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Technologies for treatment of API contaminated waste waters

MASTER'S THESIS

to achieve the university degree of
Diplom-Ingenieurin
Master's degree programme: Chemical and Pharmaceutical Engineering

submitted to

Graz University of Technology

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Graz, November 2018

AFFIDAVIT

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Abstract

Active pharmaceutical ingredients (APIs) and especially antibiotics (AB) are of great interest due to the large consumption in medication and agriculture. Through each usage of an antibiotic, antibiotic residues even in low concentrations end up in the environment with tremendous implications. Antimicrobial resistances (AMR) in living organisms are developed in water and soil, which are then repetitive introduced into natural cycles. Therefore, the world health organization (WHO) reports more frequently about AMR and its danger to society. As a result, an increase of interest concerning multiclass methods for analysis of antibiotic residues in environmental samples in literature is recognizable. Due to the complexity of ABs, measurements in strong matrixes are still challenging. Hence, one aim of this thesis was to develop and modify commonly used sample preparation techniques for various surface water, effluent, waste water, and sludge samples to analyse ABs as representatives of APIs more efficiently. Another main goal was to lower the limits of quantification (LoQ) of the target ABs for liquid chromatography-mass spectrometry (LC-MS/MS) as analysis of choice.

It was possible to develop and modify different approaches for sample preparation within this thesis. The goal was to compare the newly developed techniques to commonly used sample preparation methods such as reversed-phase solid phase extraction (RP-SPE) and the standard sample preparation technique used by Wetsus laboratory, which is based on dilution. Electromembrane extraction (EME) and reactive extraction were introduced as experimental and new techniques and tested for their applicability on higher polluted samples. Waste water and sludge samples were obtained from three different waste water treatment plants throughout west and north Netherlands, whereas all surface waters were drawn from locations of without anthropogenic influences in Friesland province.

Through the exploratory nature of this thesis, surprising and reliable data was obtained throughout the selected target analytes. Overall the obtained data within this thesis showed clearly that it is nearly impossible to measure all ABs at once. Nevertheless, satisfactory recoveries were possible to achieve for macrolides in sludge samples with a modified dilution method and for fluoroquinolones with RP-SPE. EME turned out to be a promising sample preparation technique due to less solvent and time consumption, but the results of the experiments indicate that further investigation is necessary.

Kurzfassung

Aktive pharmazeutische Wirkstoffe (API) und insbesondere Antibiotika (AB) sind aufgrund der hohen Verbrauchszahlen in Medizin und Landwirtschaft von großem Interesse. Bei jedem Einsatz eines Antibiotikums gelangen Antibiotikarückstände bereits in geringen Konzentrationen in die Umwelt mit enormen Auswirkungen. Antimikrobielle Resistenzen werden von in Wasser und Boden lebenden Organismen entwickelt, welche dann wiederum in die natürlichen Zyklen eingeführt werden. Daher ist ein Wachsen des Interesses an Breitbandmethoden zur Analyse von Antibiotikarückständen in Umweltproben in der Literatur zu erkennen. Aufgrund der Komplexität von Antibiotika sind Messungen in Proben mit starken Verunreinigungen immer noch eine Herausforderung. Ein Ziel dieser Masterarbeit war daher die Entwicklung und Modifikation von häufig verwendeter Probenvorbereitungstechniken für verschiedene Oberflächenwasser-, Effluent-, Abwasser- und Schlammproben, um Antibiotika als Vertreter von APIs effizienter zu analysieren. Ein weiteres Hauptziel war die Quantifizierungsgrenzen (LoQ) der Zielantibiotika für die multidimensionale Flüssigchromatographie-Massenspektrometrie (LC-MS/MS) zu senken.

Im Rahmen dieser Arbeit konnten verschiedene Techniken zur Probenvorbereitung entwickelt und modifiziert werden, welche dann mit den gängigen Methoden der Probenvorbereitung wie Festphasenextraktion mit Umkehrphase (reversed-phase solid phase extraction-RP-SPE) und der Standard-Probenvorbereitungstechnik des Wetsus-Labors (die auf Verdünnung basiert) verglichen wurden. Die Elektromembranextraktion (EME) und die reaktive Extraktion wurden als experimentelle und neue Techniken eingeführt und auf ihre Anwendbarkeit an Abwasser- und Schlammproben getestet. Abwasser- und Schlammproben wurden von drei verschiedenen Abwasserbehandlungsanlagen in West- und Nord-Niederlande bezogen, während alle Oberflächengewässerproben von Orten ohne anthropogenen Einfluss in der Provinz Friesland gezogen wurden.

Durch den explorativen Charakter dieser Arbeit wurden interessante und zuverlässige Daten für die gesamten Zielanalyten erzielt. Insgesamt zeigten die erhaltenen Daten im Rahmen dieser Arbeit deutlich, dass es nahezu unmöglich ist, alle ABs gleichzeitig zu messen. Dennoch konnten zufriedenstellende Wiederfindungsraten für Makrolide in Schlammproben mit einer modifizierten Verdünnungsmethode und für Fluorochinolonantibiotika mit RP-SPE erzielt werden. EME erwies sich aufgrund des geringeren Lösungsmittel- und Zeitverbrauchs als eine Probenvorbereitungsmethoden der Zukunft, jedoch zeigen Ergebnisse der Experimente, dass weitere Untersuchungen notwendig sind.

Acknowledgment

Before I thank everybody officially, I want to thank EVERYBODY for their PATIENCE!

To begin with, I would thank the Graz University of Technology for giving me the opportunity as well as for the scholarship to work for my thesis in the Netherlands and especially I would like to thank Prof. Siebenhofer for supervising me.

Beside TU Graz, I want to thank Wetsus for providing an interesting topic and surrounding for this work. Special thanks goes to my supervisor Jan-Willem Schoonen for his enthusiasm, his patience and guidance throughout my stay in Leeuwarden. Another special thanks goes to my second supervisor Ton van der Zande for his scientific input, for sharing his knowledge (and wisdom) about LC-MS/MS and sample preparation as well as his support and patience throughout data evaluation, even though it took me some time to understand. Additionally, I want to thank the whole Wetsus analytical team for letting me "play" with their instruments, for answering all my questions, for assisting, for the fun we had in the laboratory and consequently for creating a great working environment, without them it would not have been the same experience.

An extraordinary thanks goes to my family for their emotional as well as financial support throughout my whole studies, their understanding even when studies took longer than expected, and for being available no matter what.

One ultra-special thanks goes to my boyfriend Tobias for not only being on my side for over 4 years through fun and hard times, but also for his unfailing support and encouragement throughout my thesis (even when I seemed to act crazy sometimes). Thank you for everything!!! Additionally, I want to thank the parents of Tobias for letting me occupy the dining table more than once without questioning and of course for their kind support.

I also want to thank all my friends for enduring and tolerate me throughout my studies, but especially throughout the process of writing this "never-ending" thesis and for their input as well as for pushing me when I needed it. A special thanks goes to my CPE-Task Force- friends for lending me their lecture notes without dooming me and for helping me through my master studies. I would not be writing this acknowledgment if it were not for the help of my friends!

Last but not least, I want to thank all my proof readers for reading my thesis and their help throughout this process!

I. Table of contents

II. List of abbreviations

General abbreviations		Abbreviations of target analytes	
µL	Microliter	ACSUL	Acesulfame – human marker
ACN	Acetonitrile	AMP	Ampicillin
$Al_2(SO_4)_3$	Aluminium sulphate	ATLd7	Azithromycin-D7
AMR	Antimicrobial resistance	AZI	Azithromycin
ARG	Antibiotic resistance gene	BAC-Cl 12 or 14	Benzalkonium chloride 12 or 14– disinfectant
CAS	Unique numerical identifier assigned by the Chemical Abstracts Service (CAS) to every chemical substance described in the open scientific literature	CFT	Cefotaxime
CO_2	Carbon dioxide	CFX	Ciprofloxacin
$CuSO_4$	Copper(II) sulphate	CFXd8	Ciprofloxacin-D8
dw	Dry weight	CHEX	Chlorhexidine – disinfectant
EME	Electromembrane extraction	CLAR	Clarithromycin
EU	European Union	CM	Clindamycin
g	gram	COLIST	Colistin sulfate
GC	Gas chromatography	DIA	Diaveridine
h	Hours	DM	Dimetridazole
H_2O	Water	DOX	Doxycycline
HCOOH	Formic acid	ERYT	Erythromycin
IS	Internal standard	FLUMEQ	Glumequine
LC-MS	Liquid Chromatography coupled with mass spectrometry	GAPE	Gabapentine – human marker
LoD	Limit of detection	LINCOM	Lincomycin
LoQ	Limit of quantification	NEOMYC	Neomycin
MeOH	Methanol	OFLX	Ofloxacin
MilliQ	Ultrapure water from Millipore Milli-Q®	OTETR	Oxytetracycline
min	Minute(s)s	PENG	Penicillin G
mL	Millilitre	PENV	Penicillin V
mm	Millimetre	SMO	Sulfamethoxazole
mmHG	A millimetre of mercury is a manometric unit of pressure, generated by a column of mercury one millimetre high. Now defined as precisely 133.322387415 Pascals	SPECTI	Spectinomycin
N_2	Nitrogen gas	SUCLOP	Sulphachloropyidazine
pH	Potential or power of hydrogen	SUCRAL	Sucralose – human marker
PNEC	Predicted no effect concentration	SUDOX	Sulphadoxine
RE	Reactive extraction	SUDOX-D3	Sulfadoxin-D3
RE-SPE	Reversed phase – solid phase extraction	SULFAM	Sulphamethazine
rpm	Revolutions per minute	SULPYR	Sulfapyridine
RSD	Relative standard deviation in %	TETR	Tetracycline
sec	Seconds	TILMIC	Tilmicosin
SPE	Solid phase extraction	TRC	Triclorsan - disinfectant
St.Dev.	Standard deviation	TRIM	Trimethoprim
STD	Standard	TRIM-D9	Trimethoprim-D9
SUPERMOD	modifier	TYLOS	Tilosin
ww	Wet weight		

1. Introduction

"Antibiotic resistance is one of the biggest threats to global health, food security, and development today" – World Health Organization [1]

"'Antibiotic apocalypse': doctors sound alarm over drug resistance" – The Guardian [2]

These are only two statements representing the current situation around the globe concerning antibiotic resistance crisis. Antimicrobial active pharmaceutical ingredients (API) were the products of the medical breakthrough many decades ago, where bacterial infections were a horrendous threat for human kind. After decades of successfully treating these infections, antibiotics (ABs) became a magic bullet not only in medicine but also in food industry to mortify all unwanted bacteria. Nobel prize winner and penicillin discoverer Alexander Fleming forecasted already in 1945, that there might come a time, when exposing microbes to non-lethal quantities of the ABs, microbes would become educated and resistant[3], [4]. Decades passed, and he proved to be right. Nowadays, misuse, overuse, and lack of new drug development by the pharmaceutical industry naturally lead to resistant bacteria and therefore a decrease of the antibiotic effectiveness regarding treatment of a growing number of infections such as tuberculosis or gonorrhoea [5]. Another indication of the urgency of this topic is the nearly thirteenfold increase of publications and studies in the field of antibiotic resistance in environment within the last 20 years (from 600 to 7600 publications per year) [6].

After every usage of ABs in food industry, in hospitals or at home prescribed by a physician, antibiotic residues, even in low concentrations, end up in the environment. As a result not only resistances in living organisms are developed but also in water and soil, which are then repetitive introduced into natural cycles. To avoid reintroduction of antibiotic residues into a natural cycle, antibiotic monitoring as well as proper treatments of waste and waste waters are necessary. Halling-Sorensen *et al.* stated in 1998 that API have not been adequately monitored in the environment and were not considered as dangerous micropollutants by then [7]. From that point on several studies reported that APIs are not entirely removed in sewage treatment plants and therefore are found in treated effluents, surface waters and ground waters [8], [9], [10]. Prior to removal, detailed monitoring and screening are necessary to understand the impact of these compounds on the environment.

Due to the complexity of APIs, especially ABs, measurements of those compounds in strong matrixes are challenging. One aim of this thesis was to develop or modify current sample preparation techniques for various samples such as different waste waters, effluent, surface water and sludge to analyse ABs as representatives of APIs more efficiently, less time consuming and more cost-effective.

Another main goal was to lower the limits of quantification (LoQs) of the chosen analytical method liquid-chromatography tandem mass spectrometry (LC-MS/MS) for the target analytes. The used sample preparation technique by Wetsus is already an easy method to measure ABs in low concentration for low polluted samples, but with relatively high LoQs. Table 1 lists the currently

achievable LoQs by Wetsus, which should be improved in order to reach lower limits below the PNEC (predicted no effect concentration) and the desired LoQ.

Table 1: List of PNEC, current limit of quantifications (LoQ) and wished LoQ of some antimicrobial API in ng L^{-1}

antimicrobial API	**PNEC*** *[ng L^{-1}]*	**current LoQ** *[ng L^{-1}]*	**desired LoQ** *[ng L^{-1}]*
AMOX	250	2000	20
AMP	250	1000	10
AZI	250	5000	50
CFT	125	1000	10
CFX	64	1572	50
CLAR	250	10	10
CM	1000	20	10
DOX	2000	10	100
ERYT	1000	610	-
OFX	500	5000	50
TRIM	500	30	20

*cited from: [11][12][13][14] [15]

Since waste water, effluent and sludge samples are highly polluted matrixes, which are challenging to analyse, no Wetsus standard preparation method was yet implemented. Therefore, new treatment approaches such as modified dilution preparation, reactive extraction and electromembrane extraction are tested, evaluated, and compared to literature. For less polluted samples like surface water, the efficiency of commonly used methods such as solid phase extraction (SPE) compared to the Wetsus standard preparation technique has to be determined. Finally, the most suitable sample treatment technique for each sample has to be selected and applied on selected samples for monitoring, screening, discussion and for comparison to literature.

2. Theoretical background

This chapter provides a theoretical insight behind the practical work that was done in this thesis. First, the terms "APIs and antibiotic" are clarified and antibiotics in general are discussed, followed by a theoretical investigation of their key role regarding micropollution and antimicrobial resistance in water. Secondly, a short overview of the treatment of these contaminations in waste water is provided and finally the principles behind the sample preparation techniques in this thesis are explained briefly.

