UNIVERSITY OF TARTU
FACULTY OF SCIENCE AND TECHNOLOGY
Institute of Chemistry

Master's Thesis (30 EAP)
Applied Measurement Science

Waseem Ahmad Iftikhar

DETERMINATION OF ANTIBACTERIALS IN RIVER WATER BY SOLID PHASE EXTRACTION USING LC-ESI-MS/MS

Supervisor: Associate Professor Koit Herodes

TARTU 2020

TABLE OF CONTENTS

ABBREVIATIONS: 4

1. INTRODUCTION 6

2. LITERATURE OVERVIEW 7

2.1. Antibiotics: Classification and Consumption in Estonia 7

2.2. Need for antibacterial environmental monitoring: 8

2.3. River water, A repository of biochemical substances. 9

2.4. The impact of the bioaccumulation of pharmaceuticals: 9

2.5. Measurements of Antibiotics in the environment: 10

2.6. Sample Extraction and Detection methods: 11

2.7. Aim: 12

3. EXPERIMENTAL 13

3.1. Chemicals and Reagents 13

3.2. Instruments and Methods 13

3.3. Preparation of Standard solutions and eluents: 13

3.4. River water collection and sample preparation. 14

3.5. LC-ESI-MS/MS 15

3.6. LC-Method development: 16

4. RESULTS AND DISCUSSIONS 17

4.1. Linearity: 18

4.2. Accuracy & Precision; River water Analysis: 19

4.3. Selectivity and Carryover effects 21

4.4. Matrix effects: 23

4.5. Recovery: 24

4.6. Process efficiency 24

5. ECOTOXICOLOGICAL IMPACT: Predicted no effect concentrations (PNECs)................. 25

6. SUMMARY... 27

Acknowledgements.. 29

References.. 30

Annexes.. 35

ABBREVIATIONS:

AB.	Antibiotics
AJS-ESI	Agilent Jet stream Electrospray ionization
AOAC	Association of Official Agricultural Chemists.
CE	Collision energy
ECDC	European Centre of Disease Control and Prevention
FA	Formic Acid
HCl	Hydrochloric Acid
HFIP	1,1,1,3,3,3-Hexafluoro-2-propanol
HPLC	High Performance Liquid Chromatography
LC-MS/MS	Liquid Chromatography – Tandem Mass Spectrometry
LLE	Liquid Liquid extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
MAE	Microwave assisted extraction
MeOH	Methanol
Min	Minute
MS/MS	Tandem mass spectrometry (MS^2)
NA	Not applicable
ND	Not Detected
PRD	Product ion scan

R^2	Regression coefficient
RSD	Relative standard Deviation
Rt	Retention time
SPE	Solid Phase Extraction

ANTIBIOTICS:

CIP	Ciprofloxacin
ENR	Enrofloxacin
FF	Florfenicol
MAR	Marbofloxacin
NOR	Norfloxacin
OFL	Ofloxacin
SDM	Sulfadimethoxine
SMX	Sulfamethoxazole

1. INTRODUCTION

Antibiotics are a group of compounds with varying classes, modes of actions, and a selective toxicity profile that are capable of destruction or inhibition of growth of bacteria but incapable of being toxic towards eukaryotic cells and are used to treat bacterial infections. Since the discovery of penicillin by Alexander Fleming in 1928, they have played an important role in advancing and revolutionizing human medicine.

However, due to the worldwide application of intensive care methods during the last two decades, antibiotic presence and resistance grew, and the scientific community has shown an increasing concern about the possible adverse effects associated with the presence of antibiotics in the environment. Classified as an emerging "Serious Threat" by the World Health Organization (WHO) [1], many analytical techniques and methods have emerged for their monitoring in the environment.

One such analytical technique that is widely used is Liquid Chromatography (HPLC) – Tandem Mass Spectrometry (MS/MS). A powerful technique that enables acceptable separation and selectivity towards individual analytes followed by their quantification at low concentration levels. Additionally, sample preparation through Solid Phase Extraction (SPE) coupled with an Agilent Jet Stream Electro Spray ionization (AJS-ESI) source enables for lower matrix effects and a better signal to noise ratio.

According to the European Commission of Disease Control and Prevention (ECDC) consumption statistics the major groups of antibiotics consumed in Estonia in 2018 belonged to the groups of Beta-Lactams, Tetracyclines, Macrolides, Quinolones, Sulfonamides and other J01 Antibiotics. Some of these Fluoroquinolones have displayed an increasing trend of antibacterial resistance whereas minimum to no data was available for Sulfonamides and Amphenicols. These three classes are low on the margin of consumption and had minimum studies about their fate in the environment. It prompted the targeted environmental study of these antibiotics at specific spots within Emajõgi; a river which flows from lake Võrtsjärv through Tartu county into lake Peipsi, crossing the city of Tartu for 10 km and a length of approximately 100 km.

2. LITERATURE OVERVIEW

2.1. Antibiotics: Classification and Consumption in Estonia.

More than 20 classes of antibiotics were produced in the 20th century. They are mainly classified based on their Chemical Structure, mechanism of action (bactericidal or bacteriostatic) or their range over the spectrum of bacteria that they can affect. Narrow spectrum is specific to Gram-positive and Gram-negative bacteria whereas wide spectrum includes a whole variety of bacteria. Further divisions or classification that help evaluate them are usually for laboratory studies such as bactericidal or bacteriostatic. The ones that target bacterial cell wall, cell membrane or interfere with their enzymatic functions exhibit bactericidal activity (that kills bacteria directly). The ones that interfere with their functionalities such as Protein synthesis inhibitors (preventing them from dividing) are considered bacteriostatic activity.

Antibiotics act by interrupting and disrupting the molecular targets within bacteria and on the cell surface, preventing them from growing or initiating killing. There are broad mechanisms of actions; Disruption of the bacterial cell wall, Blocking the production of new protein units, inhibit DNA from replicating. Antibiotics classifications based on their mechanisms are given below.

Table 1. Antibiotic Classification based on their mechanism of action

Mechanism of Action	Antibiotics
Cell Wall Synthesis inhibitors	Penicillin's, Cephalosporins, Beta-Lactamase inhibitors
Protein Synthesis Inhibitors	Inhibits 30 S: Aminoglycosides and Tetracyclines Inhibits 50 S: Macrolides, Amphenicols.
DNA synthesis Inhibitors	Fluoroquinolone inhibitors, Metronidazole
RNA Synthesis inhibitors	Rifampicin
Mycolic Acid synthesis inhibitors	Isoniazid
Folic acid synthesis inhibitors	Sulfonamides, Trimethoprim.

Throughout the years the European Centre for Disease Prevention and Control (ECDC) has monitored the consumption of antibiotics in Humans and Food Producing Animals. In 2018, the major antibiotic groups that were consumed in Community and Hospital Centers in Estonia

belonged to Beta-Lactams, Tetracyclines, Macrolides, Quinolones, Sulfonamides followed by other J01 Substances. [1]. While another report published in 2019 by European Medicines Agency (EMA) listed major antibiotic usage within Estonia in veterinary medicine belonged to Penicillin's, Tetracyclines, Sulfonamides, Macrolides. Fluoroquinolones, Cephalosporins and Amphenicols.[33]

On the local level the *Ravimiamet* or Estonian Agency of medicines published their latest report in 2016 monitoring the increase and decrease in sales of the various antibiotics for a decade. In veterinary medicine, the amount of sulfonamides sold has decreased whereas the amount of fluoroquinolones (significantly Enrofloxacin and Marbofloxacin) and Amphenicols has increased, the major increase being florfenicol [34]. While for Human medicine the *Ravimiamet* generated another report that suggests that quinolones have a steady neutral trend, which indicates that the use of fluoroquinolones had not decreased as of 2016.

2.2. Need for antibacterial environmental monitoring:

Environmental changes no matter how small, occur naturally and are caused by different levels of interactions within the framework of Earths physical, chemical and biological cycles. Water is found in three different states and is found inside and on the surface of the earth's crust, in the atmosphere and within living organisms. Naturally there are many factors that determine its quality and preservation and it makes it crucial to monitor these factors. Environmental monitoring is the observation and detailed study of the changes that occur in the environment. Scientifically we would like to assess and measure these changes to derive knowledge. The data collected from monitoring can be used in many ways. In simplest terms observed sound data produces valuable information from which comes a better understanding of the situation and increases our chances of making an informed decision. Through environmental monitoring we now know that surface soils and most bodies of waters including ice caps contain trace levels of synthetic chemicals [9]. Many surface waters consisting of rivers and lakes, contains trace amounts of antibiotics and their respective metabolites. It is reasonable then to assess and monitor these antibiotics. Antibiotics are considered pseudo persistent as well due to their continual introduction into the environmental ecosystems [10].

2.3. River water, a repository of biochemical substances.

A river is defined as a natural stream or body of water that eventually empties into the ocean, lake and is fed along its course by other intermingling smaller streams. Since this water is consistently moving, the pollutants in it are being continuously diluted and decomposing more rapidly than a body of standing water but the sources of contaminations into river water from industry, agriculture and domestic WWTPs are significantly more spread outwards as it provides a convenient mode of transportation of organic pollutants.

Antibiotics are used in large amounts in hospitals, private care and veterinary medicine. The active compounds of these antibiotics are excreted whether metabolized or not through urine and feces. Studies suggest that antibiotics are, in general, poorly absorbed by the human body, and thus are excreted either unchanged or transformed into the sewers [2]. Some of them are also widely used in veterinary medicine for the treatment of infections and as a growth agent, this indicates that they might be present in well water near farms that use natural manure from these animals as their fertilizers [3].A large number of these antibiotics are only partially eliminated in a waste water treatment plant [4] and are released into the environment as effluents [5], [6]. This in turn means that they can be present in surface waters such as lakes, rivers or wetlands [7].Other sources could be through non-sewerage systems (boat lavatories, farmhouses etcetera) and applications of biosolids onto agricultural fields [8]. .

