		-	-	-	-	-	-	(17.4)	
Ceftiofur				-	-	-	-	-		-	-	-	-	-	-	-	
Penicillin G				-	(12.9)	-	-	-		-	(25.1)	-	-	-	-	-	
Tylosin				-	-	-	-	-		-	-	-	-	-	-	-	
Erythromycin-H_20				-	-	-	-	-		-	-	-	-	-	-	-	

No sampling occurred either due to no water presence or not scheduled, Numbers in bracket indicate concentrations below the tested LOQ but above the individual LOD.

Chapter **8**: Appendices

Appendix 5 Concentrations of target antibiotic residues in environmental water samples (continued)

Table A5-1: Concentrations of target antibiotic residues detected in samples taken from sources around catchment area (continued)

Analyte	May Sampling per location																
	S1-1	**S1-2**	**S1-3**	**S2-1**	**S3-1**	**S3-2**	**S3-3**	**S3-4**	**S3-5**	**S3-6**	**S4-1**	**S4-2**	**S4-3**	**S4-4**	**S4-5**	**S4-6**	**S4-7**
Sulfadiazine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amoxicillin	-	(40.3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(13.2)
Cefapirin	-	(15.4)	5.7	-	-	-	-	-	-	-	-	-	-	-	-	(22.8)	-
Cefquinone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lincomycin	-	-	-	-	-	-	-	-	-	-	(18.5)	(21.7)	(23.3)	-	(23.1)	-	-
Cefalonium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trimethoprim	-	18.5	4.6	-	-	-	-	-	-	-	(1.8)	-	-	-	-	-	-
Marbofloxacin	-	351.4	(32.1)	-	-	-	-	-	118.6	(90.6)	(32.4)	-	-	-	-	-	-
Oxytetracycline	-	209.6	-	-	-	-	-	-	-	-	(22.2)	-	-	-	(25.3)	-	-
Sulfamethoxazole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Enrofloxacin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ceftiofur	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillin G	-	(89.7)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tylosin	-	-	81.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Erythromycin-H_20	-	543.9	(25.2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	June Sampling per location																
Sulfadiazine	-	-	-	-	-	-	-										
Amoxicillin	-	-	-	-	-	-	-										
Cefapirin	-	-	(26.0)	-	-	-	-										
Cefquinone	-	-	-	-	-	-	-										
Lincomycin	-	-	-	-	-	-	-										
Cefalonium	-	-	-	-	-	-	-										
Trimethoprim	-	-	(1.8)	-	-	-	-										
Marbofloxacin	-	1009.4	389.7	-	-	-	-										
Oxytetracycline	-	930.9	206.9	-	-	-	-										
Sulfamethoxazole	-	1659.2	493.5	-	-	-	-										
Enrofloxacin	-	-	-	-	-	-	-										
Ceftiofur	-	-	-	-	-	-	-										
Penicillin G	(36.0)	815.9	325.3	-	-	-	-										
Tylosin	-	-	-	-	-	-	-										
Erythromycin-H_20	-	-	119.9	-	-	-	-										

No sampling occurred either due to no water presence or not scheduled, Numbers in bracket indicate concentrations below the tested LOQ but above the individual LOD.

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Appendix 5 Concentrations of target antibiotic residues in environmental water samples (continued)

Figure A5-2: Oxytetracycline concentration during storm event

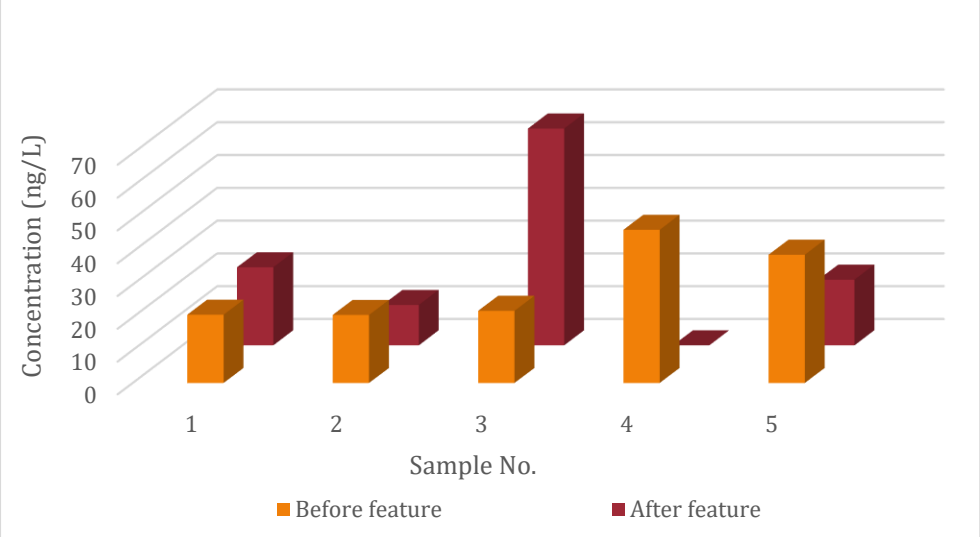
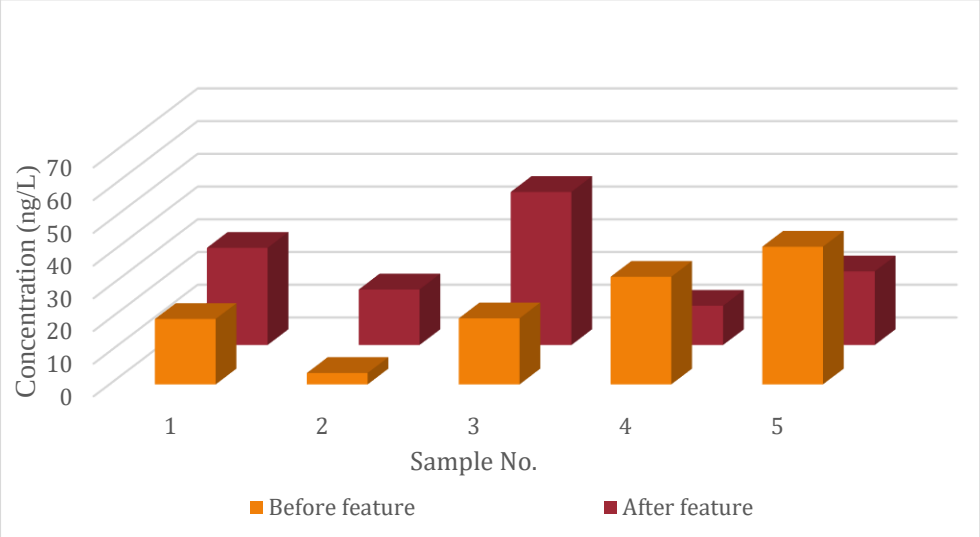


Figure A5-3: Marbofloxacin concentration during storm event



Appendix 6 Absorbance spectra for selected antibiotics

Figure A6-1: Absorbance spectra for Oxytetracycline at 1µg/L in different matrices and corresponding Xenon light irradiance from 240-500 nm

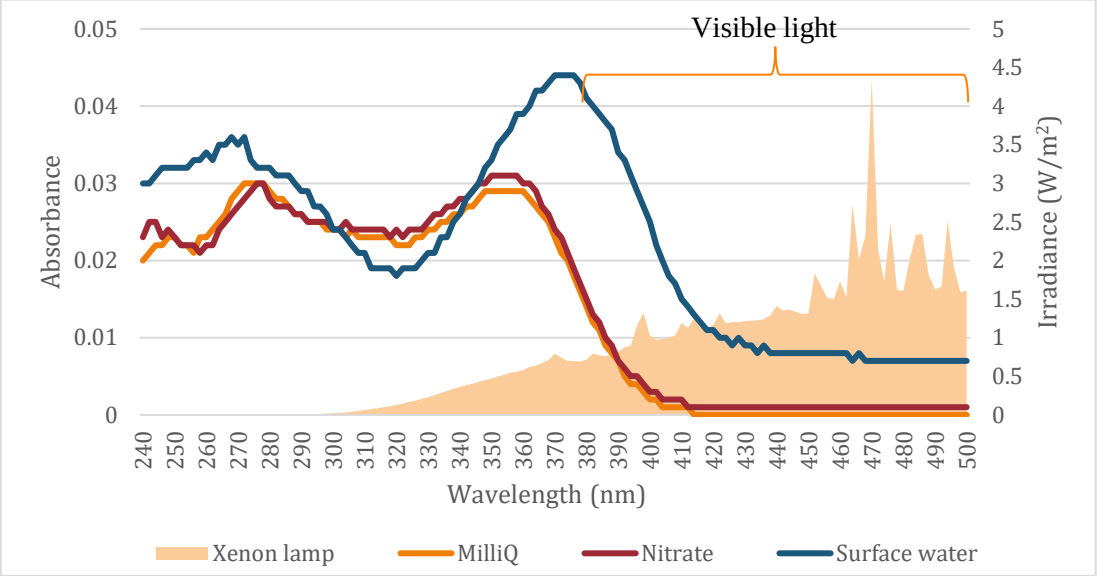
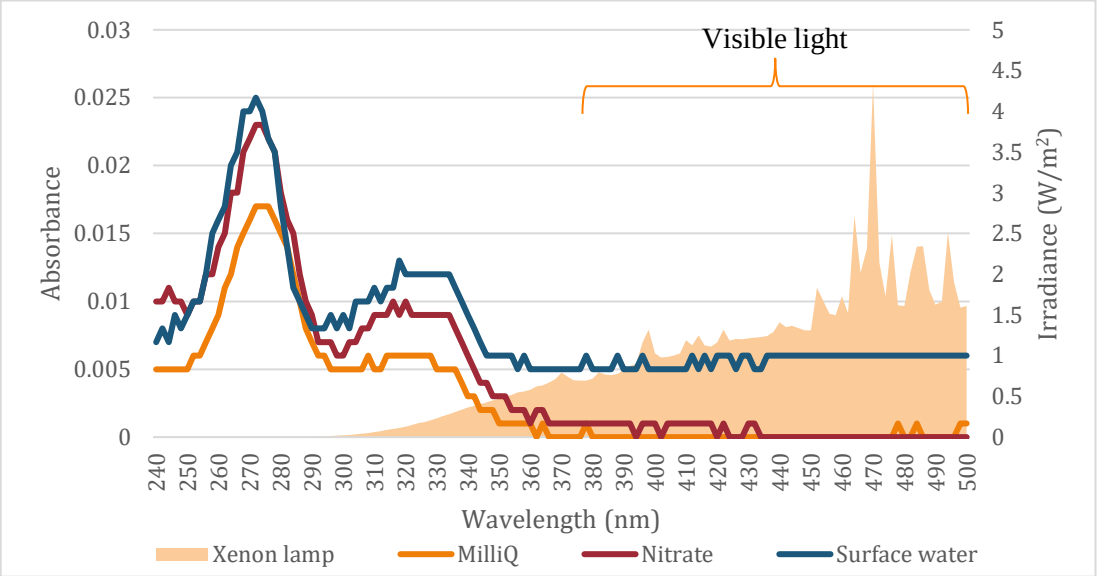