2.1. Active pharmaceutical ingredients (APIs), antibiotics (ABs) and antimicrobial resistance (AMR) in waste water

2.1.1. APIs and ABs

Generally, an API is a chemical substance in a pharmaceutical drug that is biologically active or triggers a physiological response combined with a therapeutic effect. An API is only a small portion in a drug, which achieves the desired therapeutic effect in a body, the other ingredients are therapeutically inert additives, which are optimizing the bioavailability of APIs such as fillers, disintegrants, and lubricants.

APIs are classified in two major categories of synthetic drugs, natural chemical drugs and 4 subcategories shown in Figure 1.

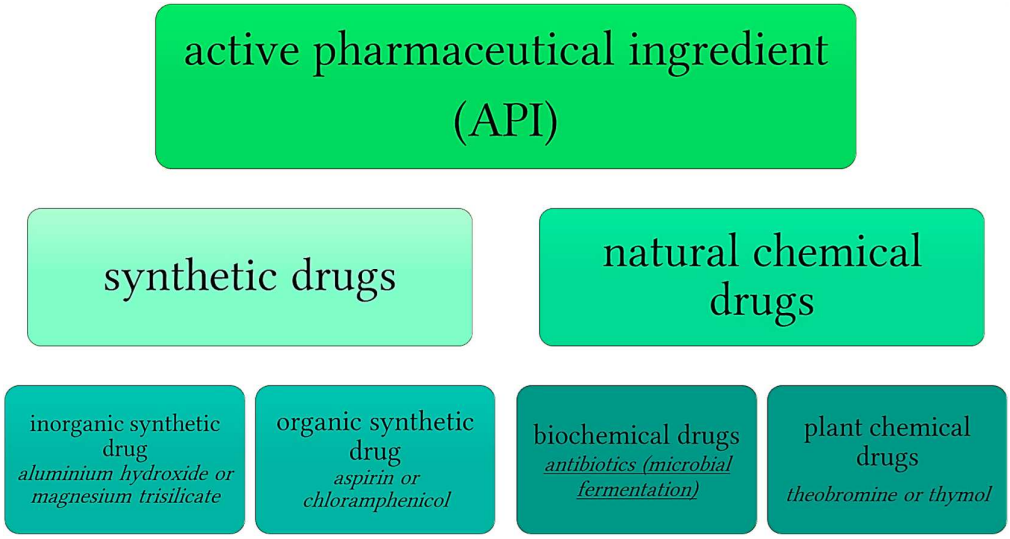


Figure 1: Classified categories of API with representatives for every subcategory

AB are representatives for biochemical drugs and belong therefore to the API category of natural chemical drugs. They are products of fermentation and isolated as unfinished or as intermediate products of limited stability. In the early 20th century Alexander Fleming discovered penicillin, which was the start of the antibiotic era, but a commercial distribution was not yet possible. Through the development of a chemical synthesis in 1940 by Ernst B. Chain and Howard W. Florey to produce penicillin in an industrial scale, it was possible to save hundreds of lives throughout World War II and therefore marked the antibiotic revolution [16]. Years went by and an array of ABs such as tetracyclines, macrolides, and (fluoro-) quinolones, saved and are still saving the lives of millions of humans and animals for over 70 years now [5], [16].

ABs can be classified according to their mechanism of action, the chemical structure or their antibiotic resistance mechanisms. Figure 2 displays schematically the classifications of ABs according to their mechanism of action.

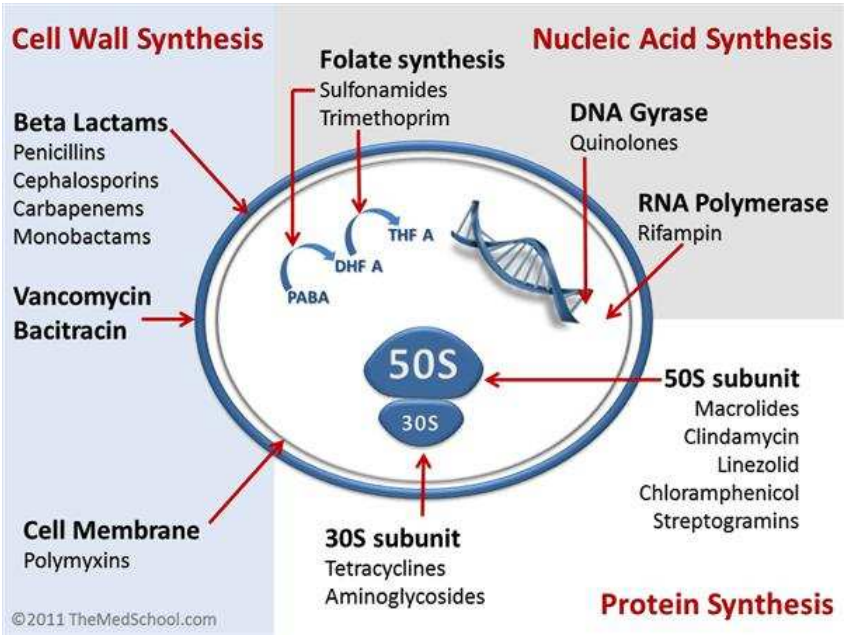


Figure 2: Overview of the antibiotic classification according to their mechanism of action adapted from Kapoor *et al.* [17]

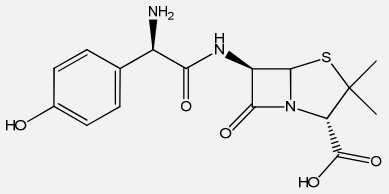
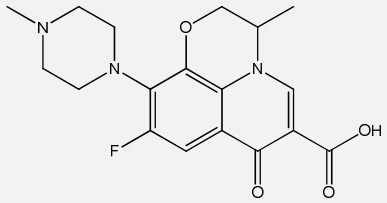
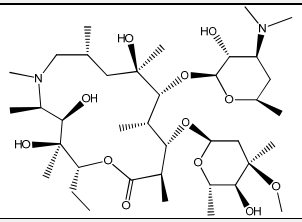
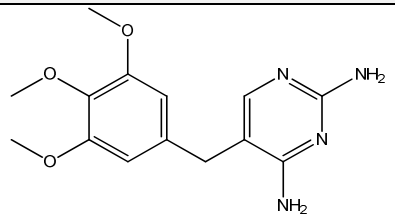
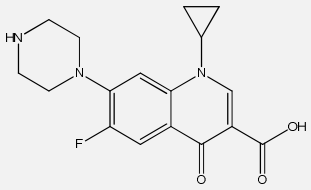
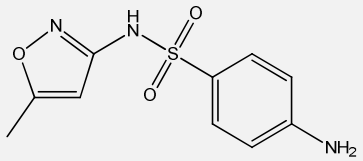
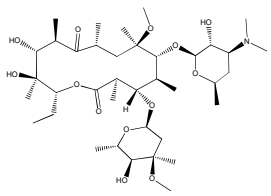
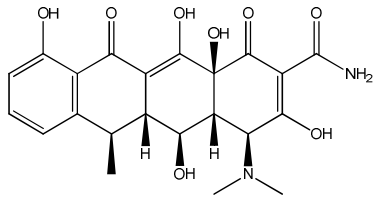
β-lactams are at the moment the most prescribed ABs according to Chem *et al.*, especially Amoxicillin appeared to be the most frequently prescribed antibiotic [18]. During cell wall synthesis, a β-lactam targets the penicillin binding proteins, which are produced during crosslinking reactions of the glycan strands and the peptide chain. β-lactam then mimics the D-alanyl D-alanine portion of the peptides chain to interact with the penicillin binding proteins with its lactam ring. This disruption of the peptidoglycan in the cell wall, as it is essential for surviving of the cell, leads to a collapse of the bacterium [17],[19].

Another important group of ABs are the macrolides including azithromycin, erythromycin and clarithromycin. These compounds are inhibitors of protein biosynthesis and targeting the sequence of the peptidyl transferase centre of the 50S ribosomal subunit at the first stage of protein synthesis (translocation). An early detachment of one or more incomplete peptide chains is the result of a macrolide attack [17].

As inhibitors of DNA replication, fluoroquinolones prevent the enzyme DNA gyrase, which notches double-stranded DNA helix, introduces negative supercoils and reseals the notched ends together. The main representatives of this antibiotic group are ciprofloxacin and ofloxacin. Both compounds are important for treating infectious diarrhoea and urinary infections [17], [20], [21].

Each antibiotic possesses a particular mechanism of action, a specific chemical structure and is therefore usable for definite infections. Table 2 lists some representatives of different antibiotic groups with chemical structures and gives examples for which indication they are usable.

Table 2: Examples of important antibiotics with according information about their antibiotic group, their pKa, PNEC (predicted no effect concentration in ng L^{-1}) and medical usage

	*Name*	Amoxicillin		*Name*	Ofloxacin
	Abbreviation	AMOX		*Abbreviation*	OFX
	Antibiotic group	β-lactam		*Antibiotic group*	Fluoroquinolone
	pKa [22]	3.2; 11.7		*pKa* [22]	5.97; 9.28
	PNEC [ng L^{-1}] [11]	250		*PNEC [ng L^{-1}]* [11]	500
	Treatment of [20]	Middle ear infections, pneumonia, skin infections, strep throat and urinary tract infections		*Treatment of* [20], [23]	Pneumonia, urinary tract infections, prostatitis, plague, infectious diarrhoea, multidrug resistant tuberculosis and eye infections
	*Name*	Azithromycin		*Name*	Trimethoprim
	Abbreviation	AZI		*Abbreviation*	TRIM
	Antibiotic group	Macrolide		*Antibiotic group*	Diaminopyrimidine
	pKa [22]	8.74		*pKa* [22]	7.12
	PNEC [ng L^{-1}] [11]	250		*PNEC [ng L^{-1}]* [11]	500
	Treatment of [24]	Respiratory, enteric, genitourinary and sexually transmitted infections		*Treatment of* [20]	Bladder infections, middle ear infections and travelery diarrhoea
	*Name*	Ciprofloxacin		*Name*	Sulfamethoxazole
	Abbreviation	CFX		*Abbreviation*	SMO
	Antibiotic group	Fluoroquinolone		*Antibiotic group*	Sulpha drug
	pKa [22]	6.09; 8.74		*pKa* [22]	1.6; 5.7
	PNEC [ng L^{-1}] [11]	64		*PNEC [ng L^{-1}]* [11]	16000
	Treatment of [20]	Bone, joint, intra-abdominal infections, infectious diarrhoea, respiratory and urinary tract infections, skin infections and typhoid fever		*Treatment of* [20], [25]	Urinary tract infections, bronchitis and prostatitis; Often in combination with TRIM: cholera and prevent pneumocystis pneumonia and toxoplasmosis in people with HIV/AIDS
	*Name*	Clarithromycin		*Name*	Doxycycline
	Abbreviation	CLAR		*Abbreviation*	DOX
	Antibiotic group	Macrolide		*Antibiotic group*	Tetracycline
	pKa [22]	8.99		*pKa* [25]	2.93; 7.46
	PNEC [ng L^{-1}] [11]	250		*PNEC [ng L^{-1}]* [11]	2000
	Treatment of [20]	Strep throat, pneumonia, skin infections, H. pylori infection and Lyme disease		*Treatment of* [20]	Bacterial pneumonia, acne, chlamydia infections, early Lyme disease, cholera and syphilis

2.1.2. Antimicrobial Resistance (AMR) and its impact on environment

In 1950ies ABs were added to animal feed to increase the growth rate of livestock and decrease animal diseases. The introduction into the agricultural industry increased the ABs demand up to 80%, ABs which are ingested again by humans when food is consumed [5]. According to Klein *et al.* the total global antibiotic consumption rate increased by 39% from 2000 to 2015 and will increase, if no policy change occurs, up to 200% until 2030 [26]. Eventually, ABs will partly end up in aquatic environment and are therefore, if not separated properly, reintroduced to the ecosystem, as seen in Figure 3.

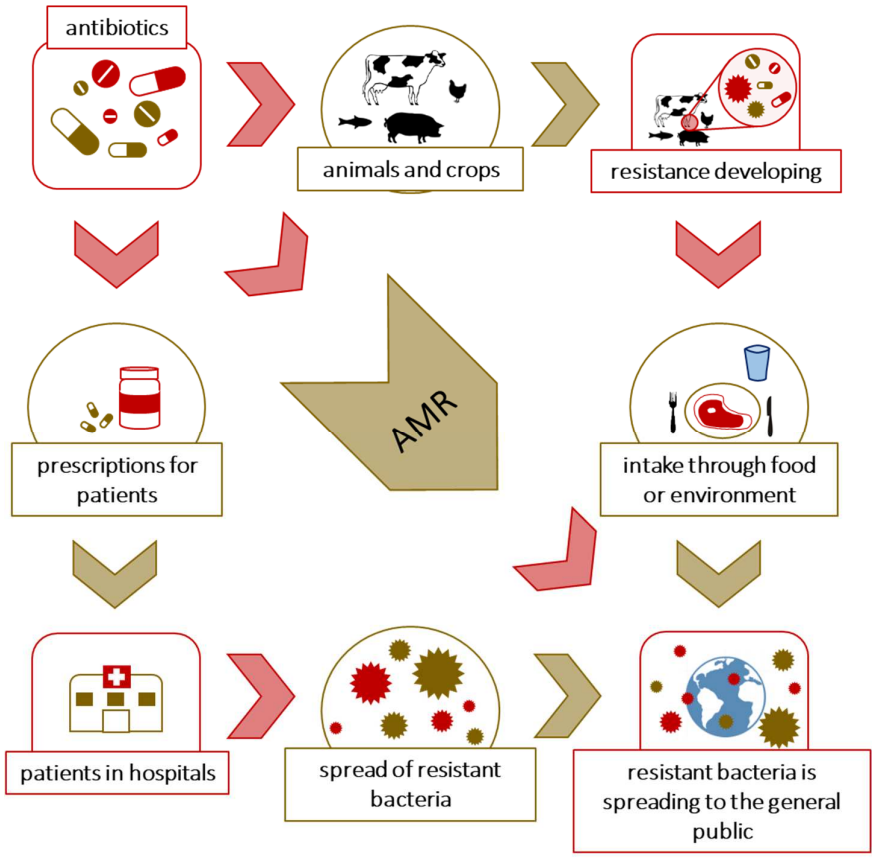


Figure 3: Spreading of antibiotic resistance based on WHO [1]

Due to the wide availability of ABs, an overuse or misuse is often the case and now humankind find themselves in the post-antibiotic era, which started in the end of the 20th century. Through overuse and misuse of ABs microorganisms have started to develop antimicrobial resistances (AMRs). An effect, that was already mentioned by scientists in the 40ies. Even Fleming himself warned about AMR in his Nobel lecture 1945, when he said: *"The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant"* [4].