2.4. The impact of the bioaccumulation of pharmaceuticals:

Once the treated water from these waste water treatment plants WWTPs enter the rivers and lakes, they pose a risk to the natural environment and the extent of the long term ecotoxicological effects are not known [21]. While there are studies on the determination of these antibiotics there are also studies done on their ecotoxicological impact. There are various endpoints, inhibition of growth, adverse reproductive effects and histopathological changes amongst others [22]. Freshwater crustaceans such as Daphnia magna showed toxicity towards Sulfamethoxazole (SMX) [23]. Two aquatic plants *lemna gibba* (Küürlemmel) and *Myriophyllum sibiricum* have shown to exhibit strong phytotoxic responses to Sulfamethoxazole and levofloxacin (fluoroquinolone)[24]. Sulfadimethoxine (SDM) has shown to be phytotoxic towards terrestrial plants and weeds [25] and has shown to be toxic *to Lythrum salicaria* or purple loosestrife (Harilik kukesaba) [26]. Ciprofloxacin and it's degraded products are not cytotoxic but still do exhibit genotoxic effects

from human cell cultures [27] and shown to degrade photosynthetic pathway by inhibition of DNA gyrase [28]. Norfloxacin has demonstrated toxicity profile for aquatic organisms such as cyanobacterium anabena [29]. Moreover studies suggests that besides bacteria, algae, rotifiers, microcrustaceans and fish have also been affected by sulfonamides and fluoroquinolones.[30] Their presence in the aquatic systems has led to the development of a future research project plan for the scientific community in aquatic ecology [31]. There are adverse consequences from antibiotic overuse and misuse. An inevitable negative and unexpected ecotoxicological effect that was recognized is that the increasing consumption of human antibiotics can lead to an increase in antibiotic resistant bacteria's in the environment [32]. Except for fluoroquinolones, there is minimum data available on the monitoring of antibiotic resistance towards Sulfonamides and Amphenicols as they form the lower portion of use. Some of the Fluoroquinolones that were recorded for consumption and monitoring of antibiotic resistant isolates by ECDC in Estonia displayed an increasing trend. A concise graph with data has been placed on Annex 3.

2.5. Measurements of Antibiotics in the environment:

With an increase in antimicrobial resistance it is also crucial to ensure that their use and further pathways into the environment are monitored and controlled. I.e. Wastewater effluents emptying into river water and the resulting impact. Fluoroquinolones and sulfonamides have shown to be stable [36] in nature and present in sewage sludge samples. Previous concentrations of Norfloxacin have found to be 0.048 ± 8 ng/g and 0.076 ± 6.8 ng/g for sulfonamides [35].Ciprofloxacin was found to be present within river Vantaa in Finland at low concentrations. [4]. The sewage sludge from the WWTPs in Estonia have previously been assessed in two cities, Tartu and Tallinn. [17] . The major components from sewage sludge samples from Tallinn were Ciprofloxacin (CIP), Norfloxacin (NOR), Sulfadimethoxine (SDM) and Sulfamethoxazole (SMX) where Ciprofloxacin (CIP) was found to be 4 times over the threshold limits set for manure. In the sample originating from Tartu, the highest to lowest concentrations were that of CIP – NOR – OFL – SDM – SMX. On average the fluoroquinolones were detected at a higher concentration in the sewage sludge sample originating from Tartu than Tallinn. No studies regarding the fate of Fluoroquinolones, Sulfonamides and Amphenicol in river water environments of Estonia have been performed. Eight antibiotics representing three main classes; Fluoroquinolones (Ciprofloxacin, Enrofloxacin, Norfloxacin, Ofloxacin, Marbofloxacin), Sulfonamides (Sulfamethoxazole, Sulfadimethoxine),

and Amphenicol (Florfenicol) were selected for this study. Their structures have been placed on Annex 2.

2.6. Sample Extraction and Detection methods:

There are a wide variety of extraction techniques available for the extraction of antibiotics and other harmful drugs within environmental matrices using SPE cartridges. Generally, for environmental matrices with a more solid nature, SPE is used only as a cleanup step and the main extraction is carried out through pressurized liquid extraction (PLE) and liquid-liquid extraction (LLE). The extraction methods determine the extraction efficiency and recovery of the analyte from the matrix considering that the matrix concentrations ranges are generally in ng kg^{-1} ranges. Several methods of extractions have been reported; liquid – liquid micro extraction (LLME) [11], solid phase extraction [12], ultra- sonication [13], multiple cartridge solid phase extraction (SPE extraction cartridges in series) or rapid resolution RR-LC-MS/MS [14], dynamic microwave assisted extraction (MAE)[15], molecularly imprinted polymers/molecular imprinting based extraction methods for the extraction of fluoroquinolones from soils [16], pressurized liquid extraction (PLE)[17], dispersive solid phase extraction based on nuclear magnetic fields and capillary electrophoresis for the analysis of fluoroquinolones[18].

River water matrices are complex and to accommodate for the large number of interferences i.e. the WWTP sludge. Only the mass spectrometer can offer reliable mass accuracy and selectivity. There are simpler detectors that are used for the detection of antibiotics such as UV/Vis[19], and Diode array detectors[20] however the majority of detection techniques used to quantify naturally very low levels of antibiotics at the ng kg^{-1} level are based on mass spectrometry.

Initially in order to detect non-polar and polar pharmaceuticals, gas chromatography was the preferred analytical method. If the analytes can vaporize and be stable at a temperature of roughly <300 °C, they can be analyzed. The main advantage of using GC-MS as compared to UV-Vis, or DAD would be the level of selectivity that the GC-MS can provide by ionizing the precursor ions into fragments of a given analyte. For the environmental analysis of antibiotics in the environment, liquid chromatography-mass spectrometry (LC-MS) has been the better analytical detection method than gas chromatography – mass spectrometry (GC-MS) because most of the antibiotic families are polar and have very low volatility making them more than ideal for LC-MS.

2.7. Aim:

The purpose of this project was twofold:

First to develop an analytical method for the simultaneous determination of 3 therapeutic classes of antibiotics in river water by offline Solid Phase Extraction and Liquid Chromatography tandem mass spectrometry (SPE-LC-MS/MS). The objectives were to adequately separate these 3 classes by varying the gradient and composition of the mobile phase using 5 mM HFIP buffer adjusted to pH 9 and to optimize the sensitivity of the method by adjusting parameters of the mass spectrometer such as collision energy.

Secondly to optimize and validate this method and make sure it is fit for purpose and could be potentially used for consistent monitoring of these antibiotics within the environment. Validation of the analytical data obtained from samples is of fundamental importance as it could be used to make critical decisions for the safety of the environment. For a better understanding of the workflow please review figure 1.

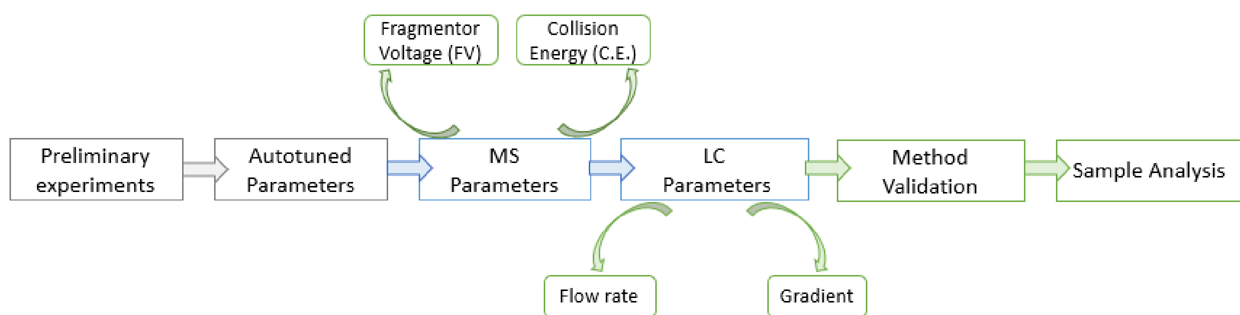


Figure 1. Flow of the LC-MS Project.

Mass spectra parameters and recommended settings referred by literature were used for initial runs to assess the antibiotics and their interactions within the column. The first step was the development of Liquid chromatography- mass spectrometry method and that began with the choice of optimizing the collision energy (CE) and fragmentor voltage (V_f) for the MS2 and PRD ion scans. Agilent's optimizer program was used for this requirement. Once preliminary conditions were established, the next parameters to be considered were mobile phase compositions and choice along with creating a gradient elution method. After the method was successfully developed, a validation was performed to ensure that the results were reliable.

3. EXPERIMENTAL

The LC-MS method was developed in cooperation with another project titled "*Analysis of antibiotics in sewage sludge samples by PLE LC-MS/MS by Joshua Osagu*"

3.1. Chemicals and Reagents

All the Chemical substances purchased were of analytical grade. Antibiotics: Ciprofloxacin (CIP), Ofloxacin (OFL), Norfloxacin (NOR) and Florfenicol (FF) were purchased from Sigma-Aldrich, whereas Enrofloxacin (ENR) was purchased from Dr. Ehrenstorfer Gmbh (Germany) and Marbofloxacin (MAR) was purchased from Honeywell (U.S.A).

1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was obtained from ACROS Organics (U.S.A).

Formic Acid and HPLC grade methanol were purchased from Honeywell (U.S.A.).

Aqueous solutions were prepared with deionized and double distilled water (Resistivity > 18 MΩ) from Millipore MilliQ Advantage A10 (milliQ water).

LC eluents: HPLC grade Methanol (MeOH) (Sigma-Aldrich), and HFIP buffer (ACROS organics).

Other chemicals: Hydrochloric acid (HCl) was obtained from Sigma-Aldrich. Ammonia solution was obtained from LiChropur (Germany).

3.2. Instruments and Methods

Sartorious GENIUS analytical balance (0.00001 g readability). Solid phase Extraction (SPE) carried out in an Agilent vacuum manifold. SPE cartridges were obtained from Waters (U.S.A). SPE elution and storage vials were BluCAPP 15 mL Polypropylene (PP) conical tubes. Syringe and needles were from NORM-JECT. Syringe filters were 0.2 um Sartorius (U.K). pH meter used was Evikon E6115.

3.3. Preparation of Standard solutions and eluents:

All Stock solutions of Sulfamethoxazole (SMX), Florfenicol (FF), Marbofloxacin (MAR), Ciprofloxacin (CIP), Enrofloxacin (ENR), Norfloxacin (NOR), and Ofloxacin (OFL) were prepared by dissolving 10 mg of each standard in 0.1 M Formic Acid (FA) while Sulfadimethoxine

(SDM) was prepared in 0.1M FA : 35% methanol (MeOH) to obtain a concentration range of 0.9 - 1.1 mgg^{-1}.Intermediate stock solutions were prepared by diluting stock solutions in order to obtain a concentration range of 10 ug g^{-1}.

Mobile phase preparation was carried out using HFIP and ammonium hydroxide. 527 ul of HFIP buffer was added to 1 liter of milliq water under a magnetic stirrer. The pH of the 1-liter eluent was adjusted to a basic pH=9 with ammonium hydroxide solution (~600ul) and Evikon pH meter. Finally, vacuum filtration was carried out with 0.45 um PVDF membrane filters into eluent bottles.

0.1% of Formic acid used for the preparation of standards and dilutions was prepared by pipetting 1 ml of pure Formic Acid into 1 liter of milli-Q water on a magnetic stirrer to achieve 0.1% V/V followed by filtration through 0.45 um PVDF membrane filters (durapore).