Figure A6-2: Absorbance spectra for Enrofloxacin at 1µg/L in different matrices and corresponding Xenon light irradiance from 240-500 nm



Appendix 6 Absorbance spectra for selected antibiotics (continued)

Figure A6-3: Absorbance spectra for Sulfamethoxazole at 1µg/L in different matrices and corresponding Xenon light irradiance from 240-500 nm

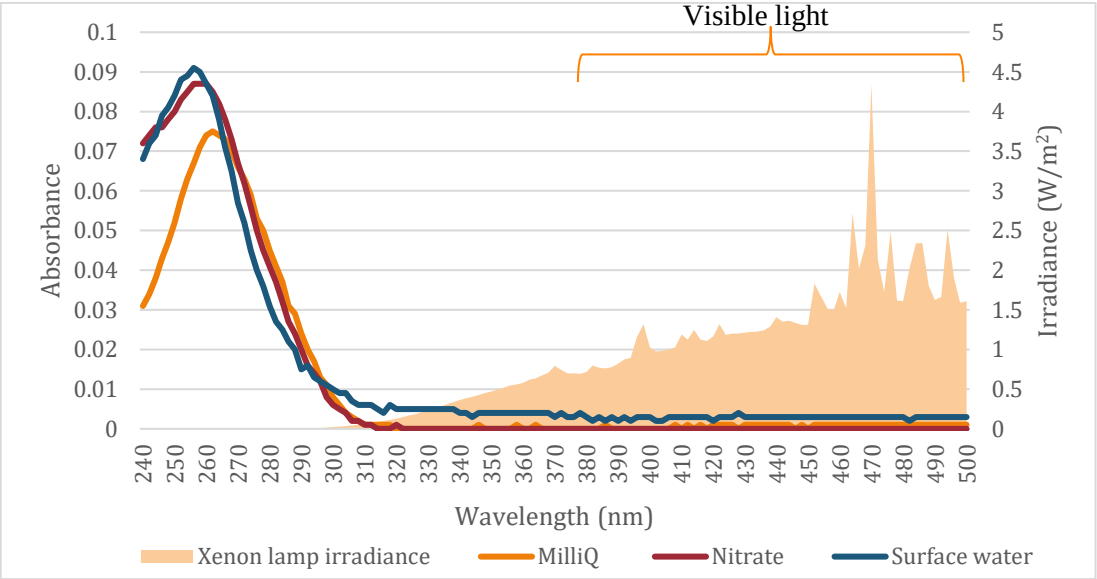
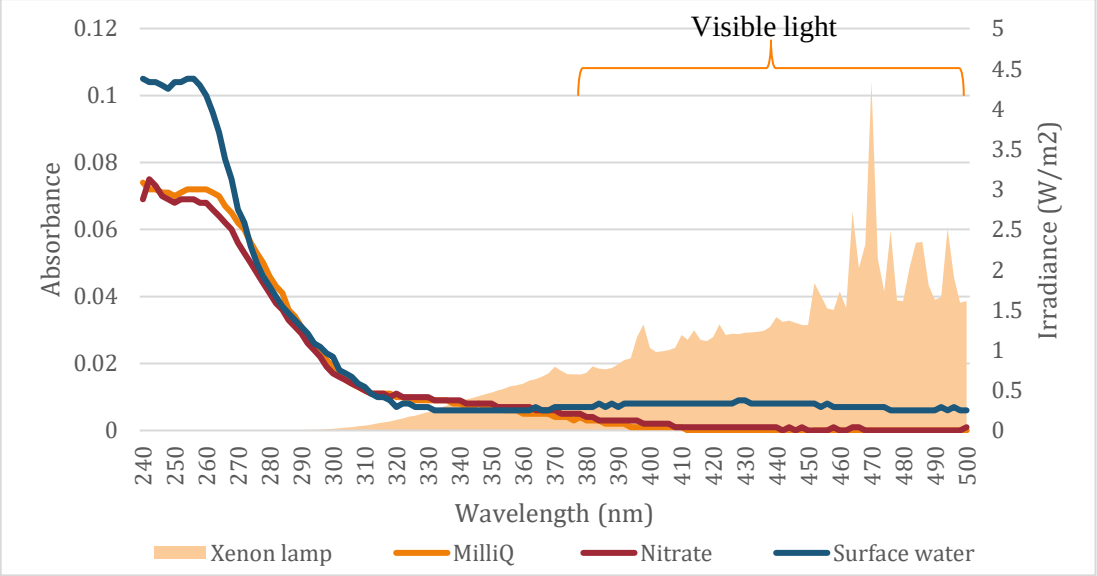


Figure A6-4: Absorbance spectra for Sulfadiazine at 1µg/L in different matrices and corresponding Xenon light irradiance from 240-500 nm



Appendix 6 Absorbance spectra for selected antibiotics (continued)

Figure A6-5: Absorbance spectra for Lincomycin at 1µg/L in different matrices and corresponding Xenon light irradiance from 240-500 nm

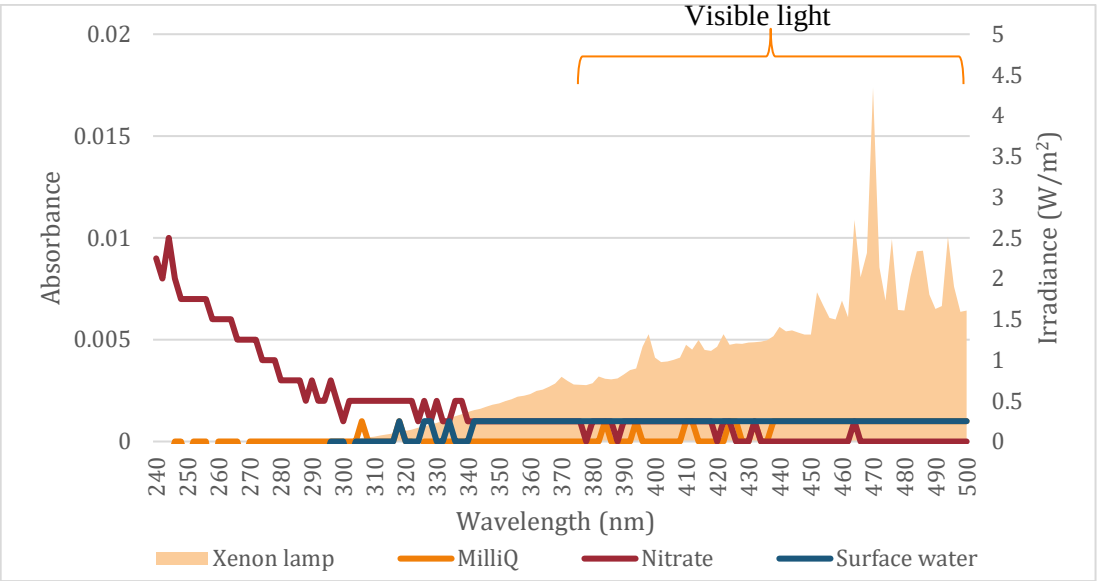
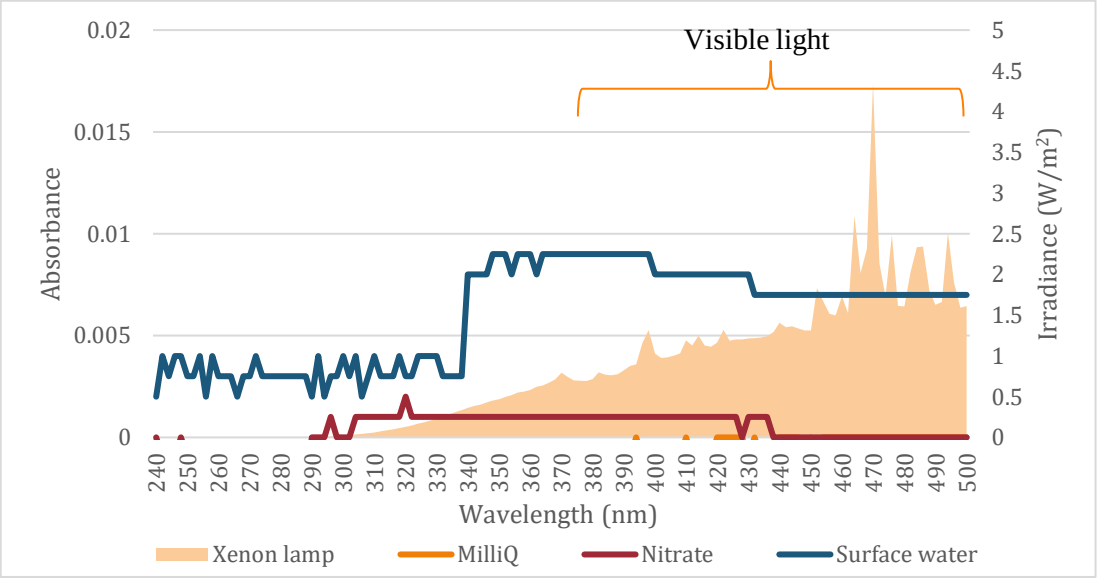
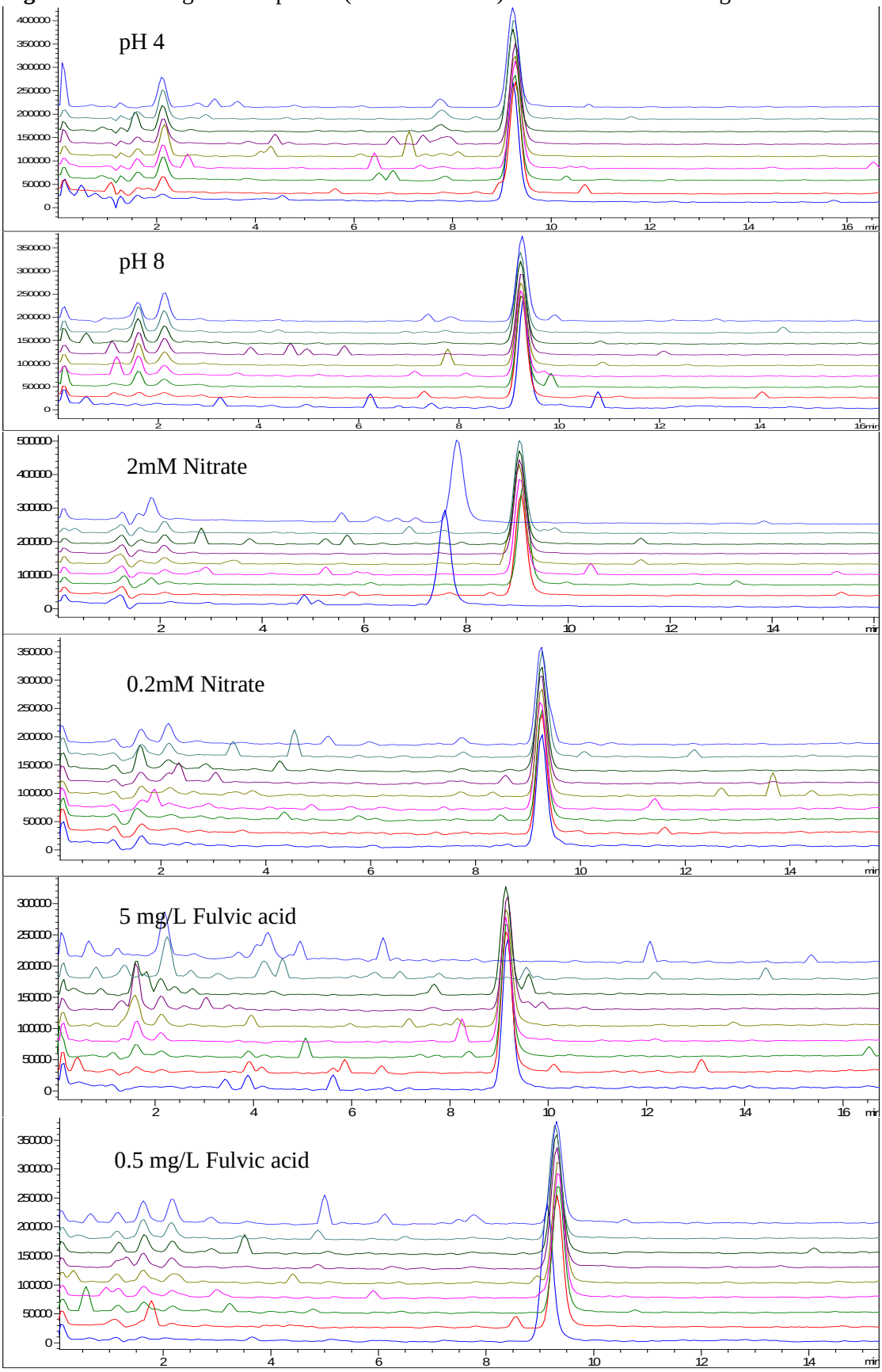