AMR in general is the ability of a microbe to resist the mechanism of action of one or more antibiotic [27]. It is an evolutionary adaption of a microbe to an antibiotic. AMR follows Darwinism in a way, that some strains of the bacteria survive and adapt to the antibiotic due to genetic trait

exchange among genera and species [21]. As already mentioned in the previous section every antibiotic group owns the ability to tackle specific species. These species therefore develop different strategies and mechanisms of resistance depending on the mechanism of action of the applied antibiotic. In Table 3, the resistance mechanisms of individual ABs are represented.

Table 3: Overview for type of resistances and resistance mechanisms for various antibiotic groups (based on Kapoor *et al*.) [17]

Antibiotic class	**Type of resistance**	**Mechanism**
Aminoglycoside	Decreased uptake	Changes in outer membrane
	Enzymatic modification	Aminoglycoside modifying enzymes
β–lactams	Altered penicillin binding proteins	penicillin binding proteins 2a
	Enzymatic degradation	Penicillinase
Macrolides	Altered target	Methylation of ribosomal active site with reduced binding
	Efflux pumps	*mef* gene type pump
Quinolones	Altered target	Mutation reduce binding to active site(s)
	Efflux	Membrane transporters
Tetracyclines	Efflux	New membrane transporters
	Altered target	Production of proteins, which bind to ribosome and alter the conformation of the active sites
Sulpha drugs	Altered target	Mutation of genes encoding *dihydropteroate synthase* (DHPS)

The evolution of AMR and the lack of development of new ABs or pharmaceuticals in recent years lead to serious consequences. According to the world health organization (WHO), AMRs have damaging effects across the globe, causing an increase of mortality rates for patients with resistant bacteria infections [1]. Global estimations are made that about 214,000 people yearly die due to multidrug-resistant and super drug-resistant tuberculosis (2016) plus 700,000 people in consequences of drug-resistant strains (HIV, malaria etc.) [28], [29].

In order to assess the negative impact of compounds, especially ABs, on the environment researchers introduced estimated limit concentrations:

⇒ minimal inhibitory concentration (MIC),

⇒ minimal selective concentration (MSC), and

⇒ predicted no-effect concentration (PNEC) [11].

Whereas MIC is described as lowest concentration of an antibiotic, which inhibits the visible growth of a microbe (in mg/L), MSC represents the lowest concentration of ABs that gives the resistant strains a competitive advantage on the basis of growth rates [30]. These limits help to determine the percentage of resistance of a particular bacterium and calculate the resistance rates. Since bacteria may develop resistances below MIC and the reliability of MSC is limited because it is obtained from competitive trails between only two closely related strains and not for complex systems, another assessment factor is necessary. PNEC is established for ecological risk assessment [31]. It marks the limit of concentration at which the exposure of the chemical or

compounds starts to have an effect on the environment or ecosystem. The PNEC data used in this thesis are based on Bengtsson-Palme *et al.*, who used EUCAST database for their calculations (European Committee on Antimicrobial Susceptibility Testing) [11]. For deriving the PNEC a flat assessment factor of 1-1000 depending on the type of environment (surface water, marine water, soil, sewage plant etc.) is applied on each size-adjusted "observed lowest MIC" ($MIC_{1\%}$), i.e. 1% of the reported MIC [11].

Especially waste water treatment plants (WWTP) are considered to be the crucial sources of antibiotic and API release into the ecosystems. Effluents showed in 28 out of 100 samples that the antibiotic concentrations exceed the PNECs. As a result WWTP may facilitate a selection of AMR, and an advanced treatment strategy is necessary to avoid accelerated antibiotics-resistance [11], [32], [33].

2.2. Treatment of API contaminated waste water

2.2.1. Municipal waste water treatment plant (WWTP)

The first biological wastewater treatment plant (WWTP) was built in Worchester, Massachusetts 1890. Fortunately, much improvement has been done since then regarding removal of biodegradable organic pollutants, and WWTP are now able to reduce the level of phosphorus concentration to low levels of under 0.1 mg L^{-1} [34]. Nevertheless, an increase of attention regarding micropollutants and their removal is recognizable. Micropollutants consist usually of mineral substances, APIs, pesticides or detergents, which are discharged into the ecosystem at ng L^{-1} concentrations but of increasing environmental impact. Even though advanced treatment steps are developed, the state of the art municipal waste water treatment systems are not designed for removal of micropollutants and antimicrobial resistant genes [34].

Figure 4 shows two types of waste water treatment plants, a standard municipal WWTP (A) and a newly developed plant Nereda® (B), which is named after a water nymph and daughter from Nereus in Greek mythology. Samples for this thesis were collected from these types of WWTP. Nereda® is a technology, which is based on aerobic granulation and a modification of the activated sludge process. It is an alternative to conventional activated sludge treatment plants and was invented by Mark van Loosdrecht of TU Delft, Netherlands [35]. The granular of slow growing organisms are formed during specific process conditions. The Nereda® technology consists of three major cycle components, in particular a simultaneous fill and withdraw, aeration or reaction and settling phase, whereas the commercial WWTP at least consider primary clarification, aeration, final clarification, thickening, anaerobic digestions and in some cases disinfection. According to Pronk *et al.* the new developed system uses 58-63% less energy consumption per year and 33% lower specific volume compared to a commercial activated sludge plant. Given the relatively novelty of the Nereda® system no antibiotic monitoring was yet carried out but nevertheless scientists are forecasting that the system does not solve the problem with micropollutants [36].

Frequently, the occurrence of antibiotic resistant genes (ARG) or strains in WWTP gained more attention. Sczepanowski *et al.* for example identified 140 ARGs and micropollutants especially in activated sludge and final effluent in a WWTP in Germany.

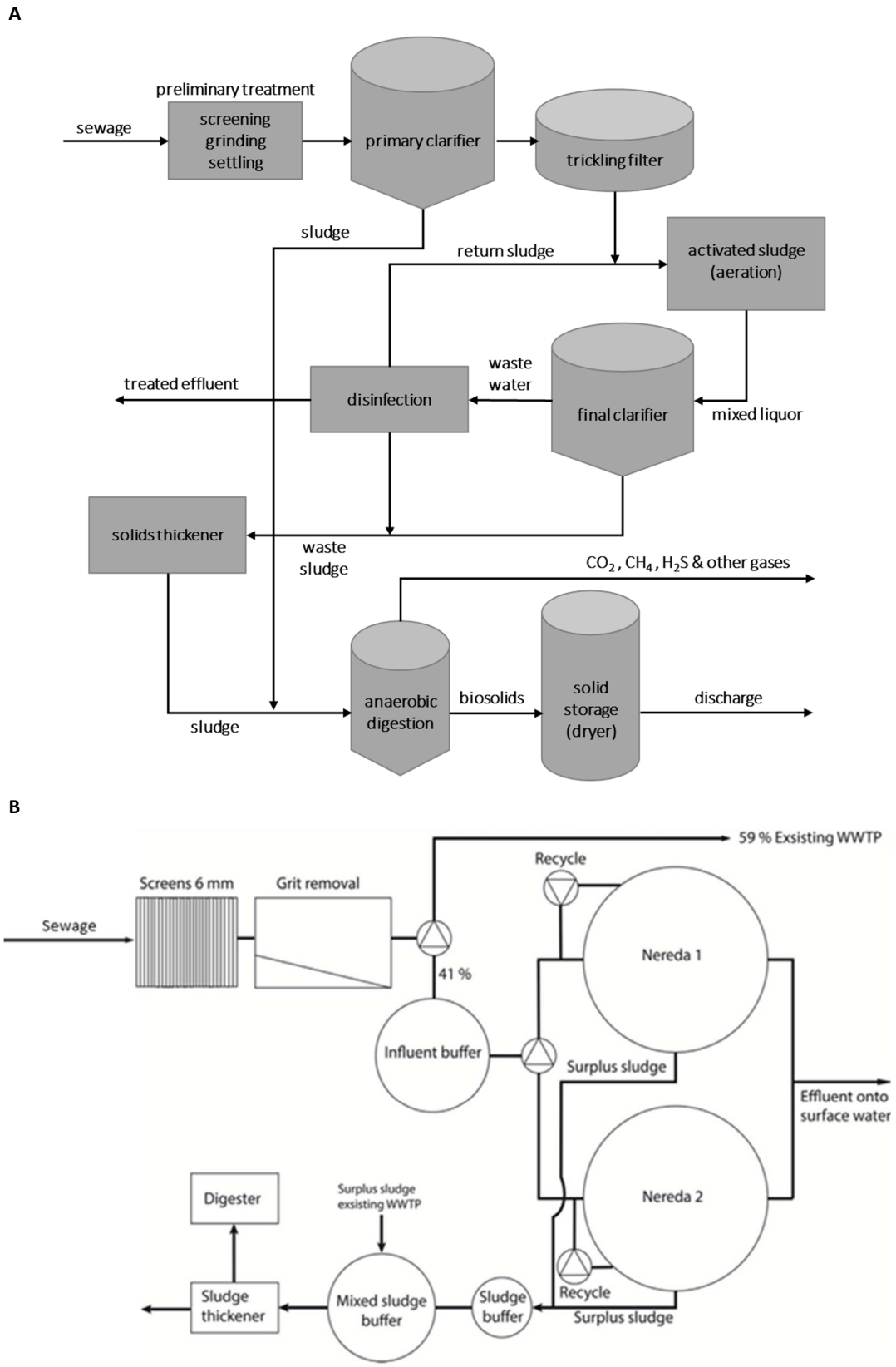


Figure 4: General structure of a municipal waste water treatment plant (A) and a Nereda® plant from Gramerwolde (B) adapted from Pronk *et al.* [35]

Through that survey it was proven that such WWTPs may play an important role in development of multidrug-resistant bacteria [37].

The AMR bacteria and ARG occur mainly in the two main products of WWTPs namely biosolids and effluent discharge. Through the discharge of effluent, the AMR and ARGs are transported into receiving waters like rivers and lakes [38]. Biosolids are usually used for fertilizing plants, because they are nutrient-rich organic residues, which are treated and stabilized. Possible ARG and AMR bacteria in biosolids are then transported through application to plants and soils [34].

The avoidance of AMR developments in WWTP can be the prolonging of the solids retention time (SRT) in the activated sludge process. Through SRT the net growth rate of the whole system is controlled and is one of the key factors influencing the composition of the wastewater microbe composition. Nevertheless, the prolonging of SRT is a balancing act, because although the degradation of ABs is increased by higher SRT, the enhanced exposure of bacteria to ABs may cause an increase in development of AMR [34],[39].

Consequently, the release of AMR bacteria and not degraded ABs through WWTP outputs are suspected to be one of the most important sources and therefore further purification steps are necessary.

2.2.2. Advanced treatment or alternative treatment plants

There are two kind of antibiotic removal: advanced treatment for improving the quality after the secondary clarification or waste water treatments with the aim to replace the conventional activated sludge treatment.

2.2.2.1. Advanced treatment

Advanced treatment is often necessary to destroy harmful bacteria or other toxic micropollutants, which are present after final clarification and to improve the quality of the discharged or reused effluent. These tertiary treatments include sand filtration, adsorption, membrane separation, disinfection with chlorination or UV radiation and advanced oxidation processes (AOPs) [10], [34], [40]. Commercial used disinfection and alternative AOPs will be discussed in more detail.

2.2.2.1.1. Commercial used disinfection

Generally, disinfection as waste water treatment was introduced to protect the microbial water quality. Chlorine is commonly used as disinfection agents, because it is seen as one of the safest and most reliable agents [41], [42]. When chlorine is dissolved in water, it converts to an equilibrium mixture of chlorine and subsequently disassociates in water as followed:

$$Cl_2 + H_2O \rightarrow Cl^- + HOCl + H^+$$

$$HOCl \leftrightarrow H^+ + OCl^-$$

Chlorine attacks the microbe by damaging its cell wall and membrane. Through the cell wall damage the chlorine enters the cell and interrupts the cell respiration and DNA activity, which are necessary for cell survival.

Chlorination is well known to be cost effective and effective in preventing the spread of numerous waterborne diseases in treated water, but scientists are not consistent about a significant reduction of AMR bacteria and ABs [42]. Junsik *et al.* for instance found in their study that impractical high concentrations of chlorine were necessary to remove over 90% AMR bacteria and ARG [43], whereas Destiani *et al*. describes the effectiveness of chlorination against various AMR bacteria (4 to 7-log reduction) [44]. Consequently, it is not eliminating the potential risk of a majority of ABs and is therefore not an effective process to control and monitor discharge of AMR bacteria in treated effluent.

An additional burden of chlorination could be the reaction of chlorine with organic compounds, which occurs naturally. As a result, by-products like trihalomethanes are formed, which are toxic at longer duration of exposure [45]. Due to the crucial drawbacks of chlorination, alternative advanced treatments need to be implemented.

2.2.2.1.2. Alternative advanced oxidation processes (AOPs)

Due to high solubility of ABs oxidative or other transformative processes (like photolysis) are considered more promising rather than sorption-based processes [46]. AOPs are alternative treatment methods, which are usually tertiary water treatment i.e. advanced treatment of the produced effluent of a WWTP. They are designed to remove organic material in wastewater by oxidation with oxidizing agent such as $HO^{\cdot}$. The source of $HO^{\cdot}$ production can be chemical or photochemical. Ozonation, UV/H_2O_2 photolysis and Solar Fenton oxidation are few of the most prominent representatives [34].

Chemical oxidation through ozonation takes place in an aqueous matrix. It involves the degradation of organic contaminants by O_3 and $HO^{\cdot}$ radicals and follows the reaction scheme below.

$$O_3 + OH^- \rightarrow HO_2^- + O_2$$

$$O_3 + HO_2^- \rightarrow O_2 + OH^- + O_2^{-\cdot}$$

$$O_3 + O_2^{\cdot-} \rightarrow O_3^{\cdot-} + O_2$$

$$HO_3^{\cdot} \rightarrow OH^{\cdot} + O_2$$

$$OH^{\cdot} + O_3 \rightarrow HO_2^{\cdot} + O_2$$

Ozone and $HO^{\cdot}$ own a strong oxidation potential and have therefore high oxidative capacities. While O_3 is selectively attacking organic molecules with nucleophilic moieties like carbon double bonds and aromatic rings, $HO^{\cdot}$ is non-selective and attacks organic and inorganic compounds due to hydrogen abstraction, radical reactions and electron transfer [34]. O_3 is very reactive with organic functional groups, like amines and reduces sulphur moieties in cellular membrane of gram-positive and negative bacteria, leading to an effective inactivation of AMR bacteria. In the end, the cell membrane is damaged, and the inner cell becomes exposable for the external environmental condition. Table 4 shows the advantages and drawbacks of ozonation compared to other AOPs.