Stock solutions were made by carefully weighing 10 mg each of the antibiotic standards on a 5-digits analytical balance to prepare a solution of 1 mg/g using methanol and formic acid.

LC-MS/MS optimization and method development were performed with 200 ng g^{-1} of individual stock solutions and antibiotic mixtures which were prepared from their individual working solutions (10 ug g^{-1}.).Working standard solutions were prepared in the concentration range of 0.1 – 508.7 ng g^{-1} for all antibiotics and linear calibration points were assessed.

3.4. River water collection and sample preparation.

River water samples (1.5 liters each) were collected in 2 liters Polypropylene bottles at random points upstream, midpoint and downstream of the WWTP along the Emajõgi river and stored at 7 °C. The water samples were then filtered using a general filter funnel to remove debris followed by a vacuum filtration through a 0.45 um PVDF membrane filter.

Typical concentrations of antibiotics found in the environment are in the ngg^{-1} range and that makes the pre concentration and cleanup an important step prior to detection. There are several methods for the extraction and concentration of compounds from environmental samples. Solid Phase Extraction (SPE) cartridges have been used and proven to be fit for purpose for the extraction of antibiotics during sample preparation [35]. Oasis Hydrophilic-Lipophilic-Balanced (HLB), water-wettable, reverse phased sorbent cartridges (Waters. U.S.A) composed of two monomers the hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene with a sorbent amount of 500 mg and particle diameter of 60 um were used for the purpose of sample preparation. The SPE

method was acquired from previously discussed sewage sludge studies conducted in Tartu [17]. The SPE cartridges are preconditioned with 20ml of methanol followed by 10 ml of MilliQ before use. Flow rate was maintained at 6ml/min approximately.

Sample extraction approach:

Step 1: Measure 1.5 liters of sample and adjust the pH to 2.8 (HCl). Pour the sample into the SPE cartridge and adjust the vacuum to 200 mbar, flow rate ~ 6ml/min.

Step 2: Rinse the SPE cartridge with milliQ water.

Step 3: Elution is carried out with 12ml methanol at a flow rate of 6ml/min.

Step 4: Concentrate the extract to almost dry under a stream of N2 Nitrogen flow in a water bath at approximately 50°C.

Step 5: After the sample has been almost dried, let it cool down and add 1ml 1:1 solution of methanol with buffer solution (1 mM ammonium acetate and 0.1% formic acid, pH 2.8), the sample then is stored at 7 °C until analysis.

Step 5: Dilute the sample and reconstitute into 0.1 M F.A.

Standard solutions containing all eight of the analytes were also prepared in a similar fashion to test for Recovery.

3.5. LC-ESI-MS/MS

An Agilent technology 6460 (Agilent, Germany)- Triple quadruple mass spectrometer equipped with Agilent Jet stream electrospray ionization Source (AJS-ESI) was utilized for Tandem mass spectrometric detection. The operating parameters that were used were as follows:

Sheath Gas Flow: 11 mL min^{-1}, Sheath Gas Temperature (N_2): 350° C, Nozzle Voltage: 600 V, Nebulizer Pressure: 30 psi, Chamber Voltage (cell accelerator voltage): 4 kV

Mass spectrometric measurements were carried out in scan mode over the mass range of m/z 50-500. The MS/MS analysis were carried out in positive ion mode. Upon ionization in positive ion mode all the antibiotics produced positive precursor ions [M+H]+ and [M+NH4]+ adduct in the case of Florfenicol that were then fragmented into their individual product ions. Different Collision Energies (10-40 eV) were tested individually for each compound and the most intense transitions

were monitored during the analysis. The parameters are displayed in Table 2 in the order of increasing retention times.

Table 2. LC-MS/MS Conditions for the analysis of antibiotics by MRM using Waters XBridge RP column.

Antibiotics	Rt (min)	RSD	Precursor ion (m/z)	Product ions (m/z)		Collision energy (eV)	
				Quantifier	Qualifier	Quantifier	Qualifier
SMX	4.82	0.6	254	108	156	15	15
SDM	10.36	0.4	311	156	108	18	18
NOR	11.49	0.3	320	302	282	18	30
CIPR	12.2	0.6	332	314	231	18	35
MAR	16.8	0.8	363	72	345	26	26
FF	18.36	0.7	375	340	241	10	22
OFL	19.187	0.6	362	318	261	18	18
ENR	22.37	0.3	360	316	245	25	25

3.6. LC-Method development:

The chromatographic analysis of the extracts was performed using Agilent LC system consisting of quaternary pump (1290 Flexible pump), Agilent Autosampler, and Agilent Column holder maintained at 30 °C. Initial trials were done with two organic phases Acetonitrile and Methanol individually coupled with 0.1% F.A in Milli-Q as the non-polar phase. The resulting analyte chromatograms indicated poor retention, separation and overall poor solubility. Instead a basic buffer of 5mM HFIP: Ammonium hydroxide was used. Runs were performed in reverse phase C18 Waters X bridge column (3.0 x 150 mm, 3.5µm) with a modified organic layer to accommodate for the high pH of buffer and a guard column. The high pH tolerance (1-12) of the column is required to accommodate for the high pH of the buffer at pH=9 and to prevent the dissolution of the silica support layer. The five major analytes belong to fluoroquinolones which are notorious for being zwitterionic with multiple pKa values and disagreements about said pKa values. If pH > pKa the basic centers of the fluoroquinolones will get deprotonated, providing a better retention in conjunction with HFIP and since the pKa value of fluoroquinolones are in the

basic region of ~8, we want our pH to be higher than that at ~9 to ensure better chromatographic separation along with an improved signal[37]. The injection volume was 1 ul, while the flow rate of mobile phase was set to 0.35 ml min^{-1}. Run time was set at 35 min for the analysis of a mixture of 8 compounds belonging to 3 different classes by gradient elution and a post run time of 10 minutes for equilibration. Eluent components were (A) 5mM HFIP: NH_4OH buffer at pH 9 and B: HPLC grade > 99 % pure Methanol. Data acquisition was carried out using Agilent Mass Hunter software. The individual MRMS of all antibiotics are shown in Figure 2

Figure 2. Gradient elution method showing MRM of 8 different antibiotics.

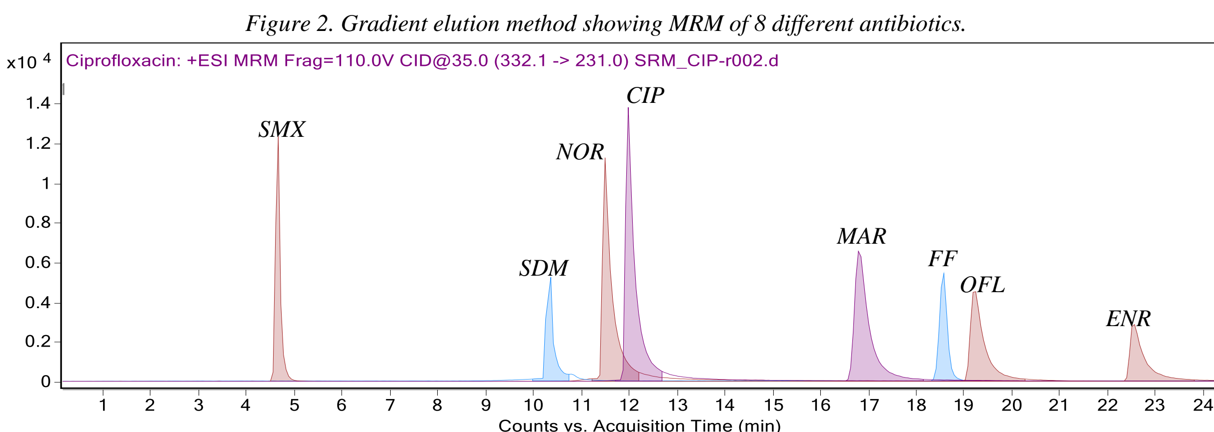


Table 3. LC Gradient Elution for the analysis of antibiotics.

Time (min)	**0**	**5**	**8**	**15**	**18**	**22**	**24**	**30**	**32**	**35**
HFIP buffer	97	97	80	80	60	60	0	0	97	97
Methanol	3	3	20	20	40	40	100	100	3	3

4. RESULTS AND DISCUSSION

LC-MS method development was performed with 0.2 ug g^{-1} of Antibiotic mixtures which was prepared from their individual working solutions (10 ug g^{-1}). The validation parameters that were optimized for the developed method were Linearity (LOD, LOQ), Selectivity, Precision, Accuracy, Recovery. Multiple samples were prepared depending on which validation parameters were being determined.

4.1. Linearity:

Linearity was studied by spiking milliq water from the final stock solution of the antibiotics at different calibration concentrations of the antibiotic mixtures. In addition, quality control samples were prepared separately from calibration solutions at low, medium and high concentration levels and analyzed randomly with calibration solutions.

Calibration curves were plotted for all eight antibiotics as peak area versus antibiotic concentration. All standards were injected in triplicates. Calibration curves for all antibiotics was found to be linear in the concentration range of 0.1-200 ng g^{-1}.

Two separate calibration curves were used, one at a lower concentration range from 0.1 – 25 ng g^{-1} for more accurate analysis of river water samples and a higher concentration range from 25 – 200 ng g^{-1}, values to be used for the individual calibration ranges. LOD and LOQ values were calculated as per the formula:

$$LOD = 3.3 \times \frac{S}{b} \quad ...(i)$$

$$LOQ = 10 \times \frac{S}{b} \quad ...(ii)$$

Where S is the standard deviation of residuals and b is the slope of the calibration curve. The linear ranges along with their regression coefficients and LOD and LOQ are expressed in Table 4. The concentrations of these calibration solutions were back calculated and the % accuracy of each antibiotic at each calibration point was calculated. % accuracy ranged from 91.97% to 99.82% for all calibration solutions which fills the SANTE criteria of ± 20 %. These calibration curve ranges provided a basis for making a matrix matched calibration curve range for the calculation of antibiotic concentration in river water samples and for comparing results obtained from other validation parameters. The regression coefficient of all calibration curve ranges antibiotics was found to be ≥ 0.99 %.

Table 4.Calibration Ranges, Regression coefficients and LOD and LOQ values.