Figure A6-6: Absorbance spectra for Erythromycin at 1µg/L in different matrices and corresponding Xenon light irradiance from 240-500 nm



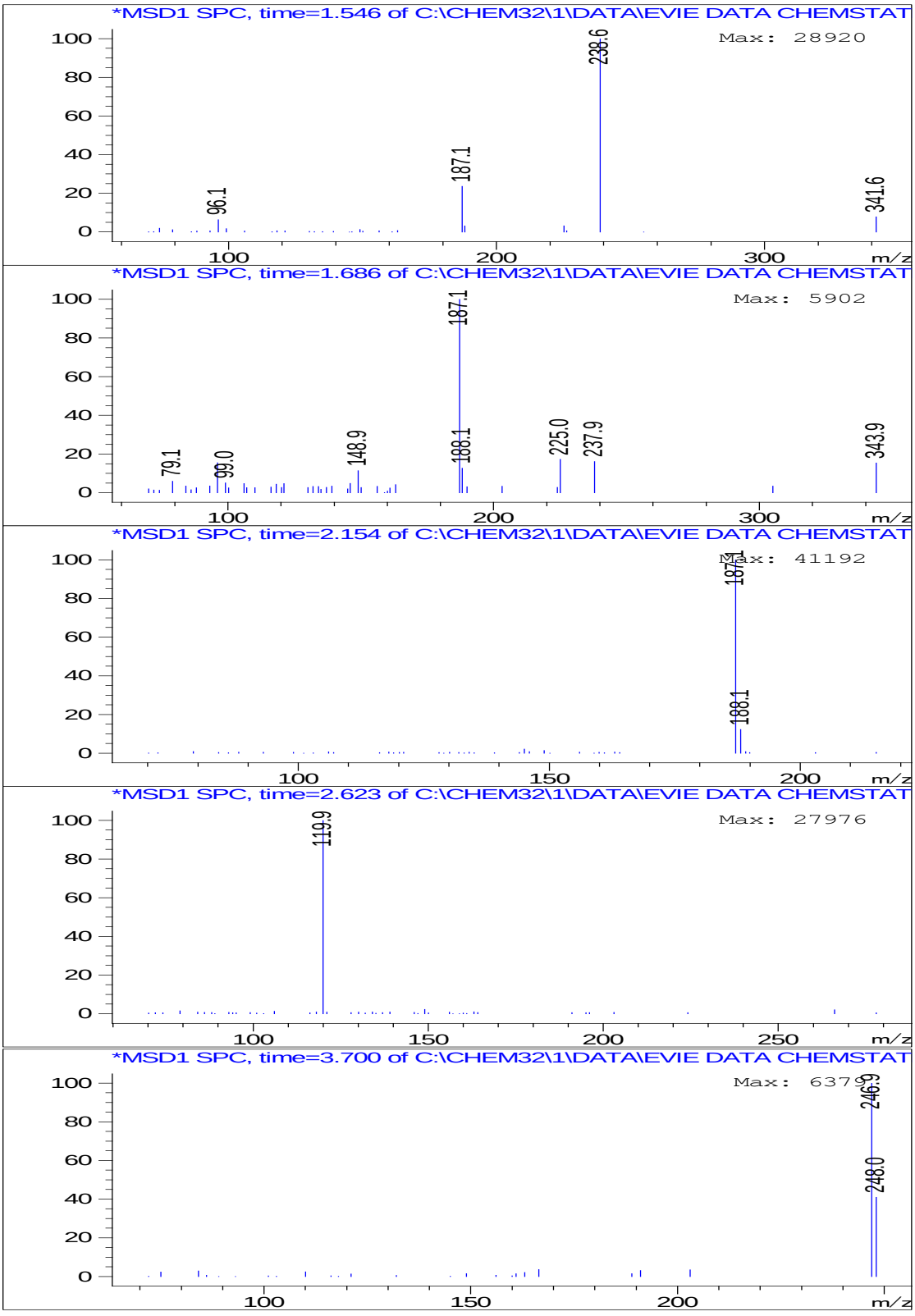
Appendix 7 Degradation profiles – transformation products

Figure A7-1: Degradation profile (30-780 minutes) – Sulfadiazine at 10 mg L^{-1}



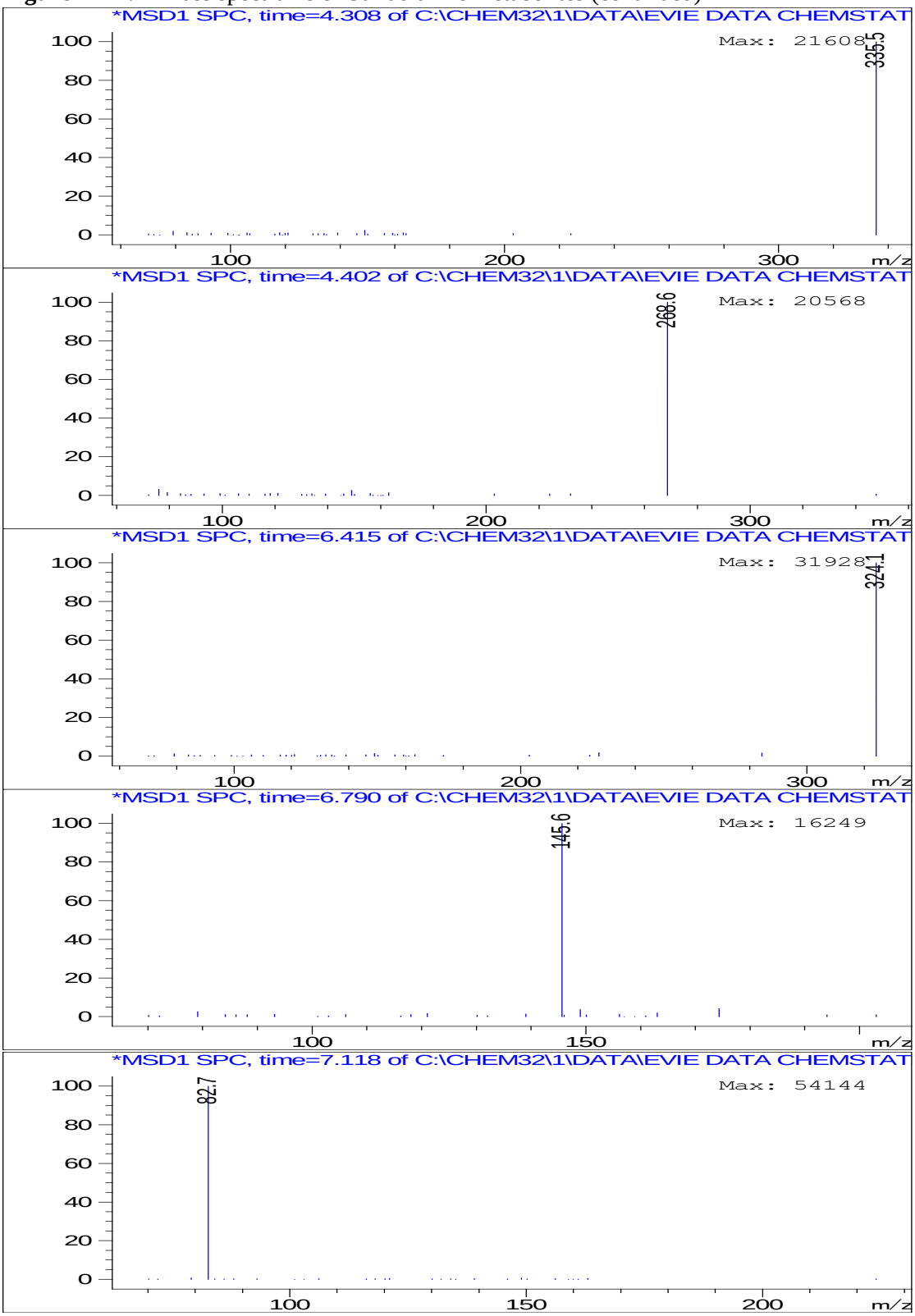
Appendix 7 Degradation profiles - transformation products (continued)