By UV radiation (absorption at wavelengths of 200-3300nm), the oxygen-oxygen bond of H_2O_2 is cleaved, which leads to a formation of $HO^{\cdot}$ radicals and these radicals contribute to H_2O_2 decomposition by secondary reactions as followed:

$$H_2O_2 + h\nu \rightarrow 2\,HO^{\cdot}$$

$$HO^{\cdot} + H_2O_2 \rightarrow H_2O + HO_2^{\cdot}$$

$$HO_2^{\cdot} + H_2O_2 \rightarrow HO^{\cdot} + H_2O + O_2$$

$$HO^{\cdot} + HO_2^{-} \rightarrow HO_2^{\cdot} + HO^{-}$$

$$2\,HO_2^{\cdot} \rightarrow H_2O_2 + O_2$$

$$HO^{\cdot} + HO_2^{\cdot} \rightarrow H_2O + O_2$$

$$2^{\cdot}OH \rightarrow H_2O_2 + O_2$$

The inactivation of AMR bacteria and ARGs are credited to the combination of UV radiation and the production of radicals from H_2O_2. H_2O_2 penetrates the lipid bilayer in the cell membrane and exposed bacteria undergo high mutation rates, growth defect and death [34]. Due to the combination of UV photolysis and H_2O_2 a non-selective attack on various ABs and AMR bacteria is possible [46].

The Solar Fenton oxidation is a homogeneous process, which is highly efficient and involves a catalytic breakdown of H_2O_2 through a reaction with ferric or ferrous iron (Fe^{3+} or Fe^{2+}). Generally, the reaction takes place in an acidic media in presence of UV-VIS sunlight to produce active species like $HO^{\cdot}$ and it follows reactions below:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{-} + HO^{\cdot}$$

$$Fe(OH)^{2+} + h\nu \rightarrow Fe^{2+} + HO^{\cdot}$$

The combination of the Fenton process (use of H_2O_2 and ferrous salts to form $OH^{\cdot}$, OH^{-} and Fe^{3+}) and solar irradiation lead to destruction, inactivation or degradation of micropollutants through penetration of the cell with the reduced form of hydrogen peroxide. Further information, advantages and drawbacks of this advanced process are also shown in Table 4.

Table 4: Further information of advanced oxidation processes (AOP) for removal of ABs or AMR bacteria in effluent

Oxidation process	Mechanism of action	Advantages	Drawbacks
Ozonation [34], [47], [10]	⇒ ozone (O_3) bonds with unsaturated chains within the membrane-bound phospholipids and lipopolysaccharides ⇒ cell wall/membrane damage ⇒ a permeability increase of cell membrane, leading to leakage of components from the cell and cell lysis to the outside	+ highly efficient (2-log reduction of ARG) + nearly complete elimination of micropollutants, viruses and bacteria + short contact time + no harmful residuals afterwards due to decomposition of O_3	- not for all types of molecules - low dosages my not effective enough - O_3 is a corrosive compound → resistant material necessary - O_3 is irritating and toxic - inner molecular ARG are less affected by O_3 - ARG remain within the cell debris after ARB loss of viability - lack of research regarding the produced $HO^{\cdot}$ - high costs
UV/H_2O_2 photolysis [34], [48], [46]	⇒ through UV radiation and H_2O_2 radicals are produced ⇒ UV radiation interacting with target moieties in bacterial cells by light absorption from chromophores such as the L-tryptophan ⇒ inactivation through photochemical reactions inside the cell ⇒ H_2O_2 has damaged impact in exposed bacteria: high mutation rates, growth defects and deaths	+ acts simultaneously as disinfectant by physically inactivation of microorganisms and photolysis of peroxide + non-selective effectiveness + eliminates the antimicrobial properties of the parent antibiotic	- gap of knowledge about the effect on AMR bacteria or ARG - less suitable for effluents with high turbidity - photosensitized process over the course of high dose wastewater disinfection may create antibacterial active transformation products
Solar Fenton oxidation [34], [49], [50]	⇒ iron and H_2O_2 penetrate cells by affecting the cell metabolism ⇒ after oxidation on the cell wall and membrane, an increase of permeability occurs, which leads to a leak of cellular components into the external environment ⇒ cellular damage due to production of reduced from of hydrogen peroxide ⇒ resulting in oxidation of loose ferrous iron inside the cell producing Fenton oxidation ⇒ through high reaction constants of H_2O_2 and iron, even submicromolar concentrations of H_2O_2 are inactivating the enzymes	+ highly efficient + effective in inactivating cultivable AMR bacteria of various ABs + reduced limit of detection and quantification (LODs and LOQs) + less tedious + eco-friendly technique with capability of degrading a wide spectrum of organic pollutants and micropollutants	- correlation of affected AMR bacteria and ARG concentrations, which are resistant to oxidation treatments are yet to be determined - no research about the effect of solar Fenton oxidation on the functional characteristics on cultivable AMR bacteria in real effluents - formation of large amount of ferrous iron sludge - formation of high concentration of anions in treated effluent

2.2.2.2. Alternative treatment plants

The aim of alternative waste water treatments is to replace the conventional activated sludge treatment to remove ABs and AMR bacteria. Alternative treatment plants include membrane processes such as microfiltration, ultrafiltration, reverse osmosis and processes, which use biological material. Examples for alternative treatment plants are the membrane bioreactor (MBR) and constructed wetlands [34]. Table 5 names the most important facts about membrane the alternative treatment plants and lists the advantages as well as the drawbacks of these treatments, including schematic drawings.

Table 5: Shortly explained information about membrane bioreactors and constructed wetlands with a schematic representation of the treatment as well as their corresponding advantages and drawbacks

Membrane bioreactor [34],[51],[52]
⇒ There are two possible operation modes for membrane bioreactors, namely aerobic (activated sludge) or anaerobic, latter is considered to be more effective due to higher removal rates of ARG, AMR bacteria and ABs.
⇒ Uses various types of biological treatment through the degradation of organic microcontaminants by microorganisms and combines physical separation of the solids through membranes and biological treatment to produce highly clarified effluent.
⇒ One of the main mechanisms behind the removal of ABs, AMR bacteria and ARGs is potentially the membrane filtration of the influent in conjunction with the biological material inside the reactor where the biodegradation processes take place, so called mixed liquor suspended solids (MLSS).
⇒ Another dominant removal mechanism is adsorption prior to filtration through the membrane of some ABs onto the sludge with charged extracellular polymeric substances and soluble microbial products due to their functional groups.
Schematic representation

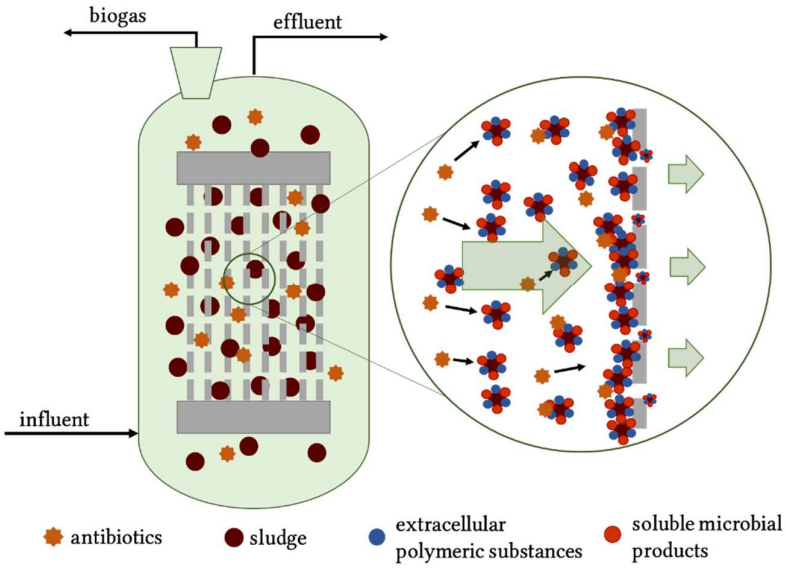


Figure 5: General representation of a membrane bioreactor

Table 5: continued

Advantages	*Drawbacks*
+ high biodegradation capacity + high biodegradation efficiency + low sludge production + low costs + simplicity of construction + solid-free effluent + low net-energy production	- still in development - potential risk of ARG transfer, may leading to AMR in new ecosystems - effect of operating conditions on the removal of AMR bacteria and on total DNA has not yet been examined in literature - impact of SRT, suspended solids, dissolved organic carbon and wastewater colloids on the total bacterial population and on the AMR bacteria has not yet been studied - membrane biofouling
Constructed wetlands [34]**,**[53]	
⇒ Natural waste water treatment, which uses natural functions vegetation, soil and organisms to treat wastewater. Due to high tolerance, Typhas and Phragmites are the most used species. ⇒ It is a semi-aquatic ecosystem and acts as biofilter, which removes more than 70% of suspended solids in incoming sewage water. ⇒ Its function is based on the large variety of microbe communities, which multiply and generate different physical and chemical reactions. ⇒ There are three types of constructed wetlands: a) free surface flow, b) horizontal subsurface flow and c) vertical subsurface flow ⇒ Depending on the type and on the flow pattern, higher removal rates of ARG and ABs are achievable, whereas according to literature surface flow pattern showed the best results	
Schematic representation	

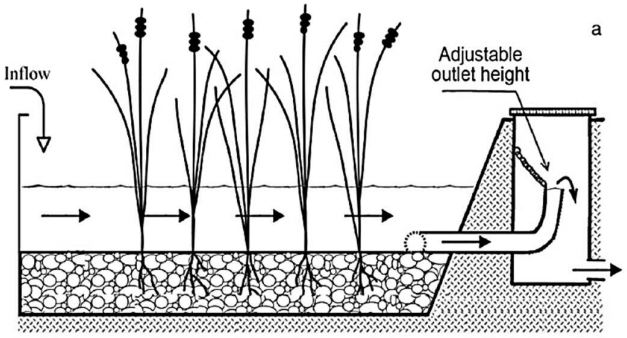


Figure 6: General representation of a surface flow constructed wetland adapted from Wang *et al.* [53]

Advantages	*Drawbacks*
+ high degree of removal of ARGs + easy operation and maintenance + low costs + good potential of water and nutrient reuse + tolerance to high variability + sustainable	- problems in cold climate - strongly depended of internal and external environment conditions - more targeted and optimized approach for removal of AMR bacteria and antibiotic necessary - high land are requirements - no standard design

2.3. Sample preparation techniques and analysis

Due to the increase of usage of antibiotics (AB), data concerning their behaviour, concentration and fate in the environment are urgently necessary. Prior to understanding how to separate ABs from wastewater effectively to avoid development of AMR, it is necessary to monitor and measure these compounds in different parts of the water cycle. As already discussed, some AMRs are developed by bacteria at very low AB concentrations and thus sensitive and robust analytical methods and appropriate sample preparation techniques are needed to analyse ABs at very low concentration in difficult matrixes, such as effluent and sewage sludge [15]. Every sample follows the analytical path depicted in Figure 7.

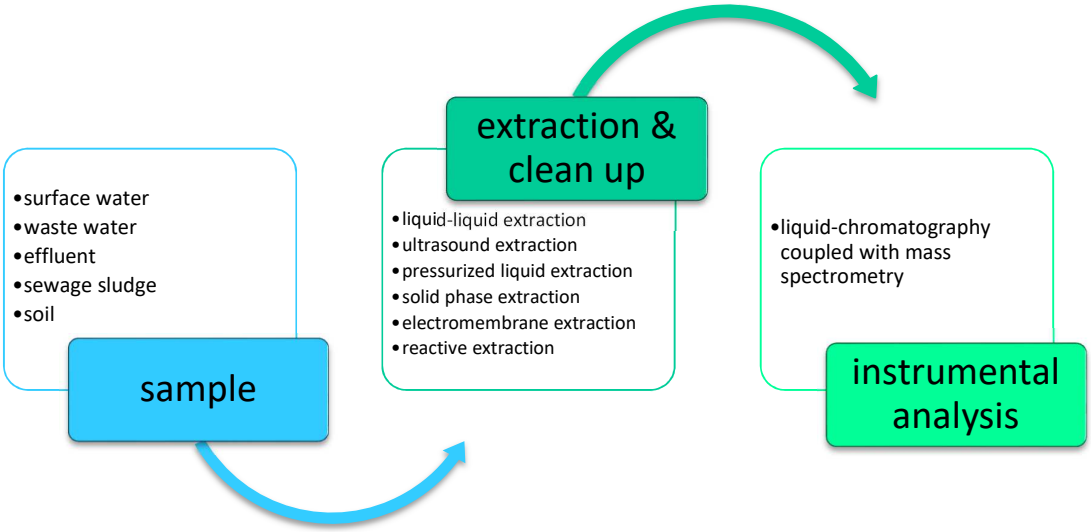


Figure 7: General analytical path of a sample from sample preparation to instrumental analysis

Over last few decades a lot of new sample preparation techniques and analytical methods have been developed and modified. This following section is focusing on the sample preparation techniques, which are used in this thesis, as well as on the LC-MS analytical method, especially the theoretical principles behind the methods is discussed briefly.

2.3.1. Most commonly used sample preparation techniques

2.3.1.1. Direct method - Wetsus standard analysis sample preparation

Wetsus standard analysis sample preparation is in general a dilution method for water samples, such as surface water, effluent or wastewater, preferably lower polluted ones. It is a rather simple preparation technique, where the ABs are analysed directly with only a few preparation steps:

1. modification of the sample with ethylenediaminetetraacetate (EDTA) in ammonium formate
2. shaking through vortex or ultrasound
3. centrifugation
4. supernatant is subsequently analysed.

According to Kim *et al.* EDTA is commonly added to remove metal ions or other compounds by chelation. In the case of the method used by Wetsus laboratory, the modifier is also added to

stabilize the pH and break bonds of the ABs to various compounds in the matrix like sediments and particles. EDTA has to be used with caution, because to high concentrations of the chelating agent can clock the electrospray ionization and suppresses the signal intensity during analysis with LC-MS.