Antibiotics	Lower linear calibration range (ng g^{-1})	Regression coefficient (R^2)	LOD (ng g^{-1})	LOQ (ng g^{-1})	Upper linear calibration range (ng g^{-1})	Regression coefficient (R^2)
CIPR	0.5 – 28.7	0.9987	0.3	0.9	28.7 – 233.5	0.9954
ENR	0.5 – 28.9	0.9975	0.2	0.6	28.9 – 200.4	0.9998
FF	0.5 – 20.7	0.9981	0.3	0.8	20.7 – 204.5	0.9998
MAR	1 – 25.4	0.9934	0.5	1.5	25.4 – 203.2	0.9997
NOR	0.5 – 25.5	0.9962	0.2	0.7	25.5 – 215.2	0.9994
OFL	0.5 – 25.0	0.9922	0.2	0.6	24.1 – 207.8	0.9999
SDM	0.2 – 27.0	0.9903	0.1	0.3	27.0 – 198.6	0.9934
SMX	0.5 – 24.6	0.9999	0.3	0.9	24.7 – 210.5	0.9998

4.2. Accuracy & Precision; River water Analysis:

The RSD of the retention times of standard solutions during chromatographic analysis provided in Table 2. of all antibiotics was less than 1, which indicates identity confirmation. As discussed in chapter 2.4, the river water samples were filtered followed by an adjustment to their pH before finally being extracted through SPE. The SPE extract obtained was further diluted before injecting into the LC-MS/MS system for quantitative analysis. The concentrations of these river water samples were back calculated along with their relative standard deviation (RSD) first in the extract and then finally within 1.5 kg of the sample

$$C._{Sample} = \frac{C_{Analyteextract} \times V_{Extract}}{V_{Sample}} \ldots (iii)$$

Where C_{sample} is the concentration in 1.5 liters of sample, $V_{extract}$ is the volume of extract after solid phase extraction (SPE) found in the extract and V_{Sample} is the volume of sample. Whereas the relative standard deviation (RSD) was calculated as per the formula:

$$\boldsymbol{RSD} = \frac{St.dev}{C_{mean}} \ldots (iv)$$

Where St.dev is standard deviation of the replicates divided by the mean concentration C_{mean}.

Samples were collected from three different locations along the river. Upstream of WWTP, midpoint of WWTP and downstream of WWTP for analysis (see annex 4 for sample collection sites). No Analytes were detected at Upstream of the WWTP. At midpoint of the WWTP the highest concentrations found were that of Sulfadimethoxine (SDM) followed by Ciprofloxacin (CIP) and Norfloxacin (NOR).At downstream of the WWTP the highest concentration that was found was that of Sulfadimethoxine (SDM) followed by Ciprofloxaxin (CIP), Norfloxacin (NOR), and Enrofloxacin (ENR). While Enrofloxacin (ENR), Marbofloxacin (MAR) and Florfenicol (FF) were not detected at midpoint and OFL, SMX, MAR and FF were not detected at downstream. The results are expressed in ug kg^{-1} for each collection point along with the RSD for repeatability. in Table 5. The MRM chromatograms of detected antibiotics are placed on Annex 5.

Table 5. Antibiotics at Upstream, Midpoint and Downstream of the WWTP. (ND: Not detected, NA: Not applicable)

Antibiotic	Upstream (ug kg^{-1})	Mid-point (ug kg^{-1})	RSD (%)	AOAC RSD (%)	Downstream (ug kg^{-1})	RSD (%)	AOAC RSD (%)
CIPR	ND	0.05	11	<15	0.0073	3	<21
SDM	ND	0.2	1	<11	0.03	1	<15
NOR	ND	0.05	5	<15	0.005	19.2	<21
OFL	ND	0.01	11	<21	ND	NA	NA
SMX	ND	0.02	13	<15	ND	NA	NA
ENR	ND	ND	31	<30	0.002	20	<30
MAR	ND	ND	ND	<15	ND	ND	ND

The RSD (%) values for assessment were obtained relative to the concentration ranges of the extracts (AOAC) and were used for assessment of matrix effects and repeatability within the samples[38]. Note that the concentrations displayed in table 5 are the concentration per kilogram of sample that were back calculated from the concentration of the extracts which are above the methods LOQ.

Furthermore, % Accuracy was tested with filtered river water extracts post SPE samples that were spiked with antibiotic mixtures at lower calibration range.

Table 6. Percentage Accuracy of spiked samples.

Antibiotic	Regression coefficient (R2)	%Accuracy range
CIPR	0.9987	84 % - 89%
ENR	0.9975	82 % - 89%
FLOR	0.9981	74 % - 84%
MAR	0.9934	75 % - 81%
NOR	0.9962	81 % - 91%
OFL	0.9922	82 % - 87%
SDM	0.9903	85 % - 91%
SMX	0.9999	82 % - 93%

4.3. Selectivity and Carryover effects

Selectivity and carryover effects were observed by injecting blank solutions after higher concentration level calibration solutions and by comparing chromatograms of blank samples and spiked samples.

After injecting multiple blank samples in between the runs and specifically after high concentration calibration solutions revealed that there was significant retention of some analytes above the LOQ, significantly SDM and NOR, followed by CIP and OFL while SMX, MAR and FF were not detected within the blanks.

Table 7. Concentrations of Analytes before and after washing.

Antibiotics	SMX	SDM	NOR	CIP	MAR	FF	OFL	ENR
Before Wash (ngg^{-1})	ND	11	10.7	7.3	ND	ND	6.3	4.4
After Wash (ngg^{-1})	ND	0.9	ND	ND	ND	ND	ND	ND

In order to avoid retention of compounds the column must be flushed regularly. Initial tests for carry over in the blank samples were conducted by flushing the column for 1 hour with 40:60 (HFIP buffer: Methanol) after the injection of a high concentration stock solution with no improvements. The isocratic run was then adjusted to a higher organic phase percentage of pure methanol for 2 hours with still considerable carry over effects. Finally substituting an acidic eluent (0.1% F.A) for the basic one significantly reduced previously observed carryover effects with only one rare occurrence of SDM which could be due to a dirty guard column. The gradient to flush to column was developed for an hour followed by a post run step to equilibrate the column to 50:50 HFIP: MeOH again.

Table 8. Gradient to flush the column

Time (min)	**0**	**10**	**20**	**30**	**40**	**50**	**60**
0.1% F.A.	90	80	70	60	40	20	10
Methanol	10	20	30	40	60	80	90

Additionally, multiple blanks were placed within the run list parameters and monitored consistently. In the blank chromatogram (Figure 3) there were no significant endogenous peaks that could interfere with the retention time of analyte. This result also indicate that the method had good selectivity. The obtained blank chromatogram showed that there is no carry-over effect.

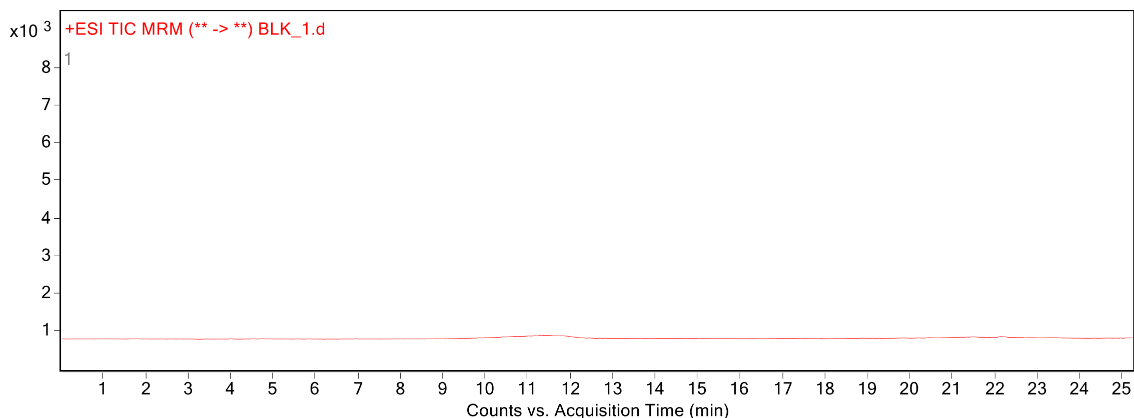


Figure 3. Blank Chromatogram to check for possible carryover effects.

4.4. Matrix effects:

Matrix effects were further evaluated by the analysis of post SPE spiked extracts. This was tested with two different samples. One sample spiked at low, medium and high concentrations post extraction. The second sample was antibiotic mixtures spiked at similar concentration levels of low, medium and high concentration. By performing LC-MS/MS analysis of blank milliQ water spiked with antibiotic mixtures at low, medium and high concentration levels, Matrix effect values were calculated by comparing the peak area in the presence of matrix and the peak area in the absence of matrix by the following equation [39]:

$$M.E. = \frac{Area_{post\ spiked\ matrix} - Area_{non-spiked}}{Area_{non-spiked}} \dots (iii)$$

Where Area $_{post\ spiked\ matrix}$ is the area obtained by the spiked sample and Area $_{non\ spiked}$ is the area of a non-spiked sample.

Table 9. Matrix Effect Evaluation at different concentration levels.

Antibiotics	Low Concentration	Medium Concentration	High Concentration
CIP	-16%	-4%	-7%
ENR	-10%	-13%	-5%
FLOR	-12%	-15%	-13%
MAR	-14%	-11%	-9%
NOR	-14%	-8%	-10%
OFL	-13%	-6%	-4%
SDM	-10%	-16%	-8%
SMX	-17%	-10%	-3%

.

Matrix effect proved to be quite significant at lower concentration limits but still acceptable as per SANTE Guidelines of ± 20%.

4.5. Recovery:

A crucial step in the validation of the LC-MS/MS method for the determination of antibiotics was to calculate recovery for the SPE method. Medium concentration point at 50 ng/g was used to spike three set of samples. The recovery % was calculated as per the equation mentioned below [39].

$$Recovery\ (\%) = \frac{Area_{Pre\ extraction\ spiked}}{Area_{Post\ extraction\ spiked}} \ldots (iv)$$

Where Area $_{\text{pre extraction spiked}}$ is the area of the spiked sample before SPE extraction and Area $_{\text{post extraction spiked}}$ is the area of spiked sample post SPE extraction.

Table 10. Recovery of antibiotics

Antibiotic	CIP	ENR	FLOR	MAR	NOR	OFL	SDM	SMX
Recovery (%)	69%	62%	68%	76%	73%	67%	74%	69%
RSD % (recovery)	9%	6%	3%	7%	2%	6%	8%	9%

Enrofloxacin (ENR) showed lowest recovery at 62 % whereas Marbofloxacin (MAR) showed the highest at 76%. Recovery as per SANTE RSD $\leq 20\%$

4.6. Process efficiency

Process efficiency is almost synonymous to trueness of an LC-MS method, in this work process efficiency was calculated as per the following equation [39]:

$$Process\ efficiency = \frac{P.E_{Spike}}{Pure} \times 100\% \dots (v)$$

Where P.E.$_{spike}$ refers to post extraction spiked sample and pure represents a pure sample at the same concentration as the spiked sample. Process efficiency was evaluated, and the average process efficiency is presented in the figure below.