Figure A7-2: Mass spectrums of Sulfadiazine metabolites



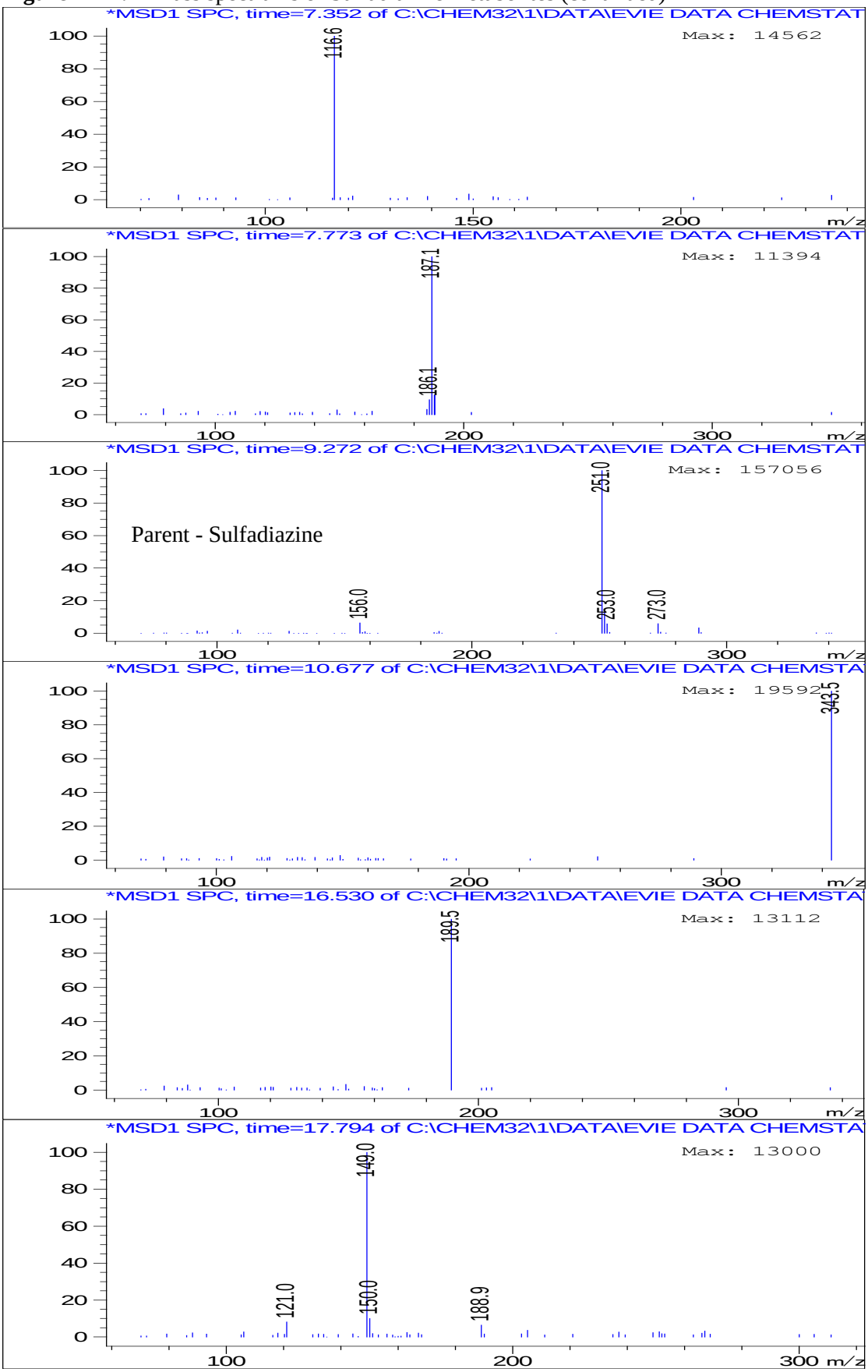
Appendix 7 Degradation profiles - transformation products (continued)

Figure A7-2: Mass spectrums of Sulfadiazine metabolites (continued)



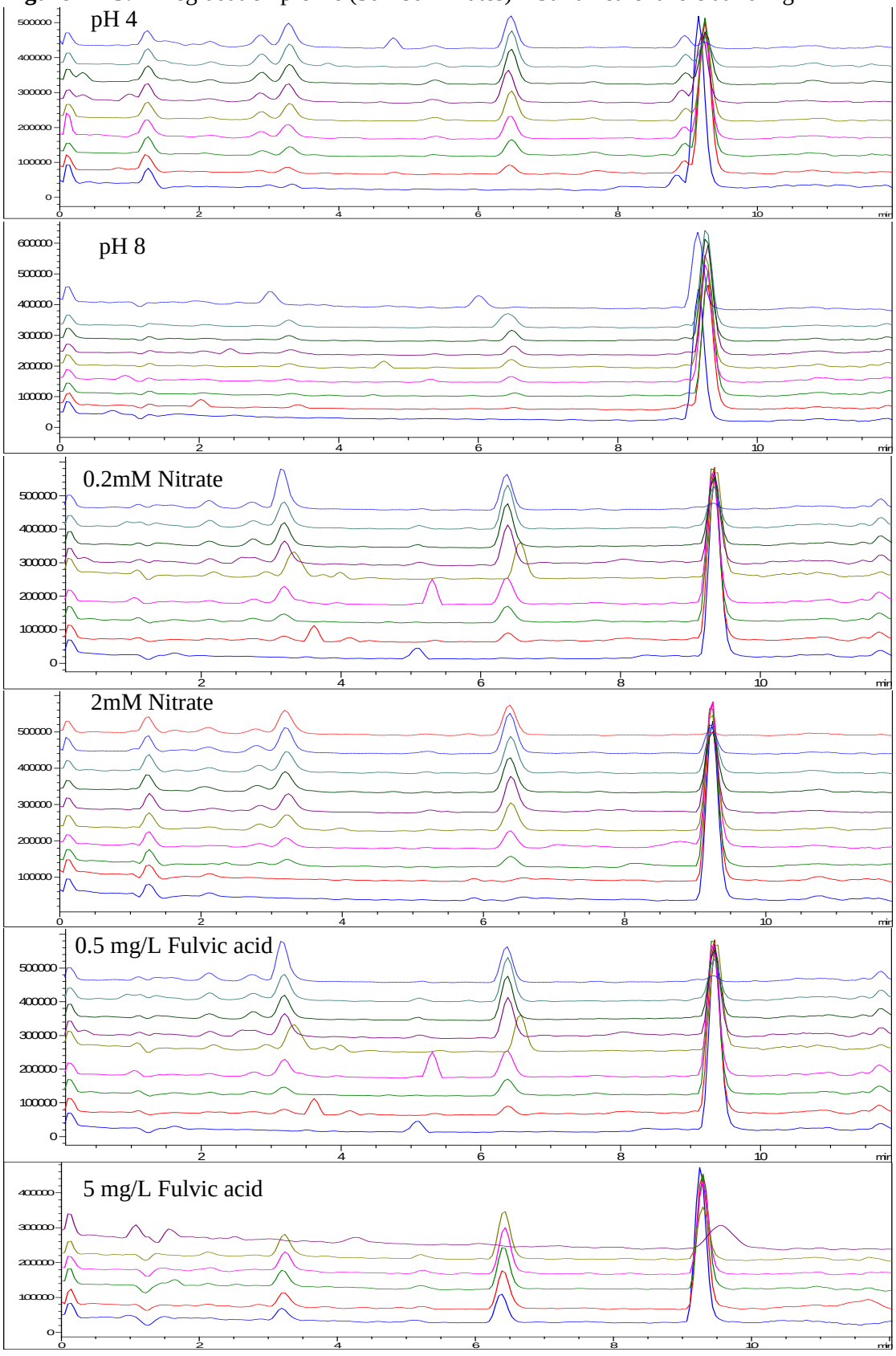
Appendix 7 Degradation profiles - transformation products (continued)

Figure A7-2: Mass spectrums of Sulfadiazine metabolites (continued)



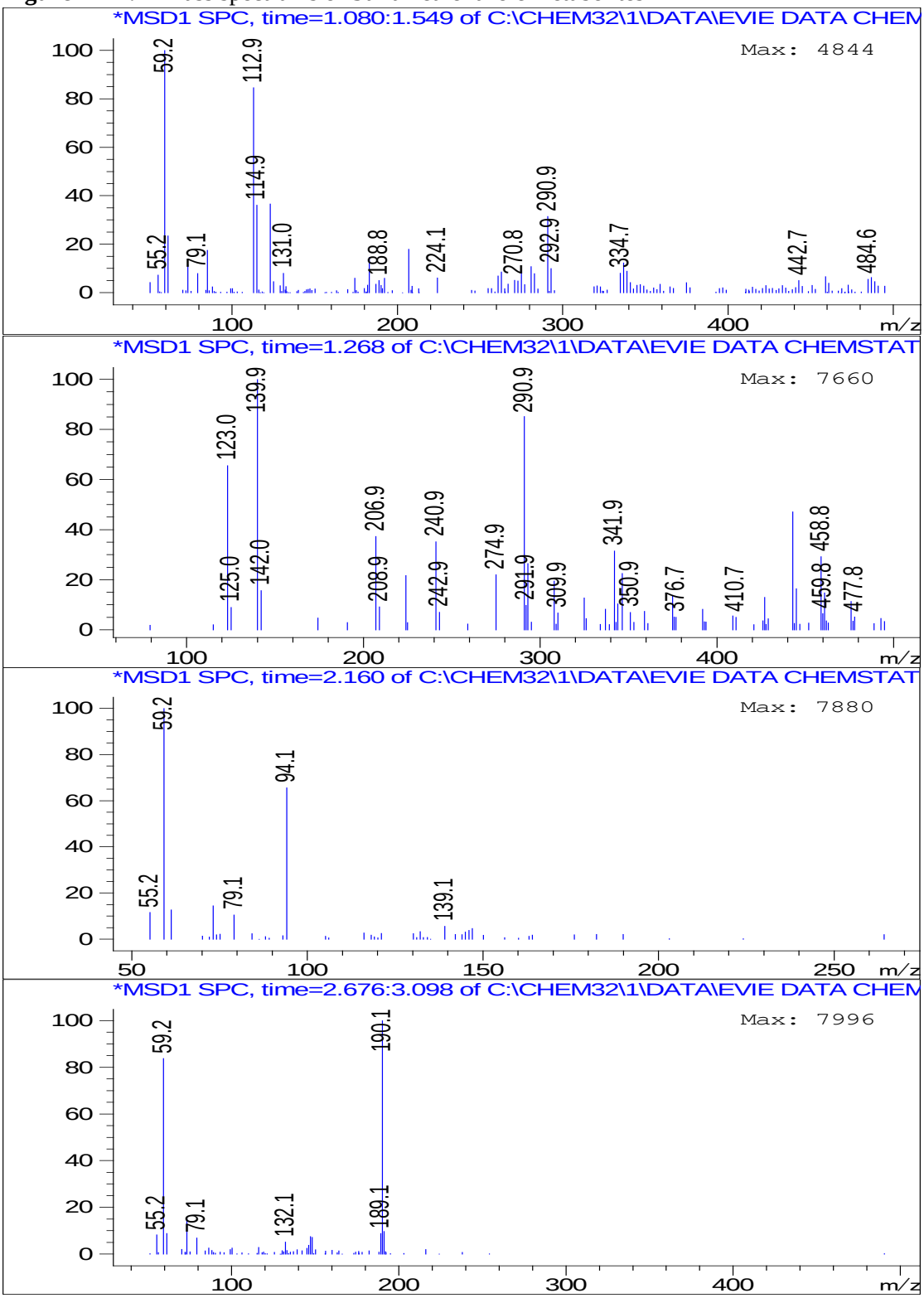
Appendix 7 Degradation profiles - transformation products (continued)

Figure A7-3: Degradation profile (30-780 minutes) – Sulfamethoxazole at 10 mg L^{-1}