The major advantage of this method is the simplicity, in a strict sense it is only a liquid extraction of ABs and the dissolved ABs are subsequently measured by LC-MS/MS. Another advantage is the broad spectrum of measurable ABs whereas all other techniques imply a certain loss of compounds, e.g. SPE works for polar OR non-polar compounds, but not for both types at the same time. Unfortunately, the achievable limits of quantification are rather high and for some ABs above the PNEC. Latter is especially for surface water and lower contaminated sample a significant drawback. For higher contaminated samples such as sludge and soil, more modifications are necessary. The detailed procedure of the Wetsus standard analysis sample preparation can be found in Appendix I.

2.3.1.2. Solid Phase Extraction (SPE) as clean-up

SPE is widely used as a sample-preparation technique and became more popular in the early 1980s, when disposable cartridges containing silica based chemically bonded sorbents were introduced into sample processing [54]. In general, SPE is used as a purification or clean-up step after extraction, where dissolved or suspended compounds are separated from a sample matrix according to their chemical and physical characteristics [55]. SPE is also commonly used to concentrate samples, due to the fact that the analytes of interest can be isolated from different kind of samples (usual liquid), such as blood, water and soil. To sum it up, the main goals of the SPE are the analyte enrichment, matrix simplification and medium exchange, i.e. transfer of the analyte from the sample matrix to another solvent [54].

For water samples mainly SPE cartridges are used, which consist of a short column (open syringe barrel) packed with absorbent material such as porous polymers or metals. The sample is transported through the solid phase with a nominal particle size of 50–60 µm where adsorption and absorption of desired analytes or undesired impurities takes place depending on the type of SPE [54]. There are different types of SPE, depending on the extraction mechanism as well as sorbents, and they have to be distinguished according to Figure 8.

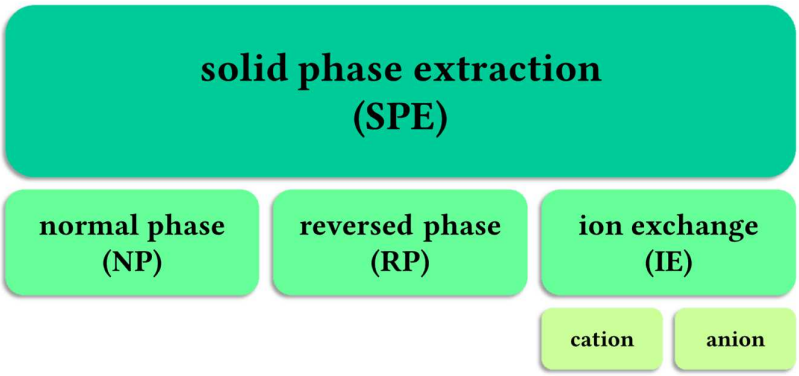


Figure 8: Classification of the solid phase extraction types

Depending on the analyte and on the sample itself the appropriate SPE mechanism and cartridge is chosen. Figure 9 gives further information about the analyte interactions and the bonding mechanism of the different SPE types.

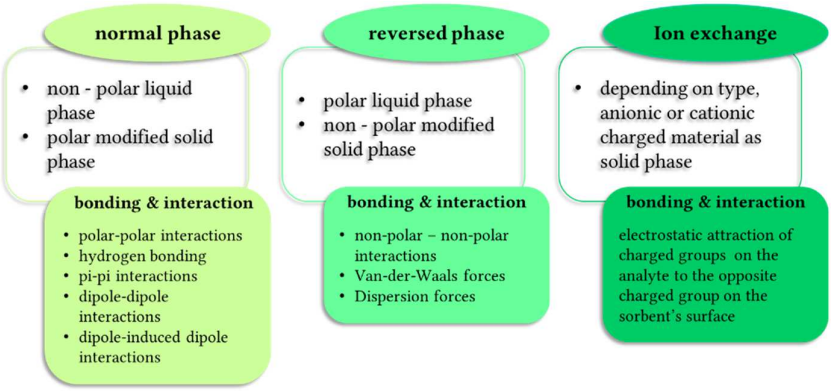


Figure 9: Interaction and bonding mechanism of normal phase, reversed phase and ion exchange

In this thesis RP-SPE is chosen to be tested for surface water and effluent. According to the "method selection guide for the isolation of organic compounds from solution" by Colin F. Poole published in 2003, RP-SPE is an appropriate approach to separate ABs from aqueous neutral solutions [54]. One popular representative of a RP-SPE cartridge is the Oasis™ HLB (hydrophilic lipophilic balance), which are specially produced for water samples. Due to the high pH value stable lipophilic divinylbenzene and hydrophilic N-vinylpyrrolidone units, they are the most frequently used cartridges according to literature to extract or separate fluoroquinolones, tetracyclines and sulphonamides, macrolides and trimethoprim from wastewater samples or water [15], [55]–[57].

The mechanism in RP-SPE is based on the interaction between non-polar groups of the desired analytes and the non-polar functional groups on the sorbent through Van-der-Waals or dispersion forces. Solvents for RP-SPE are usually polar or have very little non-polar character, whereas the eluent is more non-polar to break the bond between the analyte and the sorbent [15]. The necessary steps for a RP-SPE are shown in Figure 10.

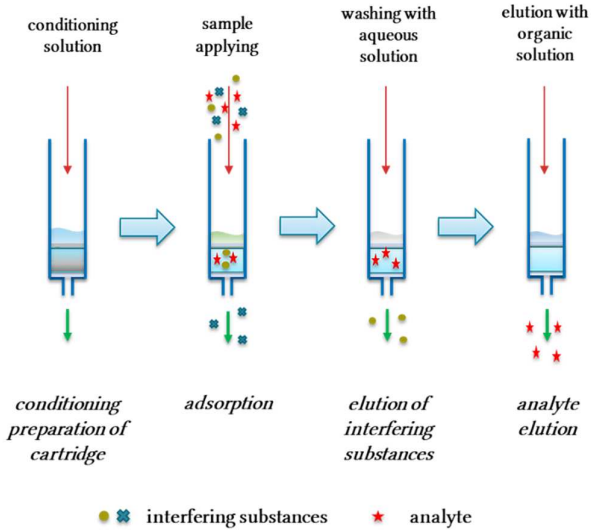


Figure 10: Basic steps of a RP-SPE: conditioning, adsorption, washing and elution of analyte

One major advantage of SPE is that an online coupling with LC-MS is possible. Camilleri *et al*., for instance, used a fully automated online SPE—HPLC-MS/MS to quantify pharmaceutical compounds at trace level in surface water with recoveries between 85-100% [58]. Even though

the automation of SPE is not convenient for all analytes and reconditioning of the cartridges is not easy, it reduces the sample preparation steps, preparation time and increases the sample throughput [59]. More advantages and drawbacks of SPE in general are listed in Table 6.

Table 6: List of advantages and drawbacks of solid-phase extraction as sample preparation according to Andrade-Eiroa *et al.* [59]

Advantages	Drawbacks
+ extraction of a wide range of organic analytes from non-polar to very polar analytes from a large variety of samples (food, environmental samples etc.)	- for higher polluted samples preparation steps prior to SPE are necessary
+ high extraction efficiency of organic compounds	- oversaturation of sorbents with at high numbers and concentration of analytes
+ high enrichment factors	- high cost due to single use
+ high reproducibility	- more solvent consumption due to more steps
+ increased selectivity	- time consuming and cumbersome
+ different operation types	
+ simultaneous extraction of a bigger number of compounds possible	

Another coupling route is a tandem SPE method to avoid interference of organic matter within the HLB cartridge. The tandem SPE set-up consists of a strong anion exchange (SAX) cartridge prior to the HLB cartridge. It is especially implemented for higher polluted sample types such as sewage sludge, high contaminated waste water and soil. The advantage of this method is the removal of natural organic matter (NOM), which in general is negatively charged due to high concentrations of humic and fulvic acids. Therefore the capacity of active binding centres in the sample is reduced and the analytes are easier detectable [60]. Due to complexity of each target antibiotic group and their different interactions with the sorbent, an elution of all analytes through the SAX cartridge is nearly impossible. Different pre-cartridges have to be used to overcome that effect and to measure all target ABs in one sample, which would lead to a higher cost per sample. A possible cheaper alternative to the SAX-HLB tandem method would be a Strata-X™ cartridge, which is tested in this thesis [61].

2.3.2. Unconventional or experimental sample preparation techniques

2.3.2.1. Reactive extraction (RE)

In general, reactive extraction is a type of liquid-liquid extraction. Its principles are based on the reactions of the desired transfer analytes with the (organic) reactive extractants. It is a combination of reaction and separation, where the reaction improves the separation substantially through enhancement of mass transfer or separation through higher reaction conversions [62]. After RE into the organic phase the product is consequently back extracted into an aqueous solution with different characteristics (higher pH, different buffer etc.) according to Figure 11.

Figure 11: General representation of a reactive extraction process

In literature, RE is often used to extract β-lactam antibiotics form dilute solutions with Aliquat 336 (example for phase transfer catalyst, see structure in Figure 12) in an organic carrier as organic phase in hollow fibres or specific glass cells [63]–[65]. Pai *et al*., for instance, used the reactive extraction to recover Penicillin G more efficiently compared to physical extraction. Pencillin G is therefore extracted by interfacial reactions at a higher pH of 6-8, where it is more stable and not decomposing, using amine extractants dissolved in an organic carrier [66].

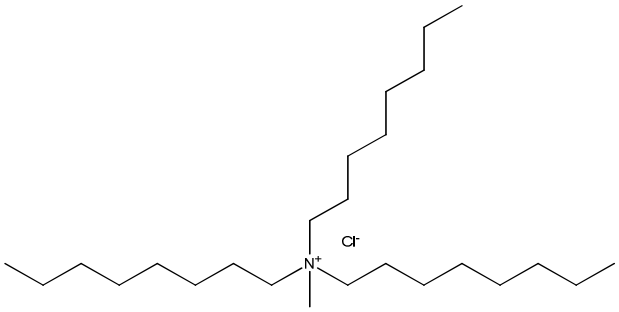


Figure 12: Structure of Aliquat 336 as an example for a phase transfer catalyst in reactive extraction

The advantages of RE include [67], [68]:

- ⇒ flexible process parameters
- ⇒ selective extraction
- ⇒ with use of hollow fibres or supported membranes no phase separation is needed
- ⇒ low energy consumption
- ⇒ large specific surface areas if supported membranes are used
- ⇒ continuous process is possible due to simultaneous extraction and back-extraction

One of its main drawbacks however is that the RE system is rather complex, and depending on the compound to extract, the process within the extraction is difficult to explain as well as to control completely [67]. Another drawback of this method is that the commonly used complexing agents or organic solutions are usually environmentally toxic [67].

The reactive extraction has not yet been tested for other ABs as well as its application as sample preparation technique in analytical chemistry, like in this thesis, is completely new and experimental.

2.3.2.2. Electromembrane extraction (EME)

Due to environmental aspects of the commonly used SPE as sample preparation technique, more alternative techniques are developed including electromembrane extraction (EME). Since the introduction of EME by Pedersen-Bjergaard *et al*. in 2006, the scientific interest in EME as a sample preparation is steadily increasing [69]. In basic terms, EME is an extraction of charged compounds from an aqueous sample (donor) through a supported liquid membrane (SLM) to an acceptor solution driven by an electrical field between two electrodes [70]. Typical examples of set-up types for EME are shown in Figure 13.

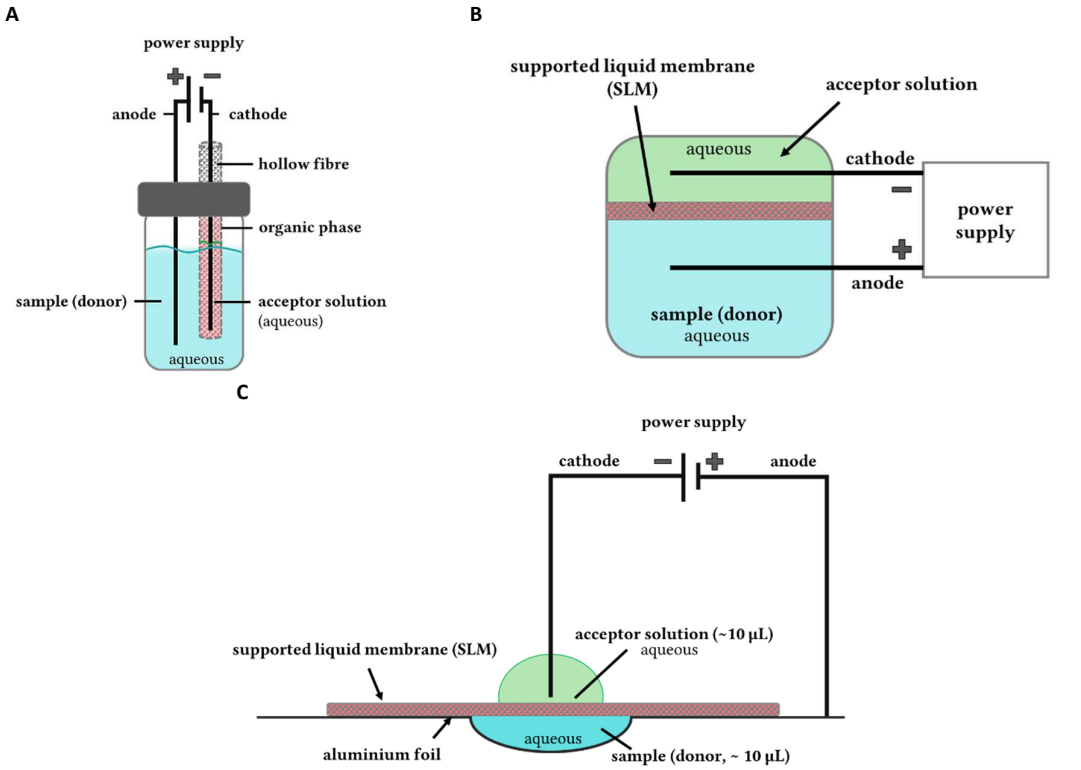


Figure 13: Examples for different types of EME: A) porous hollow fibre with immobilized organic phase set-up; B) flat sheet porous supported liquid membrane (SLM) with organic carrier set-up; and C) drop-to-drop EME set-up

An EME set-up consist in general of a donor with an adjusted pH to charge the desired analytes, a SLM such as flat porous membranes or hollow fibres, which are filled with an organic solution and an acceptor solution. The electrodes are placed in the donor and acceptor according to the desired analytes. For cations, the cathode is placed in the acceptor solution and the anode is submerged in the donor, whereas the reversed polarity is applied for anions. Afterwards a current across the electrodes is applied and the charged analytes are transported/extracted from donor to acceptor through the SLM [70].