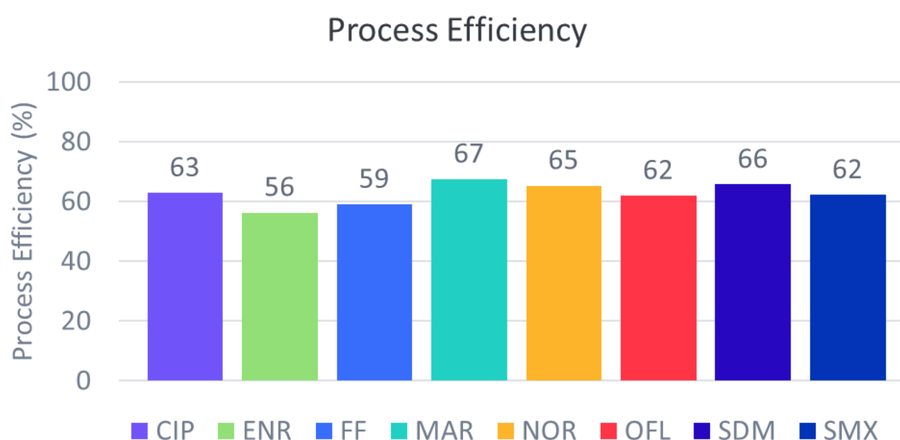


Figure 4. Process Efficiency

5. Ecotoxicological impact; Predicted no effect concentrations (PNECs).

The predicted no effect concentrations are values that are used to evaluate the environmental risk associated with discharge of antibiotics within the natural environment [41]. There are two main PNEC values, PNEC – ENV and PNEC- MIC.

The PNEC- ENV (environment) values are based on ecotoxicology data generated by peer-reviewed literatures and are calculated to be protective of the ecological species and to incorporate standard risk assessment methods whereas the PNEC – MIC (minimum inhibitory concentrations) values are based on the approach mention in the paper and are supposed to be protective and inhibitory towards antibiotic resistance.

The critical factor is calculated as $\frac{MEC}{PNEC}$, where MEC stands for measured experimental concentration. A critical factor above 1 is significant and further emphasis must be made on the

monitoring of that specific antibiotic, whereas values lower than 1 are within acceptable region. The antibiotic concentrations obtained in chapter 4.2. were used to calculate individual $\frac{MEC}{PNEC-ENV}$ values for risk assessment of the ecotoxicological affect of the antibiotics , whereas $\frac{MEC}{PNEC-MIC}$ factor was calculated to assess if any of the antibiotic concentrations detected could be considered significant in the promotion of antibiotic resistance. The Calculated values along with their significance are presented in table 11.

Table 11. Ratio of Measured values and PNEC values to assess ecotoxicological and resistance promotion impact.

AB.	PNEC-ENV (ug kg^{-1})	PNEC-MIC (ug kg^{-1})	Mid-point (ug kg^{-1})	MEC/PNEC- ENV	MEC/PNEC-MIC	Down stream (ug kg^{-1})	MEC/PNEC - ENV	MEC/PNEC-MIC
CIPR	0.45	0.06	0.049	0.108	0.811	0.007	0.016	0.122
SDM	50	N/A	0.196	0.004	NA	0.028	0.001	NA
NOR	120	0.5	0.049	0.000	0.098	0.005	0.000	0.010
OFL	10	0.5	0.009	0.001	0.019	0.002	0.000	0.004
SMX	0.6	16	0.016	0.027	0.001	ND	NA	NA
ENR	1.9	0.06	0.001	0.001	0.020	0.002	0.001	0.029

The ratio of MEC/PNEC-ENV values and the MEC/PNEC-MIC values were insignificant for all antibiotics at midpoint and downstream sample collection points which indicates minimum ecotoxicological effects.

6. SUMMARY

Antibiotics have found to show an increasing stability and consistency within environmental samples. Partially metabolized and partially removed in WWTP processes, with an increasing antibiotic resistance trend and the potential ecotoxicological effects, it has become crucial to monitor them within the environment.

In the study the aim was to develop a proficient analytical method for the assessment of low antibacterial concentration of 3 classes of antibiotics; Fluoroquinolones (Ciprofloxacin, Enrofloxacin, Marbofloxacin, Ofloxacin, Norfloxacin), Sulfonamides (Sulfadimethoxine, Sulfamethoxazole) and Amphenicols (Florfenicol) in Tartu city river water body Emajogi.

Emajõgi is a river in Estonia which flows from Lake Võrtsjärv through Tartu County into Lake Peipsi, crossing the city of Tartu for 10 km and a length of approximately 100 km. The first-time analysis of river water samples in Tartu city with the developed offline solid phase extraction (SPE), LC-MS/MS method has shown to be able to detect and quantify antibiotics at low concentration levels within river water samples with good recovery and repeatability. The work done provided a reliable data that could be used to potentially monitor the selected antibacterial compounds in other river water environments and potentially lakes.

Initial results indicated good recovery of the selected analytes through solid phase extractions from river water samples at >5 °C. Precleaning and filtration of the samples before SPE extraction is crucial to minimize all potential matrix effects, a tedious, but otherwise necessary and efficient step. After the initial results, an ecotoxicological assessment was made considering the provided impactful concentrations. All antibiotics were found to be below the threshold limit.

The novelty of the method exists in its first-time applicability and first-time assessment of river water samples from Emajõgi. It could be potentially used for routine monitoring of these analytes within the river water throughout the year and to assess different concentration ranges and influences within the water and through it, the relative ecosystem.

Antimikroobsete ainete määramine jõevees kasutades tahke faasi ekstraktsiooni ja LC-MS/MS meetodit

Waseem Ahmad Iftikhar

Kokkuvõte

Antimikroobsete ainete jääke leitakse järjest sagedamine keskkonnaproovides. Kuigi need ained osaliselt metaboliseeruvad ja osaliselt eraldatakse reoveepuhastites, siis ikkagi suureneb antibiootikumiresistentsuse ja ökotoksikoloogiliste mõjude risk. See toob kaasa vajaduse määrata nende ainete sisaldusi keskkonnaproovides.

Käesoleva töö eesmärgiks oli analüüsimetoodika arendamine kolme eri rühma kuuluvate antibiootikumide jääkide määramiseks jõevees; fluorokinoloonid (tsiprofloksatsiin, enrofloksatsiin, marbofloksatsiin, ofloksatsiin, norfloksatsiin), sulfoonamiidid (sulfadimetoksiin, sulfametoksasool) ja amfenikoolid (florfenikool).

Emajõgi on Eesti üks tuntumaid jõgesid, mis voolab Võrtsjärvest Peipsi järve ja läbib seejuures Tartu linna. Käesolevas töös arendatud metoodikat kasutati antibiootikumijääkide määramiseks Emajõe veeproovides, mis olid võetud Tartu reoveepuhastusjaama lähedalt ning sellest alla- ja ülesvoolu. Kasutatud tahke faasi ekstraktsiooni (SPE) ja LC-MS meetodit kasutav analüüsimetoodika võimaldas tuvastada antibiootikumijääkide madalaid sisaldusi hea saagise ja korratavusega. Arendatud metoodikat on võimalik kasutada antibiootikumide jääkide monitoorimiseks jõgedes ja tõenäoliselt ka järvede vees.

Valideerimise tulemusena selgus, et kasutatud SPE metoodikal on uuritud analüütide suhtes rahuldav saagis. Seejuures leiti, et maatriksiefektide alandamiseks on proovide SPE-eelne filtreerimine väga oluline. Selle mõju ekstraheerimise saagisele vajab siiski täiendavat uurimist.

Analüüsil leitud antibiootikumide kontsentratsioonidele anti ka esialgne ökotoksikoloogiline hinnang. Kõigi uuritud antibiootikumide sisaldused jäid allapoole kehtestatud piirnormi.

Arendatud metoodika oli uudne laborile, kus töö läbi viidi – varem ei ole seal uuritud ravimite jääkide sisaldust jõevees. Samuti lisandus uuritavate analüütide hulka kolm uut antibiootikumi. Metoodikat saab edasi arendada lisades veelgi analüüte ja kontrollides selle toimivust ka teiste veekogude vete analüüsil. Arendatud metoodika võimaldab keskkonna seisundit paremini hinnata.

Acknowledgements

First, I would like to thank my parents for their ever-growing love, support and for teaching me values that have always proven to be useful. To my brothers Ijaz, Fraz, Rashid, Qamar, Umar and my sisters Khadijah and Maryam for always being there for me when I needed them and for never letting me settle for less.

I would like to very humbly and sincerely thank my supervisor, Professor Koit Herodes who has taught me the immense value of understanding through patience. Even when my knowledge would fail me, he would uphold me to do better and to try and think of a solution. He will always answer my questions no matter how basic or how trivial they may seem and always hear my concerns before providing a sound solution. You have been the best mentor I could have asked to learn from and I'm forever thankful that I had the chance to learn from the best.

I would like to thank Professor Ivo Leito for his immense wisdom and guidance through all matters of my student life and for helping me solve all the uncertainties in my student life. We always had you by our side to guide us through all the deviations, and for that I am grateful.

My other sources of knowledge and support have been immense, and I would like to thank them as well. I would like to thank Christina for sharing her knowledge with me and for always making sure I had the help I need, even on weekends. I would like to thank Toiv, Hanno, Anu for molding us into practical analytical chemists with a lot of patience and care put into our learning. Ernesto and Nhung for all your advice and help and for your candid impression of my work and what could be done better.

I would like to thank my dear friends, Helmi and Zen for their honest inputs. Finally, I would like to thank my lab mate Joshua for being a valuable friend and colleague. I would have never learnt as much as I did about LC-MS if it weren't for our common passion for it and for our constant discussions back and forth. I appreciate and cherish all my time at the University of Tartu and firmly believe that my professors, colleagues and friends have imparted me with the gift of knowledge, humility and steadfastness in accepting and overcoming my limits. Thank you.

This work was supported by AMRITA project, which is financed by Estonian Research Council from ERF resources of RITA programme for Ministries of Rural Affairs, Environment and Social Affairs (contract no 7.2-2/19/5).

REFERENCES

[1] World Health Organization, Ed., *Antimicrobial resistance: global report on surveillance*. Geneva, Switzerland: World Health Organization, 2014.

[2] C. S. McArdell, E. Molnar, M. J.-F. Suter, and W. Giger, “Occurrence and Fate of Macrolide Antibiotics in Wastewater Treatment Plants and in the Glatt Valley Watershed, Switzerland,” *Environ. Sci. Technol.*, vol. 37, no. 24, pp. 5479–5486, Dec. 2003, doi: 10.1021/es034368i.