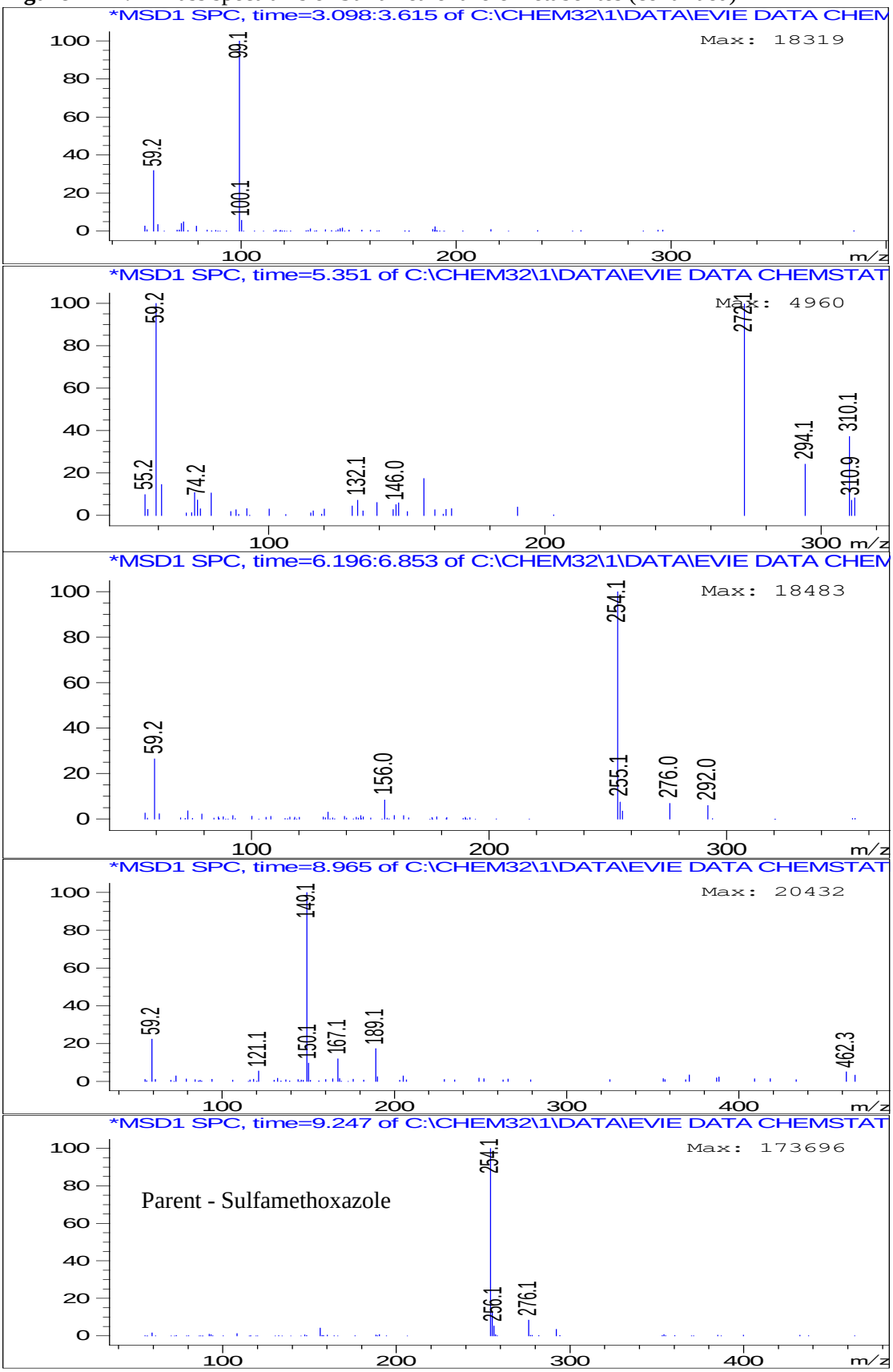
Appendix 7 Degradation profiles - transformation products (continued)

Figure A7-4: Mass spectrums of Sulfamethoxazole metabolites



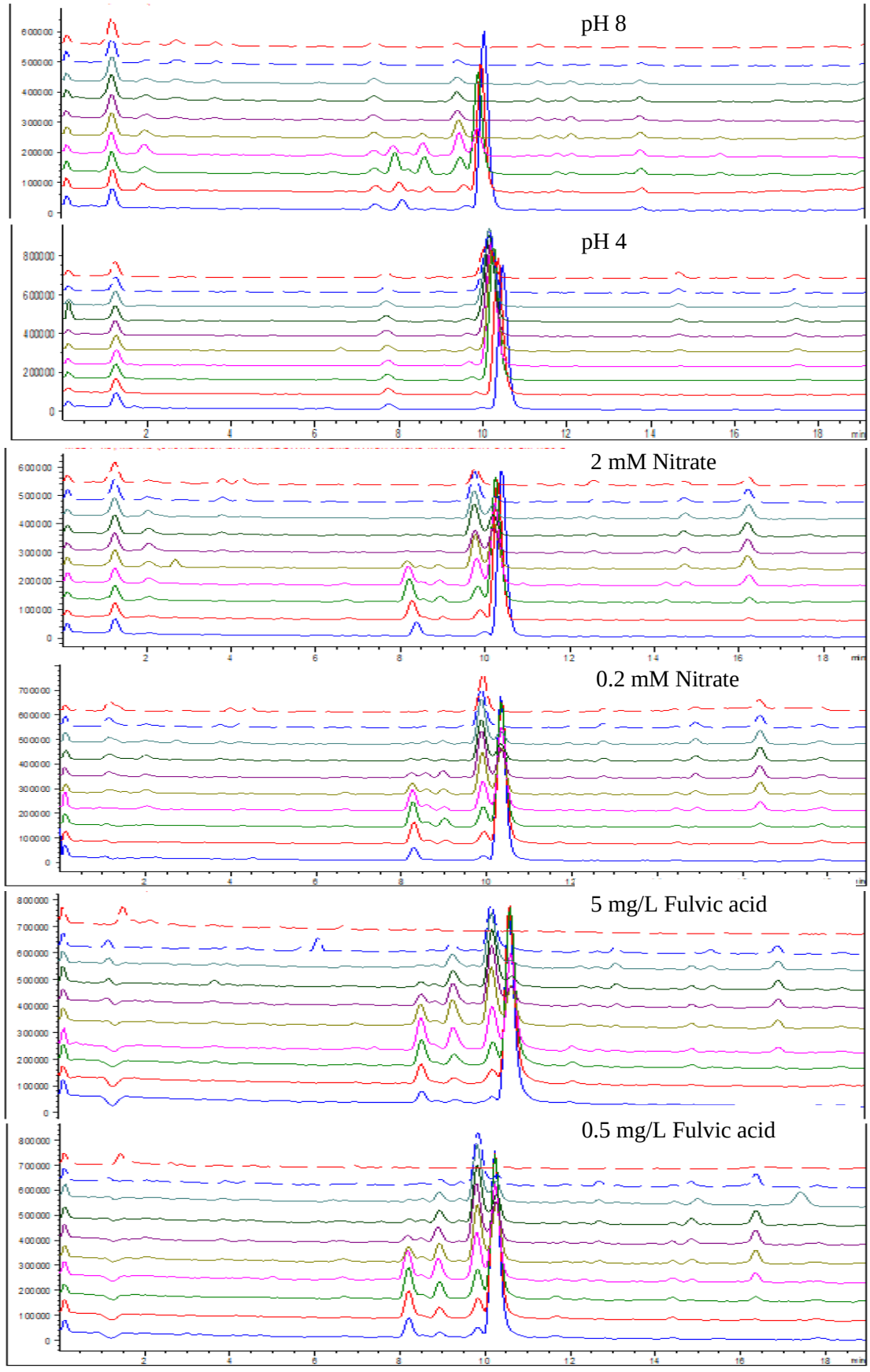
Appendix 7 Degradation profiles - transformation products (continued)

Figure A7-4: Mass spectrums of Sulfamethoxazole metabolites (continued)



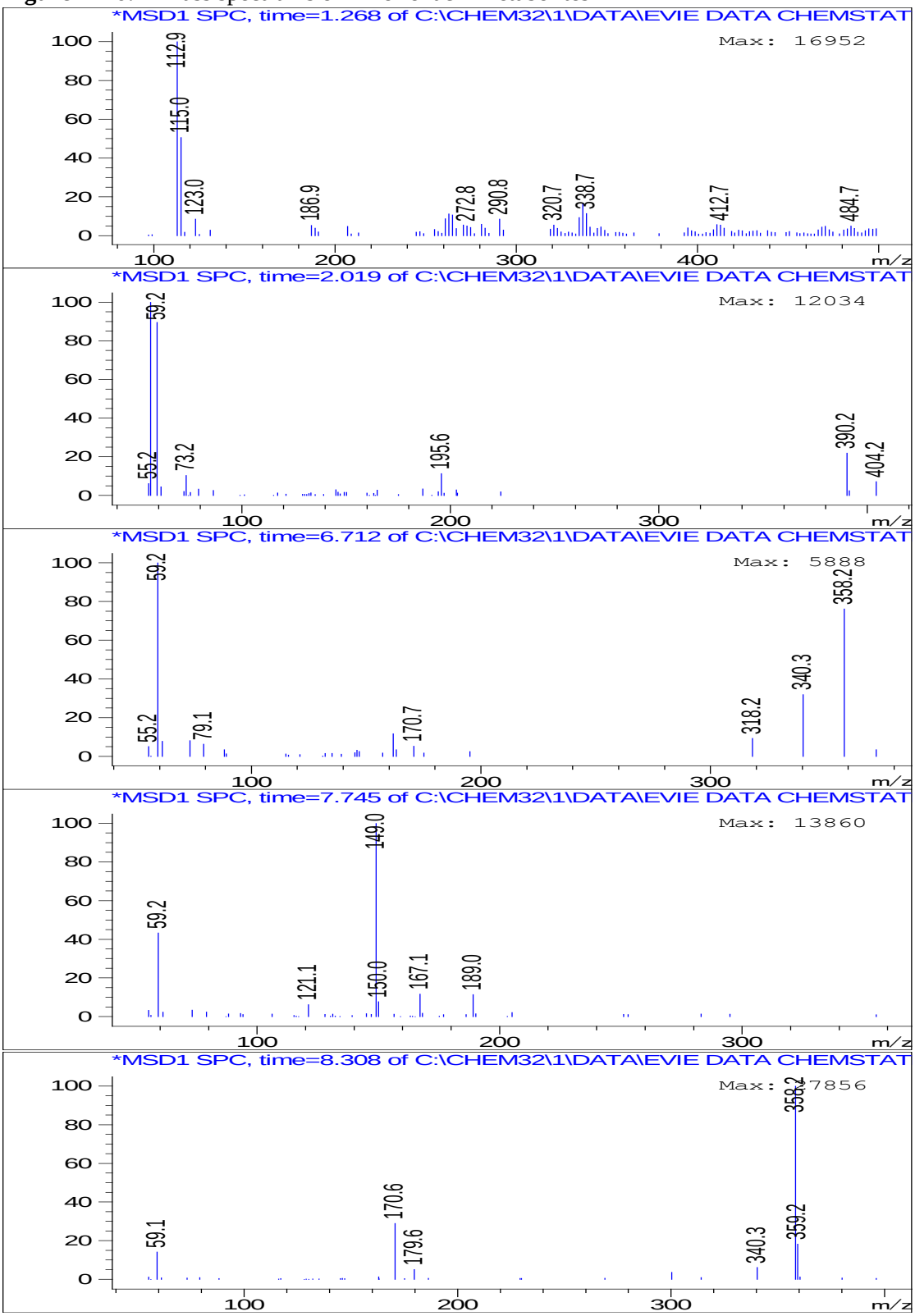
Appendix 7 Degradation profiles - transformation products (continued)