An important role in EME plays the adjustment of pH and appropriate composition of the SLM. The pH condition in the donor has to ensure efficient ionization of the target analytes in order to ease their migration in the electrical field. Additionally, the pH in the acceptor solution is a critical parameter during EME as well and should maintain the protonated state of the target analytes [71]. As carrier for the SLM different options are possible (see Figure 13) and SLM is selected according to target analyte [70]. Next to pH and SLM composition, the extraction current is also an important issue in EME. To avoid excessive electrolysis, the current should be kept as low as possible. According to Pedersen-Bjergaard *et al.*, high current leads to a major decrease of recovery and reproducability, especially in complex matrixes like plasma and urine [71].

To describe the performance of an EME the enrichment factor (EF) and extraction recovery (ER) of an analyte *i* is introduced and is calculated as followed [72]:

$$EF_i = \frac{c_{acceptor_i}}{c_{donor_i}} \; and \; ER_i = \frac{c_{acceptor_i}}{c_{donor_i}} \times \frac{V_{acceptor}}{V_{donor}} \times 100\%$$

$c_{acceptor,i}$ & $c_{donor,i}$ ..... concentrations of the analyte *i* in the acceptor and in the donor solution

$V_{acceptor}$ & V_{donor} .....volume of the acceptor and the donor solution

Table 7 : List of advantages and drawbacks of electromembrane extraction (EME) as sample preparation [70]–[74]

Advantages	Drawbacks
+ efficient sample clean-up	- limited extraction recovery
+ in theory high extraction rates possible	- highly dependent on pH and thickness of the membrane
+ no transport of undesired matrix components, such as sediments and particles	- highly dependent on the ion balance
+ direct injection of the acceptor solution into the analytical instrument (LC-MS)	- still in development
+ pre-concentration during process	- no standard EME equipment available
+ green chemistry approach due to less required solvents	- back-extraction of components possible if acceptor is saturated
+ possibility for coupling with analytical instruments	
+ possibility for miniaturization (on-chip EME, drop-to-drop EME)	
+ flexibility due to controlling by external power supply and SLM	

EME can also be applied in environmental sample preparation. Ramos-Payán *et al.*, for instance, tested various organic solutions for SLM and optimized the pH of the system to extract fluoroquinolones from waste water samples. In the end, a semi-successful extraction was possible by usage of 1-octanol as SLM in a porous polypropylene hollow fibre and fixed pH of 4 in the donor and pH 2 in the acceptor at 40 V [73]. Still some development is necessary to implement EME as a reliable sample preparation technique and a list of its advantages as well as its drawbacks is presented in Table 7. To overcome its drawbacks, issues regarding different SLMs, pH influence, extraction of larger biomolecules, back-extraction reactions of analytes and commercially available set-ups have to be addressed [71].

In this thesis, the EME method developed by Ramos-Payán *et al.* is modified and applied for the first time on more difficult matrixes, such as highly polluted waste water and sludge samples. The purpose of the newly modified EME technique is subsequently to extract as much ABs from different classes as possible simultaneously.

2.3.3. Liquid chromatography – mass spectrometry as analytical method

Due to the increase of API residues and antibiotic residues in the environment, analytical methods are required to analyse these contaminations at low concentrations, especially in aqueous and semi-solid samples, such as waste water and sludge samples. In recent years, several analytical methods for detection of pharmaceuticals are described in literature, whereas gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are reported most frequently [75]. Although lower LoQs are achievable with GC-MS, an additional derivatization of acidic compounds after the main sample preparation is required due to poor volatility or thermal stability of APIs. This additional step complicates the sample preparation and takes not only more time but also reduces the versatility of the GC-MS. To overcome this drawback, LC-MS and LC-tandem MS are introduced to analyse complex compounds. Over the last decades, LC-MS experiences a major progress in technology and application. Especially, more dimensional LC-MS (LC-MS/MS), such as LC coupled with triple quadrupole (QQQ) MS, are the choice of analysis for polar APIs and their metabolites in environmental samples [75].

In this thesis, a LC-MS/MS is applied after sample preparation and a schematical representation of the used instrument can be found in Figure 14. The LC consists of a degasser, and two pumps, in particular an isocratic pump, which is responsible for the transportation of the two mobile phases, and a binary pump, which is in charge of mixing and composition of the mobile phase. The binary pump is followed by a cooled autosampler and the temperature controlled column compartment. Through LC, the sample is separated into its different analytes in a reversed phase column using a non-polar stationary phase with a polar mobile phase. The analytes are separated according to their interactions with the stationary phase and the mobile phase. Through the separation the detectability of analytes is improved, and ion suppression can be reduced. For the analysis of APIs and ABs, the mobile phase is usually modified with an addition of formic acid or oxalic acid to a lower pH in order to improve the sensitivity of the MS detection [76].

After separation with LC, the compounds reach the electrospray ionization (ESI) where the liquid stream is passed through a metal capillary with a high voltage of 3-4 kV and large droplets are produced. During the solvent evaporation, the droplets are dispersed into highly positively or negatively charged ions (depending on the ESI mode), such as $[M + H]^+$ or $[M - H]^-$; which are further transported to the QQQ [75], [76].

In general, the QQQ is a mass spectrometer consisting of three in series coupled quadrupoles. The first set of quadrupoles oversees fragmentation and selection of the molecular ions, whereas the second one is used as collision cell where the molecular ions collide with an inert collision gas and for transporting the product ions into the third quadrupole set. Finally, the third quadrupole selects the fragments resulting from the collisions. Viewed individually, a quadrupole consists of two pairs of rods (metallic) and a voltage (radiofrequency -RF and direct-current -DC) is applied between the opposite rod pairs. At specific voltages, ions with particular *m/z* (mass-to-charge ratio) travel along the rods within a specific trajectory to reach the collision cell or in the case of the third quadrupole the detector. The QQQ it is able to identify similar eluting compounds or molecules with alike mass due to different fragmentation patterns, which is called multiple reaction monitoring (MRM) [76].

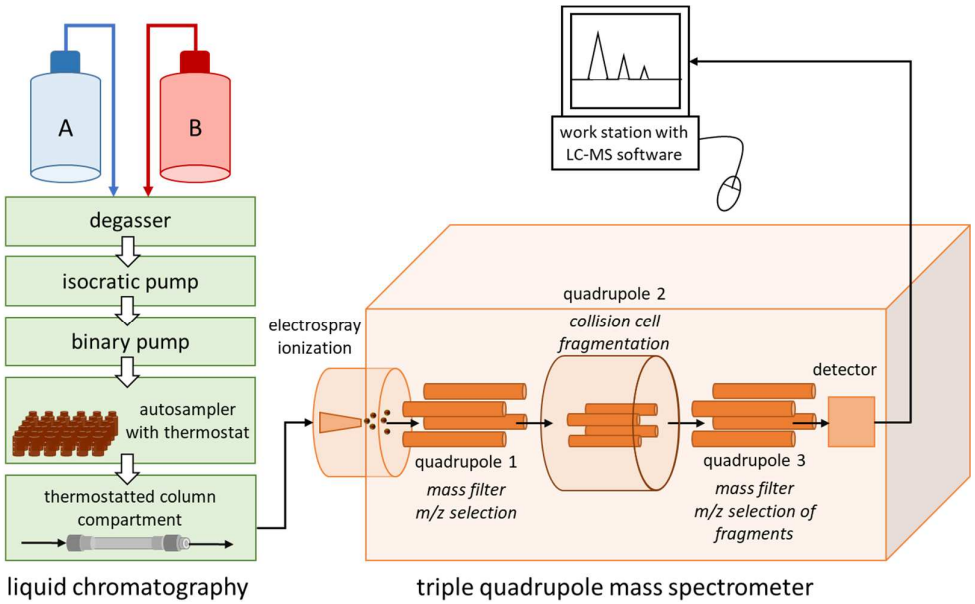


Figure 14: Schematic illustration of a LC-QQQ-MS used in this thesis

For analysis of APIs and especially ABs, LC-MS/MS is a very suitable method. It is the main applied analysis for ABs in environmental samples. One major drawback of LC-MS/MS (especially with ESI) are matrix effects. Matrix effects occur mainly due to co-eluted matrix components, which result in signal/ion suppression or enhancements of the analyte. In general, ion suppression occurs due to presence of polar/ionic interfering compounds, which are competing with the analytes for ionization. For quantification procedures, there are two possibilities how matrix effects may have an influence, firstly a loss of absolute sensitivity of some target analytes or secondly the accuracy and precision of the determination can be affected [76].

Due to matrix effects, purification and sample preparation are necessary prior to LC-MS/MS analysis. Vosough *et al.* proved that with direct analysis without purification or concentration of the sample only high detection limits are achievable and in some cases even above the PNEC [77]. Whereas, Gros *et al.* developed an offline SPE-LC-MS/MS method in 2006, where water samples were purified by SPE prior to LC-MS/MS measurements and ABs such as TRIM and OFX, were detectable with recoveries between 75 and 100% and low limits of quantification are achievable depending on the sample type [78]. Nevertheless, LC-MS/MS is still state of the art for antibiotic analysis and in future more development regarding coupling and sensitivity can be expected. For completeness, further advantages and drawbacks are summarized in Table 8.

Table 8: Advantages and drawbacks of LC-MS/MS as analytical instrument [75], [76], [78]

Advantages	**Drawbacks**
+ high selectivity	- high instrument costs
+ high sensitivity for target molecules	- sample clean-up and purification are necessary
+ high throughput	- matrix effects
+ good signal-to-noise ration allowing low limits of quantification	
+ reliable identification due to MRM (compared to single MS)	
+ wide linear range of quantification	
+ high accuracy and reproducibility at low concentrations	
+ wide range of application	

3. Experimental part

Overall six different sample preparation techniques were applied on various samples, which were subsequently measured by LC-MS. In this chapter the practical application of these techniques and the settings for the LC-MS are discussed. As mentioned above every technique has its limits, and therefore they were carried out for different sample types and different conditions to get an overview, which sample treatment is successfully applicable for a specific sample type. Table 9 summarizes the used samples, the sample type and the preparation techniques tested on the individual samples.

Table 9: Analysed samples listed according to their sample type and the applied preparation techniques.

Sample name	Sample type	Preparation techniques*
SW3 20170226	Surface water (National reserve)	RP-SPE
SW2 20170226	Surface water	RP-SPE
SW2 20170206	Surface water	RP-SPE
SW1 20170228	Surface water	RP-SPE
SW2 20170117	Surface water	RP-SPE, RE
GARMER - A 20170328	Effluent	RP-SPE
GARMER - B 20170328	Effluent	RP-SPE
GARMER - TOTAL 20170328	Effluent	RP-SPE
GARMER - NEREDA 20170328	Effluent	RP-SPE
LEEUWARDEN 20170307	Effluent	RP-SPE
DELFT 20170307	Effluent	RP-SPE
LEEUWARDEN 20170404	Effluent	RP-SPE
HARNASCHPOLDER 20170405	Effluent	RP-SPE
GARMERWOLDE TOTAL 20170420	Effluent	RP-SPE
GARMERWOLDE NEREDA 20170420	Effluent	RP-SPE
H 20170207	Hospital WW[#]	RP-SPE, PRE, W-SA, MD
N 20170228	Nursing home WW[#]	RP-SPE, PRE, W-SA, RE, EME
C 20170207	Community home WW[#]	W-SA, MD, EME
Stage A 20170727	Sludge	MD, extended MD, EME
Stage B 20170727	Sludge	MD, extended MD, EME
Digest 20170727	Sludge	MD, extended MD, EME
Granular 20170727	Sludge	MD, extended MD, EME
Delft digest 20170808	Sludge	MD, EME
Delft activated 20170808	Sludge	MD, EME
Leeuwarden digest 20170809	Sludge	MD, EME
Leeuwarden activated 20170809	Sludge	MD, EME

* RP-SPE: Reversed phase – solid phase extraction
PRE: Precipitation followed by RP-SPE
W-SA: Wetsus standard analysis sample preparation; simple dilution preparation
MD: modified dilution preparation
Extended MD: modified dilution preparation with changes in dilution fraction or pH
RE: reactive extraction
EME: electromembrane extraction

[#] WW: waste water

The samples were provided by Wetsus. Effluent and sludge samples were taken at Leeuwarden waste water treatment plant (WWTP), Delft WWTP and Garmerwolde Nereda WWTP, whereas the surface water, as well as the community, nursing and hospital waste water (WW) samples were taken in and around Sneek (SW1/2 = Sneek; SW3 = National reserve “de Deele”). All samples were stored at ≤ 4°C prior to processing. The used material and standards are stated separately for each technique and the CAS numbers can be found in Appendix II.