[3] T. Kivits, H. P. Broers, H. Beeltje, M. van Vliet, and J. Griffioen, “Presence and fate of veterinary antibiotics in age-dated groundwater in areas with intensive livestock farming,” *Environmental Pollution*, vol. 241, pp. 988–998, Oct. 2018, doi: 10.1016/j.envpol.2018.05.085.

[4] N. M. Vieno, T. Tuhkanen, and L. Kronberg, “Analysis of neutral and basic pharmaceuticals in sewage treatment plants and in recipient rivers using solid phase extraction and liquid chromatography–tandem mass spectrometry detection,” *Journal of Chromatography A*, vol. 1134, no. 1–2, pp. 101–111, Nov. 2006, doi: 10.1016/j.chroma.2006.08.077.

[5] M. Stumpf, T. A. Ternes, R.-D. Wilken, Silvana Vianna Rodrigues, and W. Baumann, “Polar drug residues in sewage and natural waters in the state of Rio de Janeiro, Brazil,” *Science of The Total Environment*, vol. 225, no. 1–2, pp. 135–141, Jan. 1999, doi: 10.1016/S0048-9697(98)00339-8.

[6] T. Ternes, M. Bonerz, and T. Schmidt, “Determination of neutral pharmaceuticals in wastewater and rivers by liquid chromatography–electrospray tandem mass spectrometry,” *Journal of Chromatography A*, vol. 938, no. 1–2, pp. 175–185, Dec. 2001, doi: 10.1016/S0021-9673(01)01205-5.

[7] D. W. Kolpin *et al.*, “Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999−2000: A National Reconnaissance,” *Environ. Sci. Technol.*, vol. 36, no. 6, pp. 1202–1211, Mar. 2002, doi: 10.1021/es011055j.

[8] T. L. Jones-Lepp, “Chemical markers of human waste contamination: Analysis of urobilin and pharmaceuticals in source waters,” *J. Environ. Monit.*, vol. 8, no. 4, p. 472, 2006, doi: 10.1039/b512858g.

[9] J. F. Artiola, I. L. Pepper, and M. L. Brusseau, Eds., *Environmental monitoring and characterization*. Amsterdam ; Boston: Elsevier Academic Press, 2004.

[10] M. Hernando, M. Mezcua, A. Fernandezalba, and D. Barcelo, "Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments," *Talanta*, vol. 69, no. 2, pp. 334–342, Apr. 2006, doi: 10.1016/j.talanta.2005.09.037.

[11] C.-Y. Lin and S.-D. Huang, "Application of liquid–liquid–liquid microextraction and high-performance liquid-chromatography for the determination of sulfonamides in water," *Analytica Chimica Acta*, vol. 612, no. 1, pp. 37–43, Mar. 2008, doi: 10.1016/j.aca.2008.02.008.

[12] A. L. Batt, I. B. Bruce, and D. S. Aga, "Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges," *Environmental Pollution*, vol. 142, no. 2, pp. 295–302, Jul. 2006, doi: 10.1016/j.envpol.2005.10.010.

[13] J.-F. Yang, G.-G. Ying, J.-L. Zhao, R. Tao, H.-C. Su, and F. Chen, "Simultaneous determination of four classes of antibiotics in sediments of the Pearl Rivers using RRLC–MS/MS," *Science of The Total Environment*, vol. 408, no. 16, pp. 3424–3432, Jul. 2010, doi: 10.1016/j.scitotenv.2010.03.049.

[14] L.-J. Zhou *et al.*, "Simultaneous determination of human and veterinary antibiotics in various environmental matrices by rapid resolution liquid chromatography–electrospray ionization tandem mass spectrometry," *Journal of Chromatography A*, vol. 1244, pp. 123–138, Jun. 2012, doi: 10.1016/j.chroma.2012.04.076.

[15] M. Ericsson and A. Colmsjö, "Dynamic Microwave-Assisted Extraction Coupled On-Line with Solid-Phase Extraction and Large-Volume Injection Gas Chromatography: Determination of Organophosphate Esters in Air Samples," *Anal. Chem.*, vol. 75, no. 7, pp. 1713–1719, Apr. 2003, doi: 10.1021/ac026287v.

[16] E. Turiel, A. Martín-Esteban, and J. L. Tadeo, "Molecular imprinting-based separation methods for selective analysis of fluoroquinolones in soils," *Journal of Chromatography A*, vol. 1172, no. 2, pp. 97–104, Nov. 2007, doi: 10.1016/j.chroma.2007.10.003.

[17] M. Lillenberg *et al.*, "Simultaneous determination of fluoroquinolones, sulfonamides and tetracyclines in sewage sludge by pressurized liquid extraction and liquid chromatography electrospray ionization-mass spectrometry," *Journal of Chromatography A*, vol. 1216, no. 32, pp. 5949–5954, Aug. 2009, doi: 10.1016/j.chroma.2009.06.029.

[18] A. V. Herrera-Herrera, L. M. Ravelo-Pérez, J. Hernández-Borges, M. M. Afonso, J. A. Palenzuela, and M. Á. Rodríguez-Delgado, "Oxidized multi-walled carbon nanotubes for the

dispersive solid-phase extraction of quinolone antibiotics from water samples using capillary electrophoresis and large volume sample stacking with polarity switching," *Journal of Chromatography A*, vol. 1218, no. 31, pp. 5352–5361, Aug. 2011, doi: 10.1016/j.chroma.2011.06.031.

[19] N. M. Kassab, M. S. do Amaral, A. K. Singh, and M. I. R. M. Santoro, "Development and validation of UV spectrophotometric method for determination of levofloxacin in pharmaceutical dosage forms," *Quím. Nova*, vol. 33, no. 4, pp. 968–971, 2010, doi: 10.1590/S0100-40422010000400037.

[20] H. Yu, H. Mu, and Y.-M. Hu, "Determination of fluoroquinolones, sulfonamides, and tetracyclines multiresidues simultaneously in porcine tissue by MSPD and HPLC–DAD," *Journal of Pharmaceutical Analysis*, vol. 2, no. 1, pp. 76–81, Feb. 2012, doi: 10.1016/j.jpha.2011.09.007.

[21] K. Fent, A. Weston, and D. Caminada, "Ecotoxicology of human pharmaceuticals," *Aquatic Toxicology*, vol. 76, no. 2, pp. 122–159, Feb. 2006, doi: 10.1016/j.aquatox.2005.09.009.

[22] L. H. M. L. M. Santos, A. N. Araújo, A. Fachini, A. Pena, C. Delerue-Matos, and M. C. B. S. M. Montenegro, "Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment," *Journal of Hazardous Materials*, vol. 175, no. 1–3, pp. 45–95, Mar. 2010, doi: 10.1016/j.jhazmat.2009.10.100.

[23] Y. Kim, K. Choi, J. Jung, S. Park, P.-G. Kim, and J. Park, "Aquatic toxicity of acetaminophen, carbamazepine, cimetidine, diltiazem and six major sulfonamides, and their potential ecological risks in Korea," *Environment International*, vol. 33, no. 3, pp. 370–375, Apr. 2007, doi: 10.1016/j.envint.2006.11.017.

[24] R. A. Brain *et al.*, "Microcosm evaluation of the effects of an eight pharmaceutical mixture to the aquatic macrophytes Lemna gibba and Myriophyllum sibiricum," *Aquatic Toxicology*, vol. 70, no. 1, pp. 23–40, Oct. 2004, doi: 10.1016/j.aquatox.2004.06.011.

[25] L. Migliore, C. Civitareale, S. Cozzolino, P. Casoria, G. Brambilla, and L. Gaudio, "Laboratory models to evaluate phytotoxicity of sulphadimethoxine on terrestrial plants," *Chemosphere*, vol. 37, no. 14–15, pp. 2957–2961, Dec. 1998, doi: 10.1016/S0045-6535(98)00336-1.

[26] L. Migliore, A. Rotini, N. L. Cerioli, S. Cozzolino, and M. Fiori, “Phytotoxic Antibiotic Sulfadimethoxine Elicits a Complex Hormetic Response in the Weed *Lythrum Salicaria* L.,” *Dose-Response*, vol. 8, no. 4, p. dose-response.0, Oct. 2010, doi: 10.2203/dose-response.09-033.Migliore.

[27] M. Garcia-Käufer *et al.*, “Genotoxic effect of ciprofloxacin during photolytic decomposition monitored by the in vitro micronucleus test (MNvit) in HepG2 cells,” *Environ Sci Pollut Res*, vol. 19, no. 5, pp. 1719–1727, Jun. 2012, doi: 10.1007/s11356-011-0686-y.

[28] L. Aristilde, A. Melis, and G. Sposito, “Inhibition of Photosynthesis by a Fluoroquinolone Antibiotic,” *Environ. Sci. Technol.*, vol. 44, no. 4, pp. 1444–1450, Feb. 2010, doi: 10.1021/es902665n.

[29] M. González-Pleiter *et al.*, “Toxicity of five antibiotics and their mixtures towards photosynthetic aquatic organisms: Implications for environmental risk assessment,” *Water Research*, vol. 47, no. 6, pp. 2050–2064, Apr. 2013, doi: 10.1016/j.watres.2013.01.020.

[30] M. Isidori, M. Lavorgna, A. Nardelli, L. Pascarella, and A. Parrella, “Toxic and genotoxic evaluation of six antibiotics on non-target organisms,” *Science of The Total Environment*, vol. 346, no. 1–3, pp. 87–98, Jun. 2005, doi: 10.1016/j.scitotenv.2004.11.017.

[31] E. J. Rosi-Marshall and T. V. Royer, “Pharmaceutical Compounds and Ecosystem Function: An Emerging Research Challenge for Aquatic Ecologists,” *Ecosystems*, vol. 15, no. 6, pp. 867–880, Sep. 2012, doi: 10.1007/s10021-012-9553-z.

[32] T. Schwartz, W. Kohnen, B. Jansen, and U. Obst, “Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms,” *FEMS Microbiology Ecology*, vol. 43, no. 3, pp. 325–335, Apr. 2003, doi: 10.1111/j.1574-6941.2003.tb01073.x.

[33] Veterinary Medicines Division, “Sales of veterinary antimicrobial agents in 31 European countries in 2017. Trends from 2010 to 2017,” European Medicines Agency, 9, EMA/294674 2019.

[34] M. Sammul, “Overview of the use of antibiotics for veterinary purposes in 2006–2016,” Ravimiamet (State Agency of Medicines), Estonia, 2016.

[35] R. Nageswara Rao, N. Venkateswarlu, and R. Narsimha, “Determination of antibiotics in aquatic environment by solid-phase extraction followed by liquid chromatography–

electrospray ionization mass spectrometry," *Journal of Chromatography A*, vol. 1187, no. 1–2, pp. 151–164, Apr. 2008, doi: 10.1016/j.chroma.2008.02.021.