Figure A7-5: Degradation profile (10-780 minutes) – Enrofloxacin at 10 mg L^{-1}



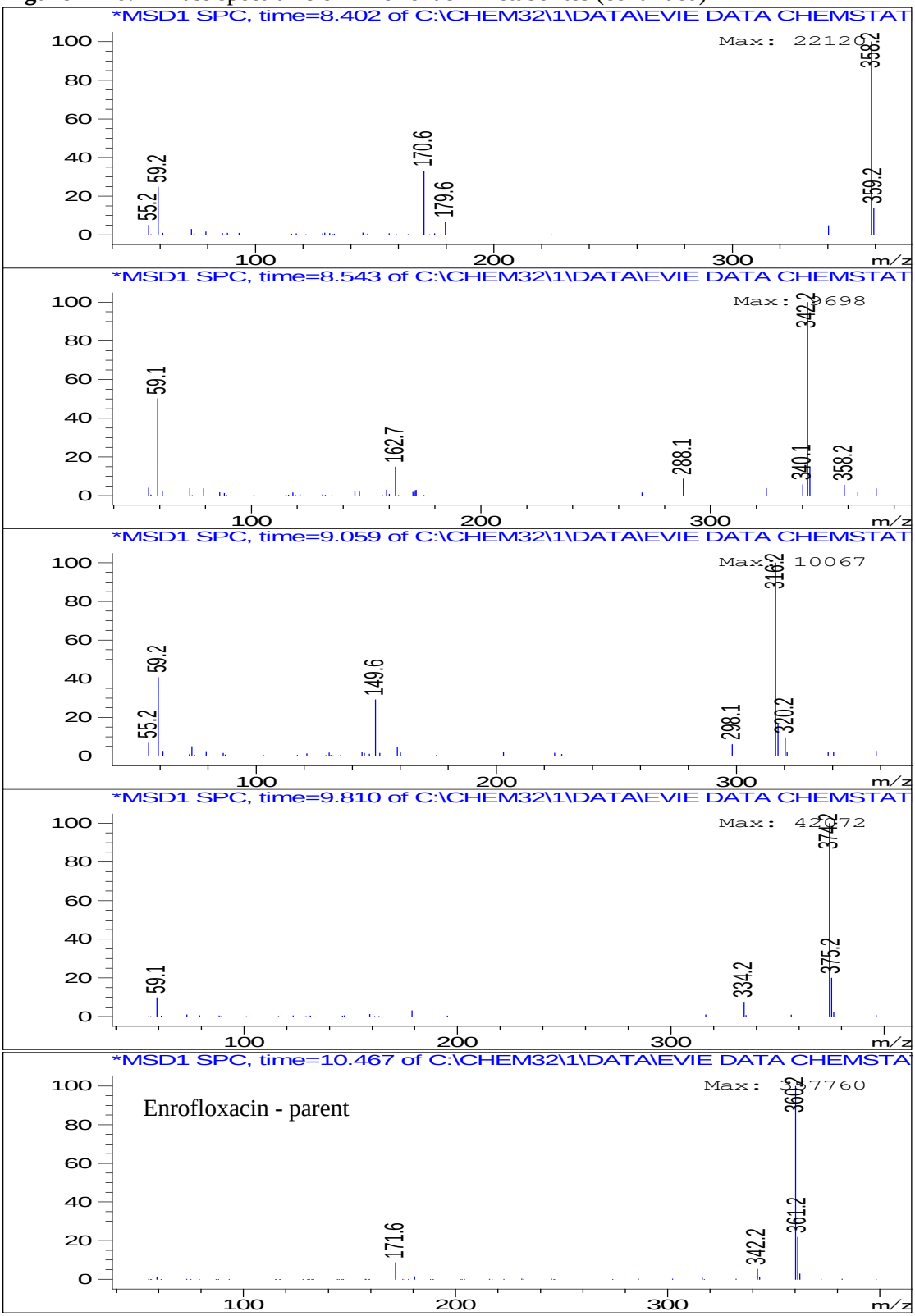
Appendix 7 Degradation profiles - transformation products (continued)

Figure A7-6: Mass spectrums of Enrofloxacin metabolites



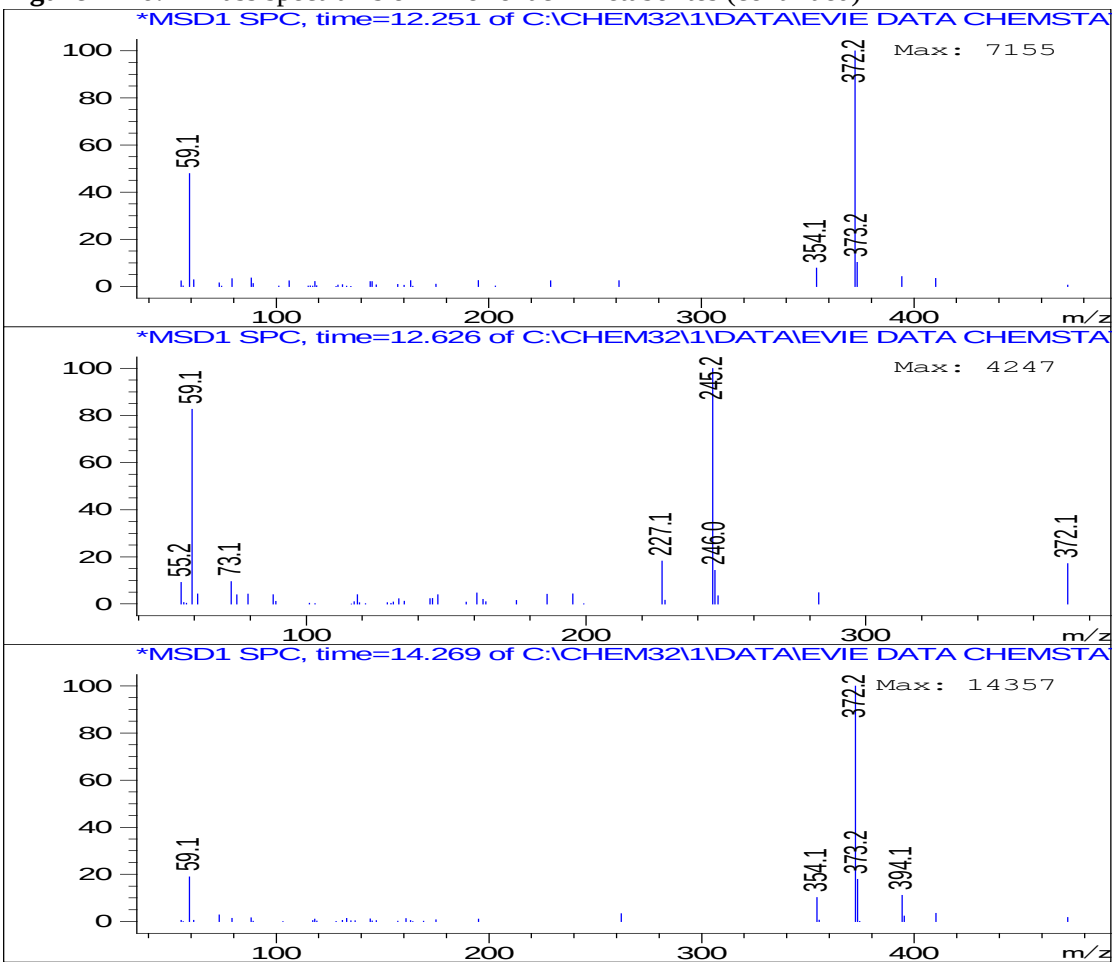
Appendix 7 Degradation profiles - transformation products (continued)

Figure A7-6: Mass spectrums of Enrofloxacin metabolites (continued)



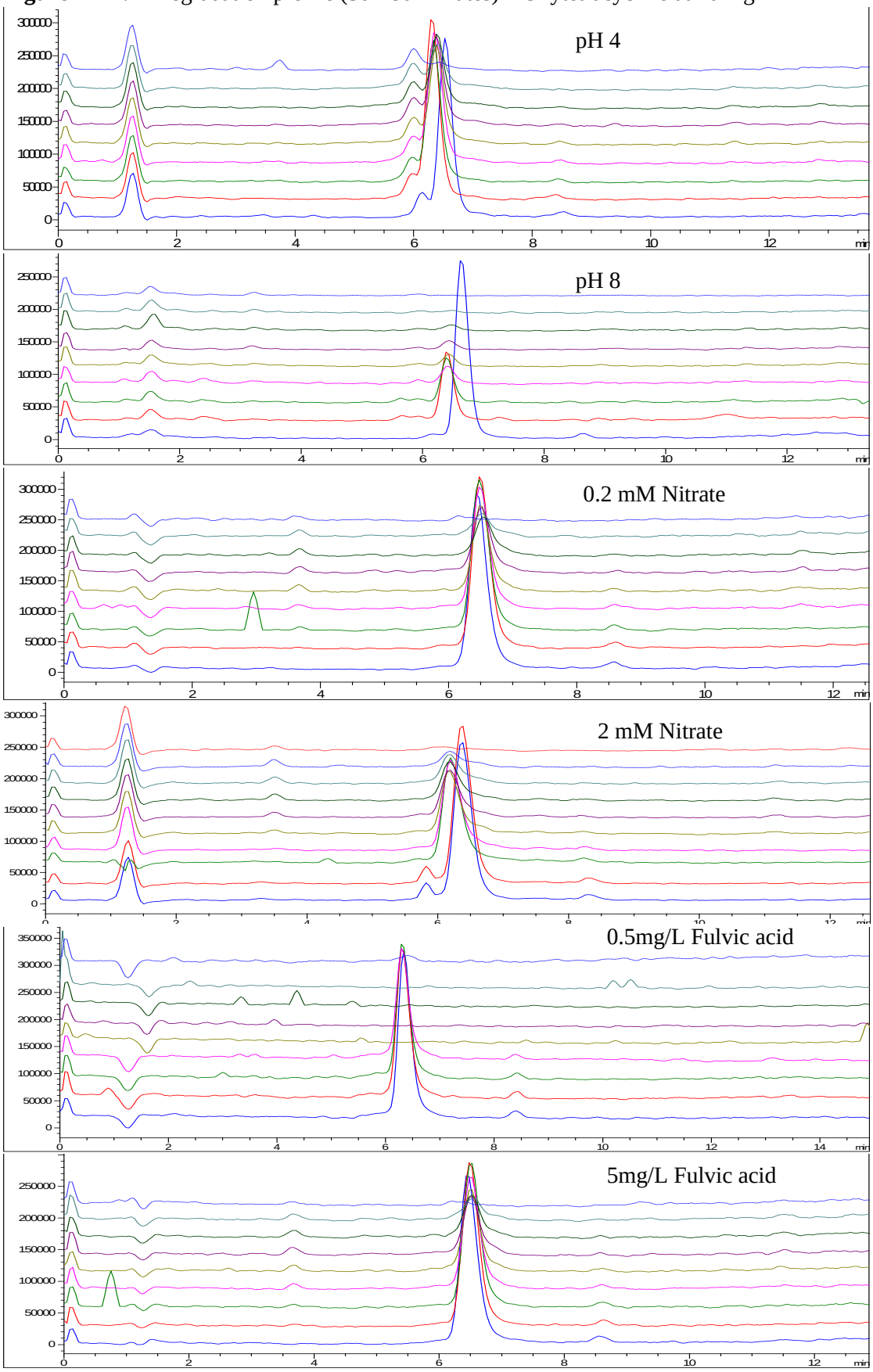
Appendix 7 Degradation profiles - transformation products (continued)