3.1. Instrumentation - Analytical method and settings for LC-MS

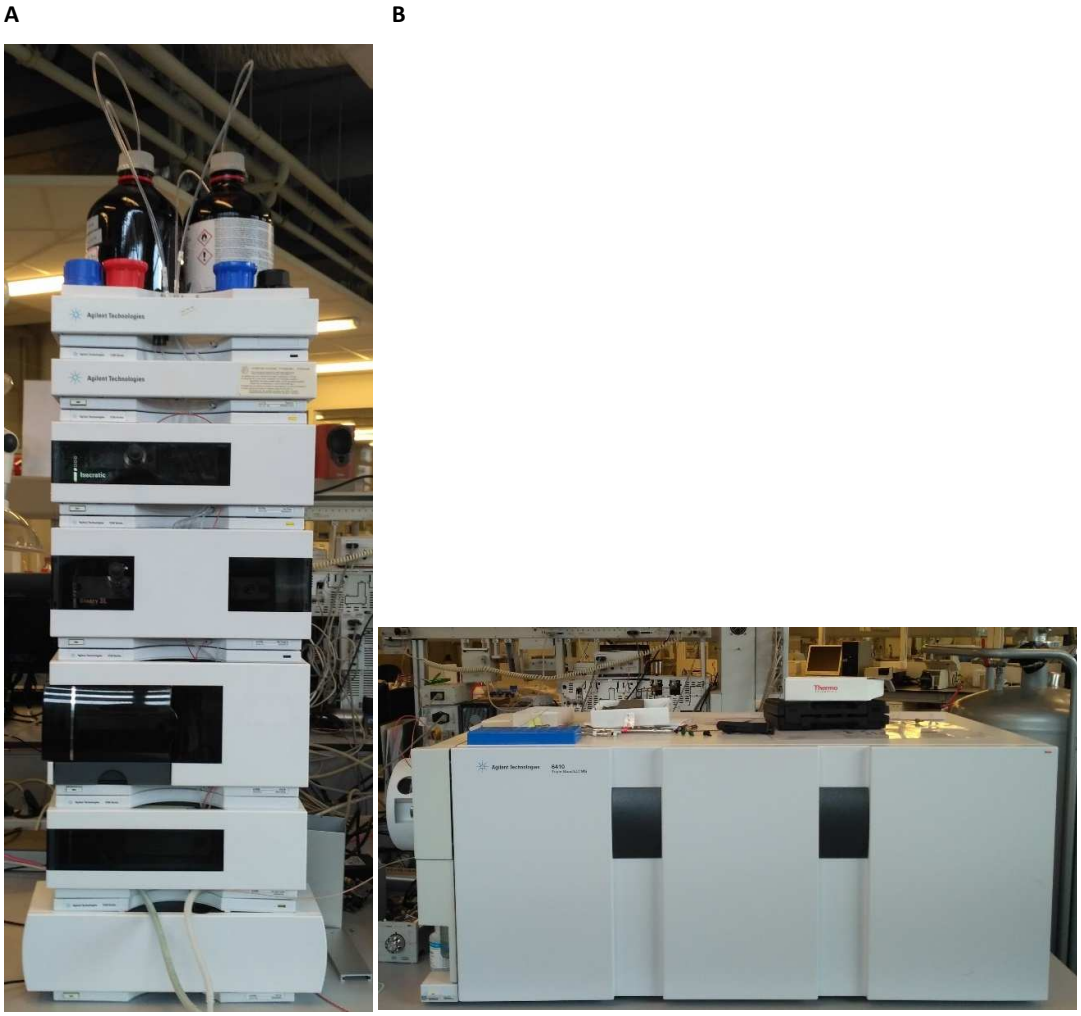


Figure 15: Agilent Technologies 6410 Triple Quadrupole LC-MS/MS; A: liquid chromatography B: triple quadrupole mass spectrometer

To analyse the samples prepared with RP-SPE, precipitation and reactive extraction, an Agilent 6410 Series Triple Quadrupole LC-MS was used and is shown in Figure 15. The LC consisted of an isocratic and a binary pump, an SL with degasser, a solvent tray and an automated sampler with a sample tray cooled to 6 °C. The compounds were separated on a Phenomenex Gemini C6-Phenyl 110 A column (150x3 mm; 5 µmm, type 00F-444-Y). The column temperature was set to 40 °C and the flow rate to 0,6 mL min^{-1} with a maximum pressure of 500 bar. A gradient elution (settings shown in Table 10) with two different solvents was used: solvent A was the aqueous eluent consisted of 0.05% oxalic acid or 0.05% heptafluorobutyric acid in ultrapure water and solvent B

was the organic eluent and consisted of 0.1% formic acid in acetonitrile. Of each sample 5 µL were injected, unless stated otherwise. LC run time was ten minutes for each analysis. The MS consisted of an electrospray ionization source and the compounds were ionized in positive mode using compound specific transitions. Data evaluation was performed with MassHunter LC-MS software.

For analysis of the samples prepared with the dilution methods and the electromembrane extraction, the LC-MS/MS was equipped with a ZORBAX RRHD eclipse plus 95 A C 18 column (2,1 x 50 mm; 1,8 µm, type 959757-902) operated at 45 °C. The instrumental settings were the same as mentioned above, except the flow rate was set to 0.5 mL min^{-1} instead of 0.6 mL min^{-1}. The gradient elution conditions are shown in Table 10.

Table 10: Gradient conditions for the LC-MS/MS measurements according to different sample preparation techniques

RP-SPE, precipitation, reactive extraction		**Dilution methods, electromembrane extraction**	
Time [min]	Organic eluent B [%]	Time [min]	Organic eluent B [%]
0.0	10	0.0	6
0.5	10	1.0	70
4.0	90	4.5	80
5.0	90	5.0	90
5.5	10	5.5	6
10.0	10	10.0	6

A total ion chromatogram (TIC) of a calibration standard STD-320 is represented in Figure 16. The standards used in this thesis contain next to ABs also human markers, like sucralose and gabapentin, and disinfectants such as benzalkonium-chloride. A TIC sums up intensities of all mass spectral peaks belonging to the same scan and therefore it includes not only the compound peaks but also background noise.

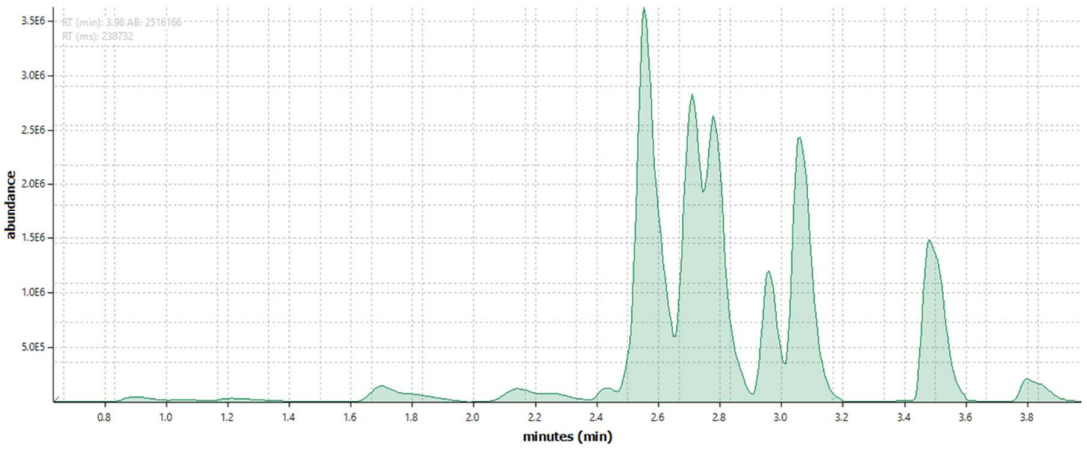


Figure 16: Example total ion chromatogram (TIC) of a STD-320 measured with an Agilent LC-MS 6410 system with a ZORBAX RRHD Eclipse Plus 95Å C18 column

As it is impossible to generate results from the TIC, the multiple reaction monitoring (MRM) mode is used for quantification of the ABs. Table 11 shows the used MRM parameter settings for the measurements with an Agilent LC-MS/MS 6410 Series system.

Table 11: Used multiple reaction monitoring (MRM) mode parameters and settings for LC-MS analysis

Abbreviations	Compound	Segment	Transition [M/z]		Fragmentor	Collision Energy
			Precursor Ion	Product Ion	[V]	[V]
ATd7	Azithromycin-D7	2	274.0 ->	145.0	125	19
CFXd8	Ciprofloxacin-D8	2	340.0 ->	322.0	130	17
DIA	Diaveridine	2	261.2 ->	245.2	155	16
SUDOXd3	Sulfadoxin-D3	2	314.1 ->	156.0	120	17
TRIMd9	Trimethoprim-D9	2	300.0 ->	264.0	145	26
AMOX	Amoxicillin	2	366.1 ->	349.1	100	3
AMP	Ampicillin	2	350.1 ->	106.0	100	22
AZI	Azithromycin	2	749.5 ->	158.1	100	30
BaC12	Benzalkonium chloride 12	2	304.3 ->	91.0	140	32
BaC14	Benzalkonium chloride 14	2	332.3 ->	91.0	140	35
CHEX	Chlorhexidine	2	505.2 ->	336.2	150	15
CFT	Cefotaxime	2	456.2 ->	396.2	110	4
CFX	Ciprofloxacin	2	332.1 ->	314.1	160	20
CLAR	Clarithromycin	2	748.5 ->	158.1	150	28
CM	Clindamycin	2	425.3 ->	126.1	110	30
DM	Dimetridazole	2	141.9 ->	96.2	100	28
DOX	Doxycycline	2	445.2 ->	428.0	150	23
ERYT	Erythromycin	2	734.5 ->	158.1	165	30
FLUMEQ	Flumequine	2	262.2 ->	244.0	100	15
GAPE	Gabapentin	2	172.3 ->	154.2	85	11
LINCOM	Lincomycin	2	407.8 ->	126.1	140	30
OFX	Ofloxacin	2	362.3 ->	318.2	120	16
OTETR	Oxytetracycline	2	461.2 ->	426.1	120	19
PENG	Penicillin G	2	335.2 ->	217.0	180	12
PENV	Penicillin V	2	351.2 ->	229.0	180	14
SMO	Sulfamethoxazole	2	253.9 ->	156.1	100	13
SUCLOP	Sulfachloropyridazine	2	284.9 ->	156.0	100	13
SUCRAL	D-Sucralose	2	414.0 ->	199.0	85	9
SUDOX	Sulfadoxine	2	311.1 ->	155.9	120	17
SULFAM	Sulfamethazine	2	279.0 ->	186.0	120	16
SULPYR	Sulfapyridine	2	250.1 ->	156.0	90	11
TETR	Tetracycline	2	445.2 ->	410.2	130	18
TILMIC	Tilmicosin	2	869.5 ->	174.2	280	54
TRIM	Trimethoprim	2	291.1 ->	261.1	140	24
TYLOS	Tylosin	2	916.5 ->	173.6	240	36

Based on the TIC in Figure 16 and the transitions described in Table 11, various ion transitions of the STD-320 are represented separately in Figure 17A-C. For a better overview, the transitions are divided in three different chromatograms according to their abundance or counts and different choice of axis scale.

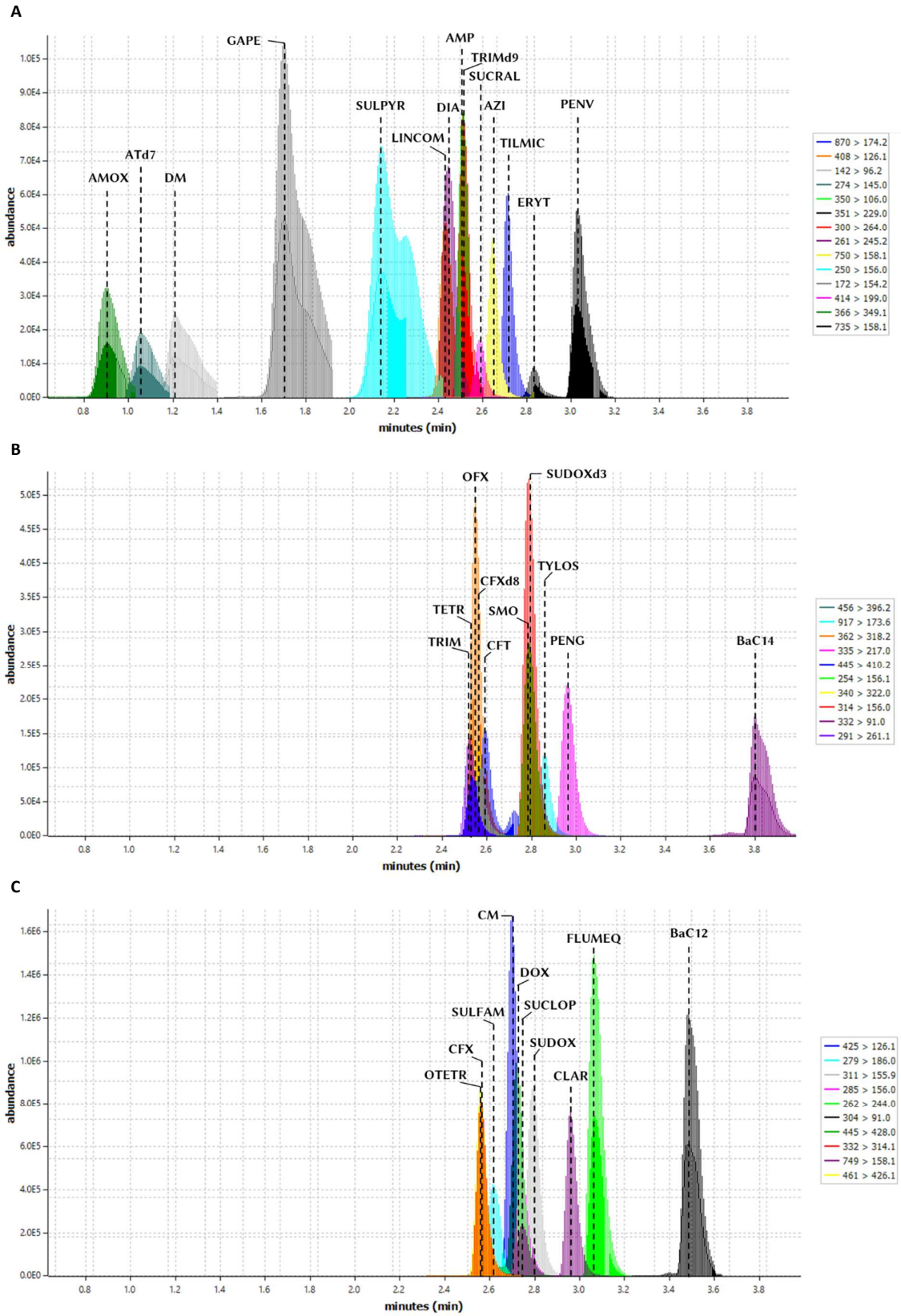


Figure 17: Example of chromatograms for various ion transitions of a STD-320 measured with an Agilent LC-MS 6410 system with a ZORBAX RRHD Eclipse Plus 95Å C18 column. A: transitions with an abundance below $1.5x10^4$; B: transitions with an abundance between $5.5x10^4$ and $5.5x10^5$; C: transitions with an abundance between $2.0x10^5$ and $1.6x10^6$

3.1.1. Calibration

For quantification of the data, simple internal standard calibrations were performed. It is an effective approach to assess the loss of signal intensity and it is simple and not as time consuming as matrix-matched calibration or standard addition. With an internal standard calibration, the instrumental response of a target compound in the unknown sample can be compared to the response from the internal standards added to the sample. Internal standard was added in the same amount to each calibration standard. Every preparation technique has its own standard and therefore different concentrations in their calibration points. The calibration standards for the different preparation methods with their approximate AB concentrations are listed in Table 12.