[36] S. Thiele-Bruhn and M.-O. Aust, "Effects of Pig Slurry on the Sorption of Sulfonamide Antibiotics in Soil," *Arch Environ Contam Toxicol*, vol. 47, no. 1, Jul. 2004, doi: 10.1007/s00244-003-3120-8.

[37] K. Kipper, K. Herodes, I. Leito, and L. Nei, "Two fluoroalcohols as components of basic buffers for liquid chromatography electrospray ionization mass spectrometric determination of antibiotic residues," *Analyst*, vol. 136, no. 21, p. 4587, 2011, doi: 10.1039/c1an15123a.

[38] AOAC, "Guidelines for Standard Method Performance Requirements." .

[39] P. J. Taylor, "Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry," *Clinical Biochemistry*, vol. 38, no. 4, pp. 328–334, Apr. 2005, doi: 10.1016/j.clinbiochem.2004.11.007.

[40] "EUROPEAN COMMISSION, Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed., SANTE/11813/2017 (2018)." .

[41] J. Tell *et al.*, "Science-based Targets for Antibiotics in Receiving Waters from Pharmaceutical Manufacturing Operations," *Integr Environ Assess Manag*, vol. 15, no. 3, pp. 312–319, May 2019, doi: 10.1002/ieam.4141.

Annexes

Annex 1: List of Figures and Tables.

Annex 2: Structure of Antibiotics.

Annex 3: Antibiotic resistance data from ECDC (European commission of disease prevention and control)

Annex 4: Sample collection points.

Annex 5: MRM Chromatograms of antibiotics in samples

Annex 1: List of Figures and Tables.

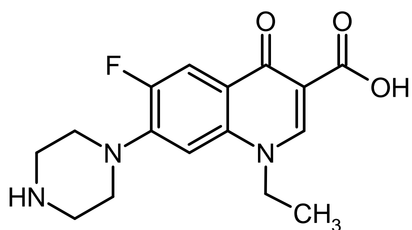
Figures:

Figure 1. Flow of the LC-MS Project. 12
Figure 2. Gradient elution method showing MRM of 8 different antibiotics. 17
Figure 3. Blank Chromatogram to check for possible carryover effects. 22
Figure 4. Process Efficiency 25

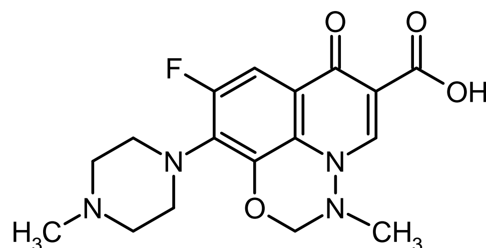
Tables:

Table 1. Antibiotic Classification based on their mechanism of action 7
Table 2. LC-MS/MS Conditions for the analysis of antibiotics by MRM using Waters XBridge RP column.......... 16
Table 3. LC Gradient Elution for the analysis of antibiotics. 17
Table 4.Calibration Ranges, Regression coefficients and LOD and LOQ values. 19
Table 5. Antibiotics at Upstream, Midpoint and Downstream of the WWTP. (ND: Not detected, NA: Not applicable) 20
Table 6. Percentage Accuracy of spiked samples. 21
Table 7. Concentrations of Analytes before and after washing. 21
Table 8. Gradient to flush the column 22
Table 9. Matrix Effect Evaluation at different concentration levels. 23
Table 10. Recovery of antibiotics 24
Table 11. Ratio of Measured values and PNEC values to assess ecotoxicological and resistance promotion impact. 26

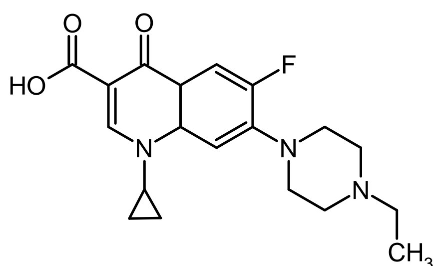
Annex 2: Structures of Antibiotic Compounds.



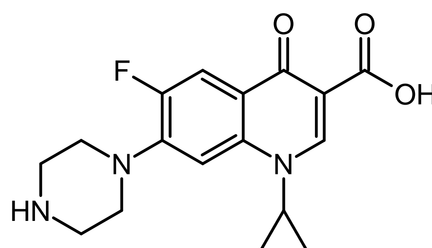
Norfloxacin (NOR) | pKa – 5.66, pKa – 8.68



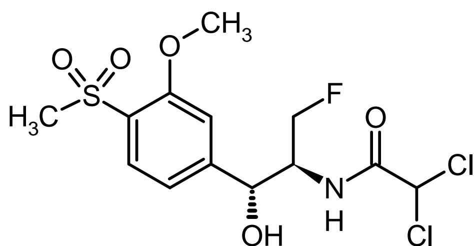
Marbofloxacin (MAR) | pKa – 5.38, pKa – 6.16



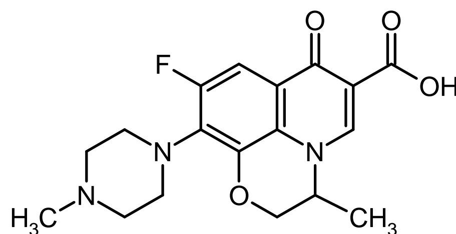
Enrofloxacin (ENR) | pKa – 5.69, pKa – 6.68



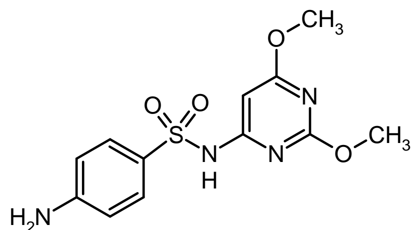
Ciprofloxacin (CIP) | pKa – 5.76, pKa – 8.62



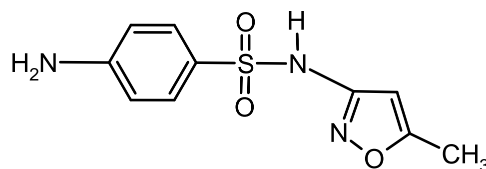
Florfenicol (FF) | pKa -3.4, pKa -8.49



Ofloxacin (OFL) | pKa – 5.45 pKa – 6.2

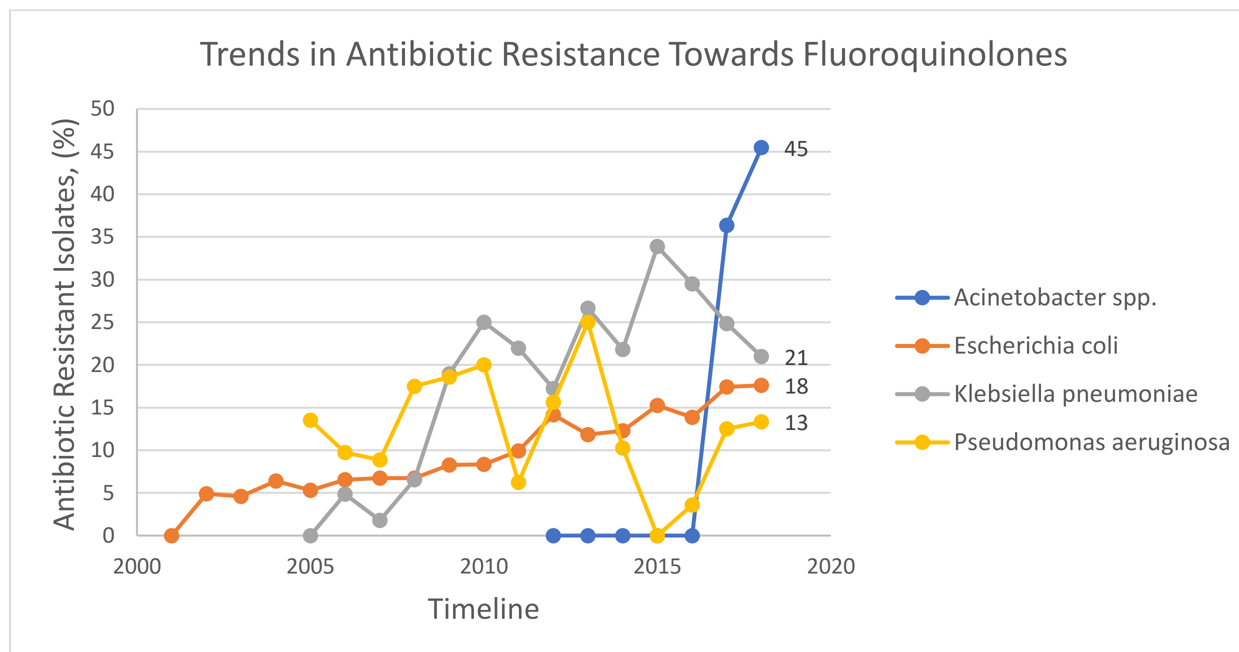


Sulfadimethoxine (SDM) | pKa – 2.11, pKa – 6.17



Sulfamethoxazole (SMX) | pKa - 1.97, pKa -6.67

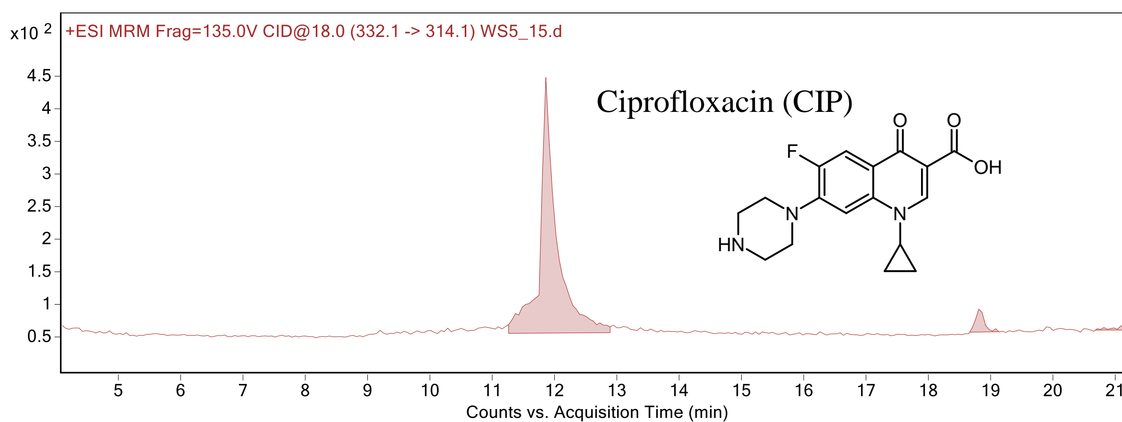
Annex 3: ECDC Data plot showing the antibiotic resistance increase of some fluoroquinolones