Figure A7-6: Mass spectrums of Enrofloxacin metabolites (continued)



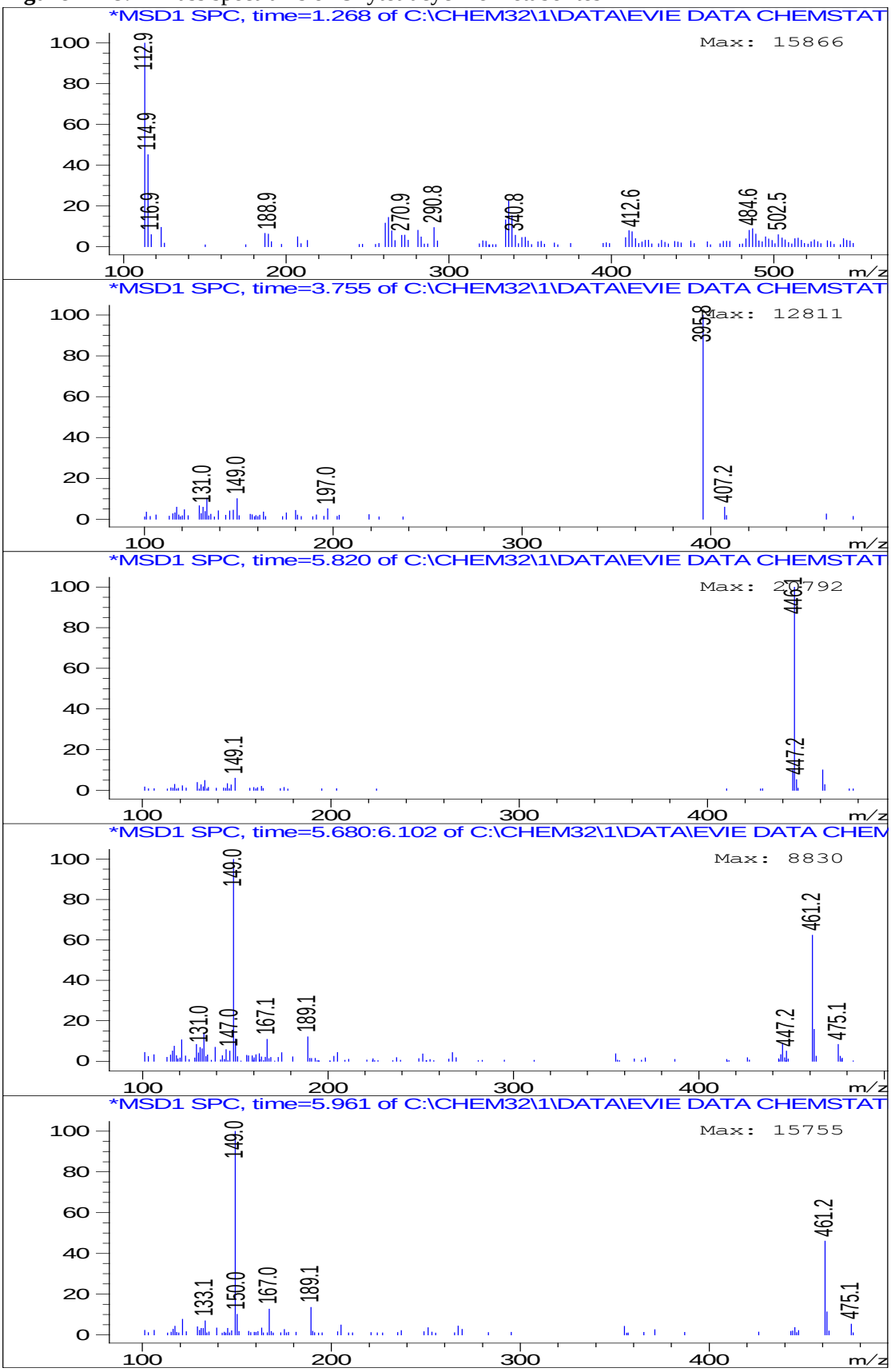
Appendix 7 Degradation profiles - transformation products (continued)

Figure A7-7: Degradation profile (30-780 minutes) – Oxytetracycline at 10 mg L^{-1}



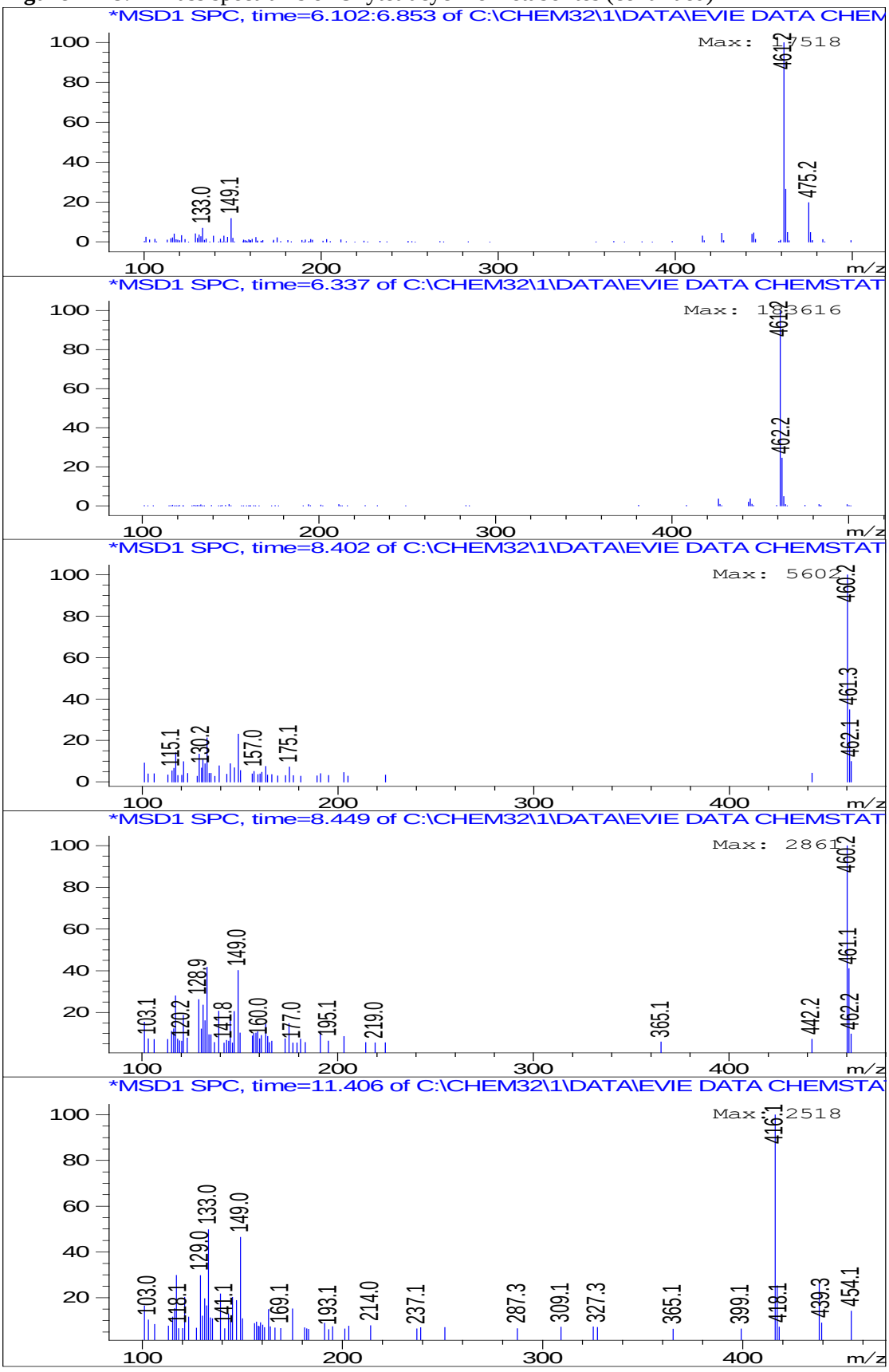
Appendix 7 Degradation profiles - transformation products (continued)

Figure A7-8: Mass spectrums of Oxytetracycline metabolites



Appendix 7 Degradation profiles - transformation products (continued)

Figure A7-8: Mass spectrums of Oxytetracycline metabolites (continued)



Appendix 8 Algae cultivation

Figure A8-1: Predominant species in the algae solution

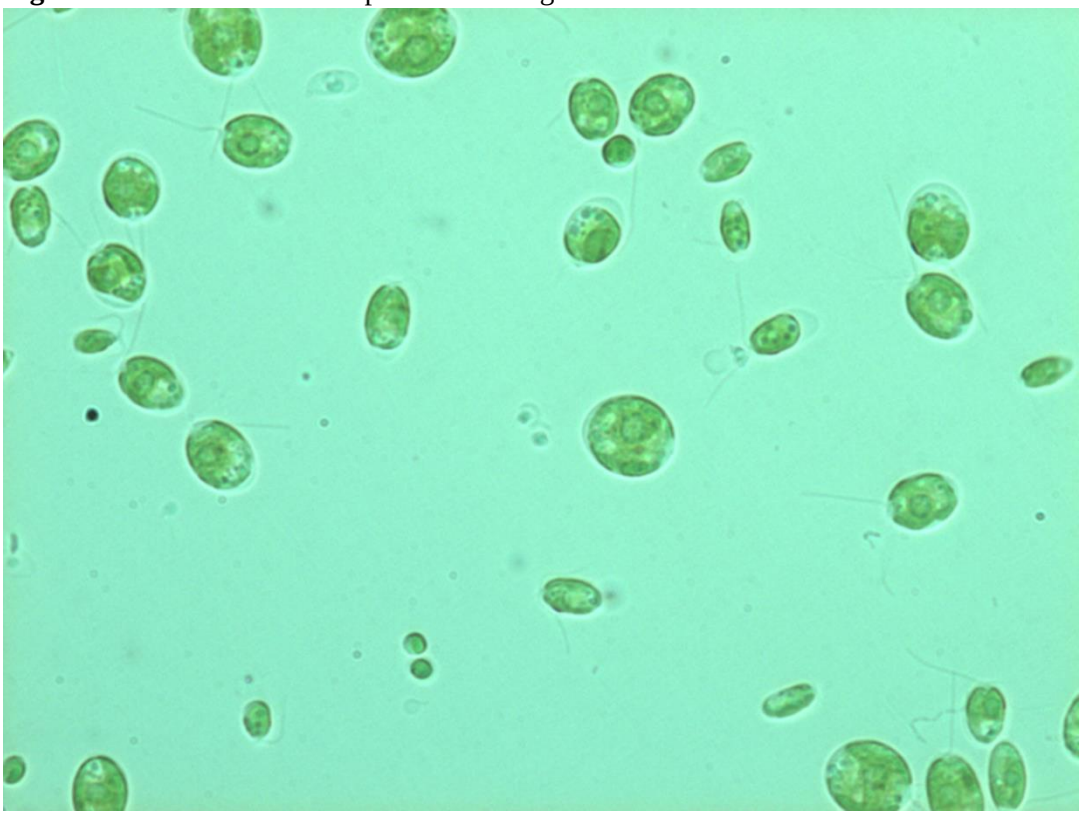


Figure A8-2: Calibration curve for growth measurement

Dillution factors	No of Cells	Absorbance at 684nm
1,00E-04	3,48E+03	0,002
1,00E-03	3,48E+04	0,01
1,00E-02	3,48E+05	0,031
1./32	1,09E+06	0,093
1./16	2,18E+06	0,161
1,00E-01	3,48E+06	0,257
1./8	4,35E+06	0,311
1./4	8,70E+06	0,598
1./2	1,74E+07	1,061
1,00E+00	3,48E+07	1,874