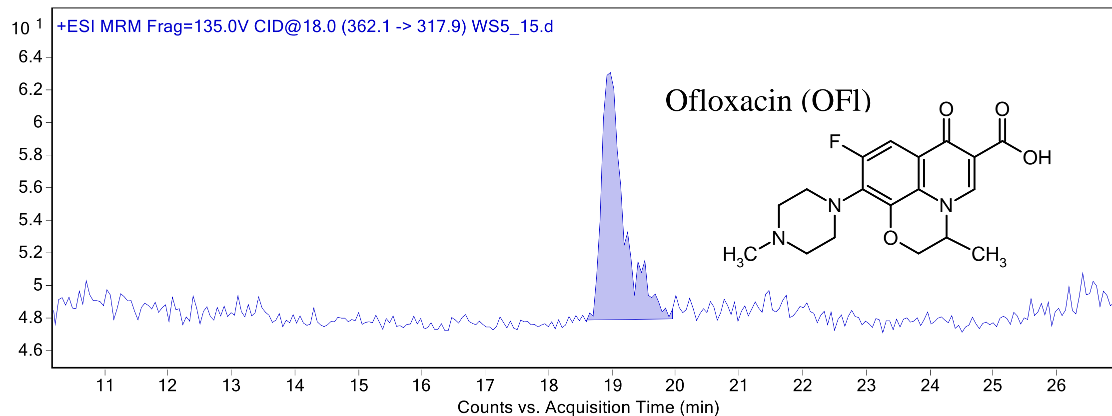


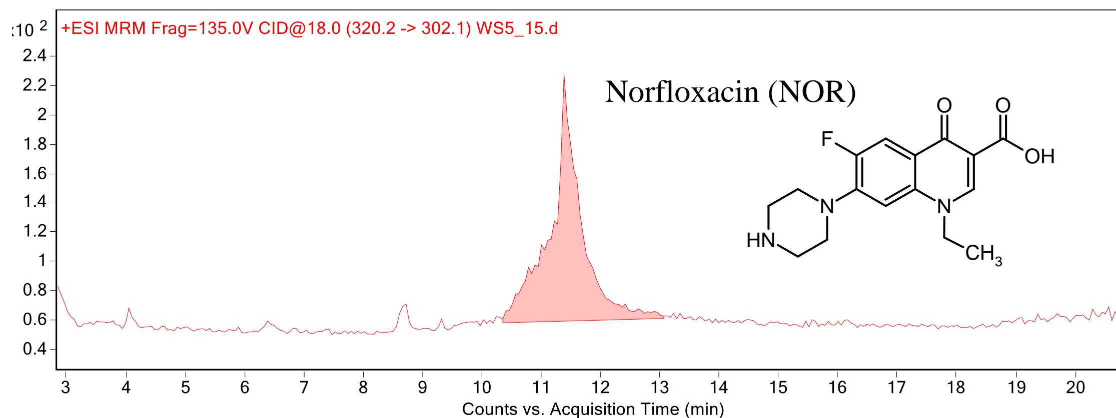
Timeline		2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018
Antibiotic Resistant isolates (%)	Escherichia coli	-	0	5	5	6	5	7	7	7	8	8	10	14	12	12	15	14	17	18
	Klebsiella pneumoniae	-	-	-	-	-	0	5	2	7	19	25	22	17	27	22	34	30	25	21
	Pseudomonas aeruginosa	-	-	-	-	-	14	10	9	18	19	20	6	16	25	10	0	4	13	13
	Acinetobacter spp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	36	45

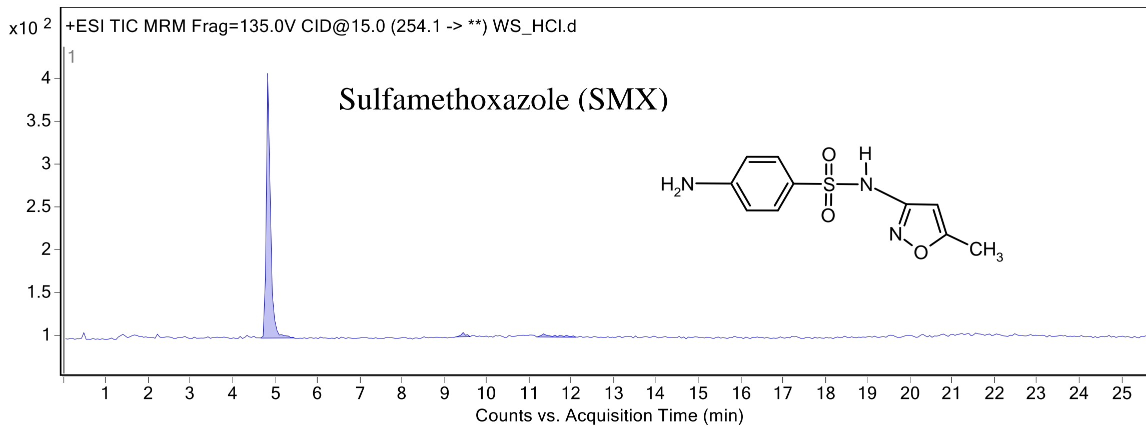
Annex 4: Sample collection points.

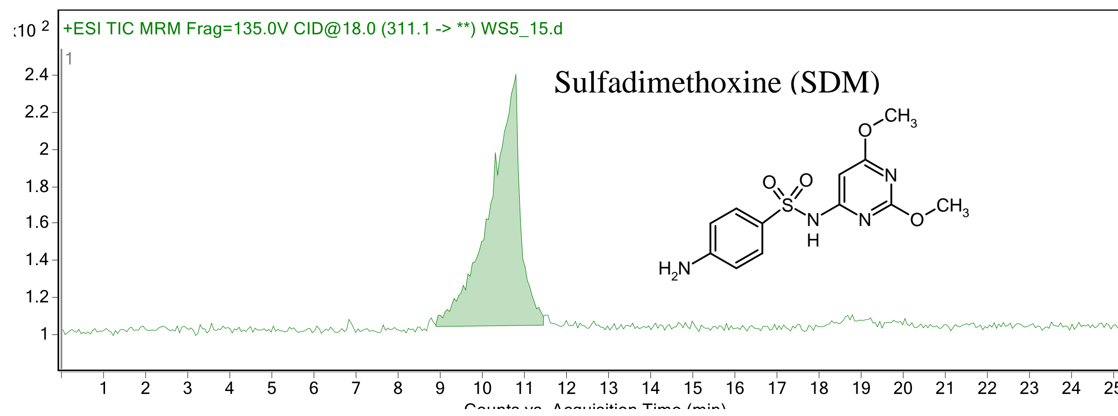
Annex 5. MRM Chromatograms of antibiotics found in samples.








x10 2
+ESI TIC MRM Frag=135.0V CID@15.0 (254.1 -> **) WS_HCl.d
Sulfamethoxazole (SMX)
Counts vs. Acquisition Time (min)


:10 2
+ESI TIC MRM Frag=135.0V CID@18.0 (311.1 -> **) WS5_15.d
Sulfadimethoxine (SDM)

Non-exclusive licence to reproduce thesis and make thesis public

I, Waseem Ahmad Iftikhar herewith grant the University of Tartu a free permit (non-exclusive licence) to reproduce, for the purpose of preservation, including for adding to the DSpace digital archives until the expiry of the term of copyright,

DETERMINATION OF ANTIBACTERIALS IN RIVER WATER BY SOLID PHASE EXTRACTION USING LC-ESI-MS/MS

supervised by Associate professor, Dr. Koit Herodes_

2. I grant the University of Tartu a permit to make the work specified in p. 1 available to the public via the web environment of the University of Tartu, including via the DSpace digital archives, under the Creative Commons licence CC BY NC ND 3.0, which allows, by giving appropriate credit to the author, to reproduce, distribute the work and communicate it to the public, and prohibits the creation of derivative works and any commercial use of the work until the expiry of the term of copyright.

3. I am aware of the fact that the author retains the rights specified in p. 1 and 2.

4. I certify that granting the non-exclusive licence does not infringe other persons' intellectual property rights or rights arising from the personal data protection legislation.

Waseem Ahmad Iftikhar

27/05/2020

INFORMATION SHEET

Determination of antibacterials in river water by solid phase extraction using LC - MS/MS

Analytical method was developed for the detection of fluoroquinolones (FQs), sulfonamides and Amphenicol in Emajogi river in Tartu City. The compounds were simultaneously extracted from river water using solid phase extraction (SPE). Identification and quantification was done through Liquid chromatography – tandem mass spectrometry (LC-MS/MS) in selected reaction monitoring (MRM) mode.The recovery of FQs ranged 62% for Enrofloxacin to 76% Marbofloxacin and 68% Florfenicol ,69% sulfamethoxazole. Limit of Quantification ranged from 0.1 ngg^{-1} for SA's ,0.6 - 1.5 ngg^{-1} for FQ's, and 0.9ngg^{-1} for FF. The method was developed and valdated for river water analysis of samples from upstream, midpoint and down stream of the WWTP. The method developed may be used for a more in-depth study on the occurence and fate of these commonly used pharmaceuticals in river water bodies.

Key words: antibiotic residue analysis, LC-MS, method development, solid phase extraction, river water analysis,

CERCS: P300 analytical chemistry.

INFOLEHT

Antimikroobsete ainete määramine jõevees kasutades tahke faasi ekstraktsiooni ja LC-MS/MS meetodit

Käesolevad töös töötati välja analüüsimetoodika fluorokinoloonide, sulfoonamiidide ja amfenikoolide rühma kuuluvate antibiootikumide määramiseks jõevees. Analüüdid ekstraheeriti proovist korraga, kasutades tahke faasi ekstraktsiooni (SPE). Identifitseerimine ja kvantitatiivne analüüs teostati vedelikkromatograafia-massispektromeetria (LC-MS/MS) meetodit kasutades valitud ülemineku jälgimise režiimis (MRM). Analüütide saagised jäid vahemikku 62 kuni 78%, mida võib pidada rahuldavaks. Määramispiirid jäid vahemikku 0,1 kuni 1,5 ng g^{-1}. Metoodika arendamisel ja valideerimisel kasutati Emajõe veeproove, mis olid võetud veepuhastusjaama lähistelt ning sellest üles- ja allavoolu. Töö tulemusi saab kasutada antibiootikumijääkide monitoorimiseks jõevees.

Märksõnad: antibiootikumijääkide analüüs, LC-MS, metoodika arendus,dsd tahke faasi ekstraktsioon, jõevee analüüs,

CERCS: P300 analüütiline keemia