Appendix 9 Example of CAKE Kinetic report

Kinetic report for Sulfadiazine – MilliQ water

Data set: MilliQ (SFO)

CE room experiment UV light

Study date: 20 March 2017
Report generated: 01 June 2020

Model Setup:

Topology: Parent only
Optimiser: IRLS (IRLS Its. 10, IRLS Tol. 1E-05, Max. Its. 100, Tol. 1E-05)
SANN Max Iterations: 10000
Extra Solver Option: Use If Required

Initial Values of Sequence Parameters:

Parameter	Initial Value	Bounds	Fixed
Parent_0	100	0 to (unbounded)	No
k_Parent	0.1	0 to (unbounded)	No

Fit step: Final

Used Extra Solver: No

Reference Table:

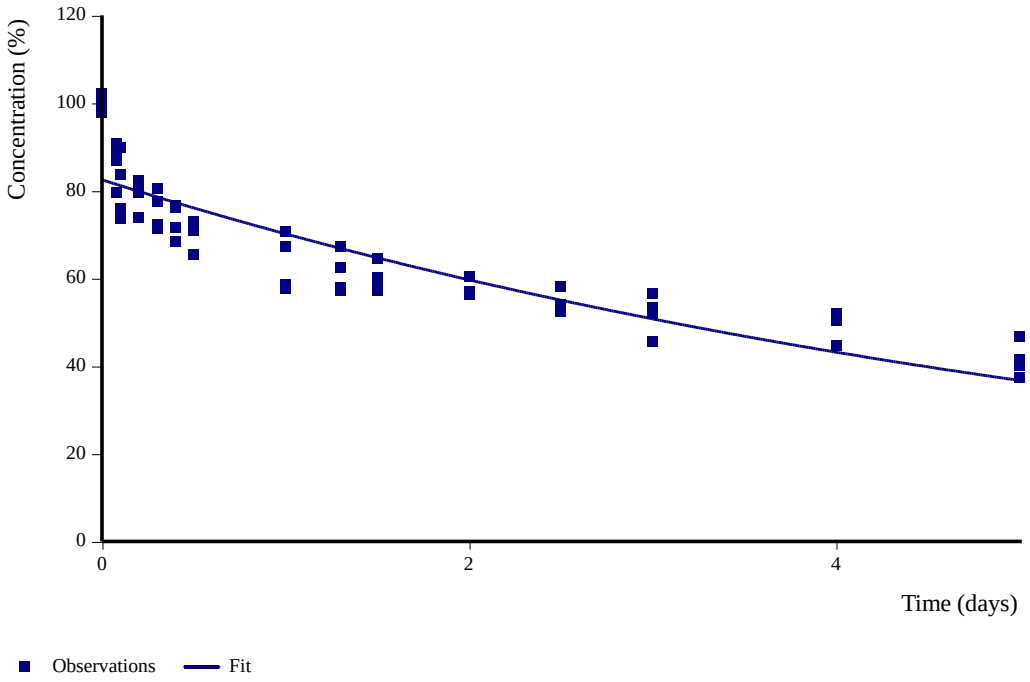
Compartment	Name
Parent	Parent

Appendix 9 Example of CAKE Kinetic report (continued)

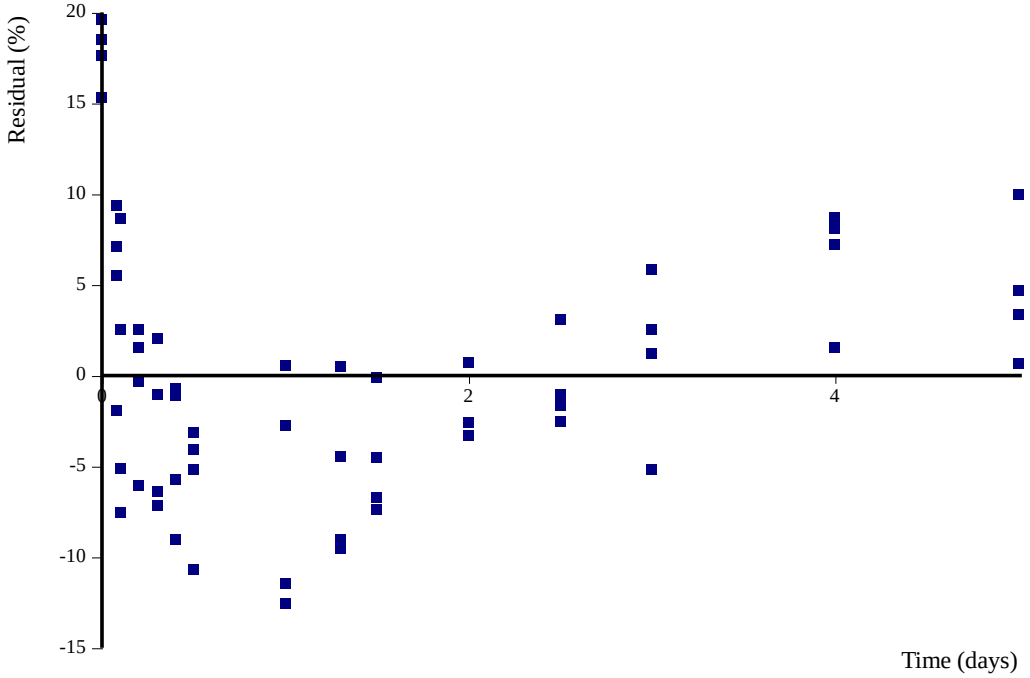
Kinetic report for Sulfadiazine – MilliQ water (continued)

Graphical Summary:

Observations and Fitted Model:



Residuals:



Appendix 9 Example of CAKE Kinetic report (continued)

Kinetic report for Sulfadiazine – MilliQ water (continued)

Initial Values for this Step:

Parameter	Initial Value	Bounds	Fixed
Parent_0	100	0 to (unbounded)	No
k_Parent	0.1	0 to (unbounded)	No

Estimated Values:

Parameter	Value	σ	Prob. > t	Lower (90%) CI	Upper (90%) CI	Lower (95%) CI	Upper (95%) CI
Parent_0	82.48	1.456	N/A	80.04	84.91	79.56	85.39
k_Parent	0.1618	0.01229	2.26E-019	0.1412	0.1823	0.1372	0.186

χ^2

Parameter	Error %	Degrees of Freedom
All data	7.51	13
Parent	7.51	13

Decay Times:

Compartment	DT50 (days)	DT90 (days)
Parent	4.29	14.2

Additional Statistics:

Parameter	r^2 (Obs v Pred)	Efficiency
All data	0.7906	0.7897
Parent	0.7906	0.7897

Parameter Correlation:

	Parent_0	k_Parent
Parent_0	1	0.6261
k_Parent	0.6261	1

Appendix 9 Example of CAKE Kinetic report (continued)

Kinetic report for Sulfadiazine – MilliQ water (continued)

Observed v. Predicted:

Compartment Parent

Time (days)	Value (%)	Predicted Value	Residual
0	102.1	82.48	19.62
0	97.8	82.48	15.32
0	100.1	82.48	17.62
0	101	82.48	18.52
0.08	86.9	81.42	5.483
0.08	79.5	81.42	-1.917
0.08	88.5	81.42	7.083
0.08	90.8	81.42	9.383
0.1	73.6	81.15	-7.554
0.1	76	81.15	-5.154
0.1	83.7	81.15	2.546
0.1	89.8	81.15	8.646
0.2	73.8	79.85	-6.052
0.2	79.5	79.85	-0.352
0.2	81.4	79.85	1.548
0.2	82.4	79.85	2.548
0.3	72.2	78.57	-6.371
0.3	71.4	78.57	-7.171
0.3	80.6	78.57	2.03
0.3	77.5	78.57	-1.071
0.4	71.6	77.31	-5.71
0.4	68.3	77.31	-9.01
0.4	76.2	77.31	-1.11
0.4	76.6	77.31	-0.7097
0.5	65.4	76.07	-10.67
0.5	70.9	76.07	-5.169
0.5	72	76.07	-4.069
0.5	72.9	76.07	-3.169
1	58.7	70.16	-11.46
1	57.6	70.16	-12.56
1	67.4	70.16	-2.758
1	70.7	70.16	0.5417
1.3	57.8	66.83	-9.035
1.3	57.3	66.83	-9.535
1.3	62.4	66.83	-4.435
1.3	67.3	66.83	0.4655
1.5	58	64.71	-6.707

Appendix 9 Example of CAKE Kinetic report (continued)

Kinetic report for Sulfadiazine – MilliQ water (continued)

Time (days)	Value (%)	Predicted Value	Residual
1.5	57.3	64.71	-7.407
1.5	60.2	64.71	-4.507
1.5	64.6	64.71	-0.1067
2	56.4	59.68	-3.279
2	56.4	59.68	-3.279
2	57.1	59.68	-2.579
2	60.4	59.68	0.7213
2.5	52.5	55.04	-2.541
2.5	54	55.04	-1.041
2.5	53.4	55.04	-1.641
2.5	58.1	55.04	3.059
3	52	50.76	1.236
3	45.6	50.76	-5.164
3	53.3	50.76	2.536
3	56.6	50.76	5.836
4	51.3	43.18	8.118
4	44.7	43.18	1.518
4	50.4	43.18	7.218
4	51.9	43.18	8.718
5	40.1	36.73	3.369
5	37.4	36.73	0.6685
5	41.4	36.73	4.669
5	46.7	36.73	9.969

Sequence Creation Information:
Fit generated by CAKE version 3.3 (Release)
running on R version 3.0.0 (2013-04-03)

Report Information:
Report generated by CAKE version 3.3 (Release)
CAKE developed by Tessella Ltd, Abingdon, Oxfordshire, UK, sponsored by Syngenta
Running on .NET version 4.0.30319.42000