Table 12: Calibration standards for all preparation methods with approximate antibiotic (AB) concentration in µg L^{-1}

Calibration Standard	RP-SPE & Precipitation AB [µg L^{-1}]	Wetsus Std. Analysis AB [µg L^{-1}]	Reactive Extraction AB [µg L^{-1}]	Modified Dilution* AB [µg L^{-1}]	Electromembrane Extraction* AB [µg L^{-1}]
STD-000	0.0	0.0	0.0	0.0	0.0
STD-020	~2.6	~2.6	~5.0	~2.7	~2.7
STD-040	~5.2	~5.2	~10.0	~5.5	~5.5
STD-080	~10.4	~10.4	~19.9	~11.0	~11.0
STD-160	~20.8	~20.8	~39.8	~21.9	~21.9
STD-320	~41.7	~41.7	~79.6	~43.9	~43.9
STD-640	~83.3	-	-	-	-

To determine the recovery of the ABs the samples were spiked with specified antibiotic concentrations. For sample spiking the same standards (STD) were used as for the calibration standards, except for modified dilution (MD) and electromembrane extraction (EME). These techniques consume more sample volume and therefore require a higher concentration of STD.

The calibration standards are prepared with the various Wetsus standard-mixes (depending on the preparation method; see following chapters), internal standards, matrix modifier (ultrapure water with 0.1 M Na_2EDTA and 1.0 M ammonium formate) and ultrapure water in 1.5 mL silanized LC-MS vials, according to Table 13.

Table 13: General composition of calibration standards

Calibration standard	Wetsus standard [µL]	Internal standard [µL]	Matrix modifier [µL]	Ultrapure water [µL]
STD-000	0	50	50	900
STD-020	20	50	50	880
STD-040	40	50	50	860
STD-080	80	50	50	820
STD-160	160	50	50	740
STD-320	320	50	50	580
STD-640	640	50	50	260

3.2. Commonly used sample preparation techniques

3.2.1. Reversed phase – solid phase extraction (RP-SPE)

3.2.1.1. Materials

Strata™-X, 33 µm Polymeric Reversed Phase, 60 mg / 1 mL (Phenomenex 8B-S100-UAK) disodium ethylenediaminetetraacetate dihydrate (Na_2EDTA; Sigma Aldrich), Methanol (≥ 99,8%, HiPerSolv CHROMANORM for HPLC/LCMS, VWR), Formic Acid (≥ 96.0%, ACS reagent, Sigma Aldrich),

ultrapure water (Millipore Milli-Q®), reconstruction solution consisting of 0.05 g ammonium acetate (≥ 98%, Sigma Aldrich), 5.0 mL Formic Acid (99.9%, HiPerSolv CHROMANORM for LC/MS, VWR) and 0.5 mL 5M ammonia solution (≥ 25% AnalaR NORMAPUR, VWR), organic solution mix consists of methanol : acetonitrile (99.8%, Sigma Aldrich) in a 1:1 v/v ratio and 1 v% formic acid, Wetsus-Standard STD-155 and Wetsus Internal Standard STD-148 (see Table 14)

Table 14: AB standard for RP-SPE experiments, standard concentrations and dilution concentrations in µg L^{-1}

Wetsus-STD	Compound	Standard concentration [µg L^{-1}]	Dilution concentration [µg L^{-1}]
STD-155	CHEX	5208	130.2
	ACSUL	5213	130.3
	SUCRAL	5208	130.2
	TRIM	5203	130.1
	SMO	5211	130.3
	CFX	5218	130.5
	CLAR	5203	130.1
	BaC12/14	5216	130.4
	GAPE	5207	130.2
	CM	5211	130.3
	DM	5216	130.4
	ERYT	5211	130.3
	AZI	5238	130.9
	OFX	5219	130.5
STD-148	TRIM-D9	5187	228.0
	DIA	4559	227.9
	ATd7	4557	227.9
	CFXd8	4562	227.7

3.2.1.2. Method

To prepare the sample, 2 mL of Na_2EDTA solution (1 mg L^{-1} in ultrapure water) and 1 mL organic solution mix and 200 µL of formic acid were each added to two Greiner tubes containing 50 mL of sample. The solutions were subsequently vortexed for 8 minutes at 1700 rpm. From these solutions, respectively 8 mL were taken and transferred into twelve 15 mL Greiner tubes, where the samples were spiked with three different standard solution concentration steps according to Table 15. For obtaining statistical data, the procedure was carried out in triplicate.

Table 15: Different spike levels of STD-155 and added standard concentration in µg L^{-1}

Sample name	Added STD-155 [µL]	Concentration in the sample after added standard [µg L^{-1}]
Blank	-	-
Spike 1	5	0.081
Spike 2	10	0.162
Spike 3	25	0.406

To activate a cartridge before the actual extraction of the samples can take place, the cartridges were washed with 750 µL of organic solution mix and with 750 µL ultrapure water. Both liquids should elute slowly, to give the material enough time to swell and equilibrate. Afterwards, the samples were filtered by applying a vacuum of 10-15 mmHg (13- 20 mbar). After filtering, the samples tubes were rinsed with ultrapure water three times, to ensure increased absorption in the filter material. A vacuum of 27 mbar was applied until visible dryness of the material.

Two times 400 µL of the organic solution mix per cartridge was used to elute analytes into 2 mL Eppendorf tubes. The organic mixture was evaporated via heated nitrogen stream and the residue

was re-dissolved in 190 µL reconstruction solution and 10 µL of internal standard (STD 148). 150 µL of the reconstructed solution was transferred into a micro vial and measured via LC-MS/MS.

3.2.2. Precipitation of higher contaminated samples with $CuSO_4$ and $Al_2(O_4)_3$

3.2.2.1. Materials

Strata™-X, 33 µm Polymeric Reversed Phase, 60 mg / 1 mL (Phenomenex 8B-S100-UAK), Disodium ethylenediaminetetraacetate dihydrate (Na_2EDTA, Sigma Aldrich), Formic Acid (> 96.0%, Sigma Aldrich), ultrapure water (Millipore Milli-Q®), reconstruction solution and organic solution mix (see Materials in chapter 3.2.1), precipitation solution consisting of 0.2 M aluminium sulphate 14 hydrate (GPR RECTAPUR, VWR) and 0.2 M Copper(II) sulphate pentahydrate (AnalaR NORMAPUR, VWR), Wetsus-Standard STD-155 and Wetsus Internal Standard STD-148 (see Table 14).

3.2.2.2. Method

For the preparation, 1 mL ultrapure water, 1 mL organic solution mix and 600 µL of precipitation solution were added to an aliquot of 5 mL high contaminated sample (hospital water) in 15mL Greiner tubes. For each sample run triplicates of the blank and of the spiked sample (5, 35 and 125µL of diluted STD-155) were performed. After mixing, the samples were vortexed for seven minutes at 1700 rpm and 10 minutes centrifuged at 3250 rpm. After decantation of the sample solutions into new Greiner tubes 3 mL Na_2EDTA solution (1 mg L^{-1} in ultrapure water), 3 mL of ultrapure water and 75 µL of formic acid were added and again vortexed for 7 minutes at the same conditions.

The preparation of the cartridges and the ab- and desorption procedure was carried out as described in the method section 3.2.1.2. The reconstructed samples with added internal standard (IS) were measured with LC-MS/MS.

3.3. Unconventional sample preparation techniques

3.3.1. Modified dilution methods

These methods are based on the standard sample preparation technique for water samples of Wetsus (Appendix I) modified by changing the pH and the dilution grade.

3.3.1.1. Dilution experiments on sludge samples with a solid content of ≤ 5%

3.3.1.1.1. Materials

“SUPERMOD” modifier consisting of 15 mL NH_3 (5M dilution from ≥ 25% ammonia solution, AnalaR NORMAPUR VWR), 50 mL oxalic acid (2M dilution from ≥98% oxalic dihydrate, Alta Aesar), 15 mL disodium ethylene diamine tetraacetic acid (≥97%, Sigma Aldrich), 7.5 mL formic acid (99%, for LC-MS VWR), 12.5 mL ultrapure water (Millipore Milli-Q®) methanol (≥ 99.9%, HiPerSolv CHROMANORM for HPLC/LCMS grade, VWR) and organic solution mix (see 3.2.1.1), Wetsus standard-mix STD-159 and internal standard-mix STD-148 with concentrations according to Table 16.

Table 16: AB standards for dilutions experiments, with standard concentrations and dilution concentration in µg L^{-1}

Wetsus-STD	Compounds	Standard concentration [µg L-1]	Dilution concentration [µg L-1]
STD-159	BaCl	9863	1644
	CFX	9867	1645
	CLAR	9874	1646
	OFLX	9872	1645
	TRIM	9891	1649
	TETR	9882	1647
	DOX	9882	1647
	AZI	9874	1646
	SMO	9875	1646
	CM	9865	1644
	DM	9854	1642
	GAPE	9873	1645
	TRC	9879	1646
	OTETR	9880	1647
	SUDOX	9880	1647
	PENV	9870	1645
	SUCLOP	9871	1645
	SULFAM	9877	1646
	PENG	9868	1645
	TILMIC	9869	1645
	FLUMEQ	9874	1646
	NEOMYC	9868	1645
	SPECTI	9866	1644
	ACSUL	9860	1643
	SUCRAL	9866	1644
	CHEX	9866	1644
	COLIST	9859	1643
	LINCOM	9869	1645
	TYLOS	9868	1645
	AMOX	9865	1644
	AMP	9870	1645
	CFT	9871	1645
	ERYT	9860	1643
	SULPYR	9881	1647
STD-148	TRIM-d_9	4517	1882
	DIA	4515	1881
	ATL-d_7	4515	1881
	CFX-d_8	4512	1880
	SUDOX-d_3	4516	1882

3.3.1.1.2. Method

For the experiments with sludge samples, the recovery was investigated in dependency of different factors such as pH changes and different dilutions. The basic process of the sample treatment remains unchanged and was carried out according to the procedure shown in Figure 18. Samples with a solid content of ≤ 5% were treated as a fluid.

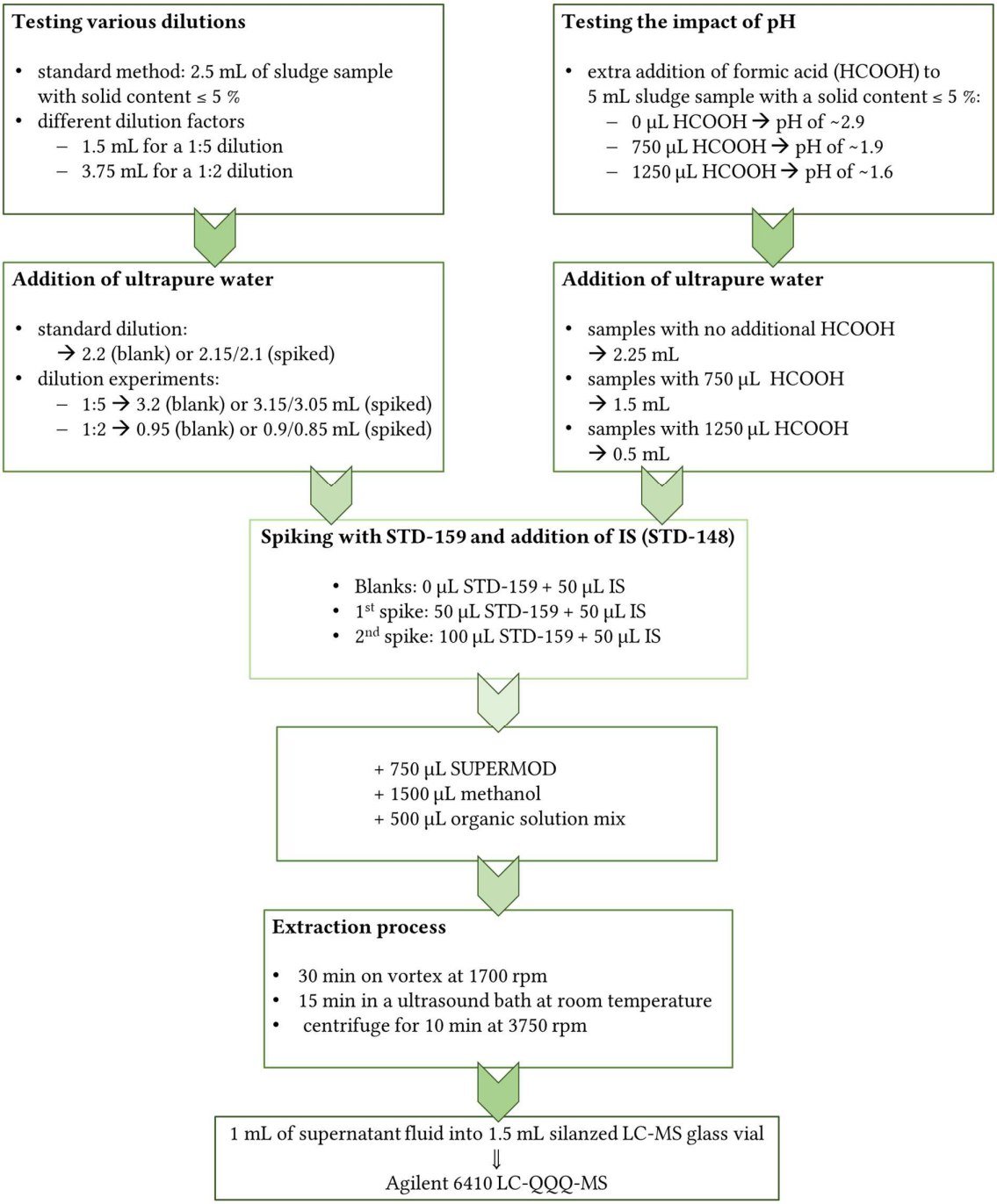


Figure 18: Flow diagram visualizing the experimental approach for handling sludge samples with a solid content of ≤ 5%

3.3.1.2. Dilution experiments for semi solid samples with a solid content of ≥ 5%

3.3.1.2.1. Materials

"SUPERMOD" modifier prepared as mentioned in materials section 3.3.1.1, methanol (≥99.9%, HiPerSolv CHROMANORM for HPLC/LCMS grade, VWR) and organic solution mix prepared as in previous chapters, Wetsus standard-mix STD-155 and internal standard-mix STD-148 according to Table 16.

3.3.1.2.2. Method

Sludge with a solids content of ≥ 5% cannot be pipetted, and therefore the water content was determined with a simple drying process to get the real solids content. This was then used to calculate the results back to the dry weight. The water content of digested sludge is estimated around 94.5% and for granular sludge 95.0%. For the dilution experiments, the same investigation of the recovery as in chapter 3.3.1.1.2 was carried out. The basic process of the sample treatment remains unchanged and was performed according to Figure 19.

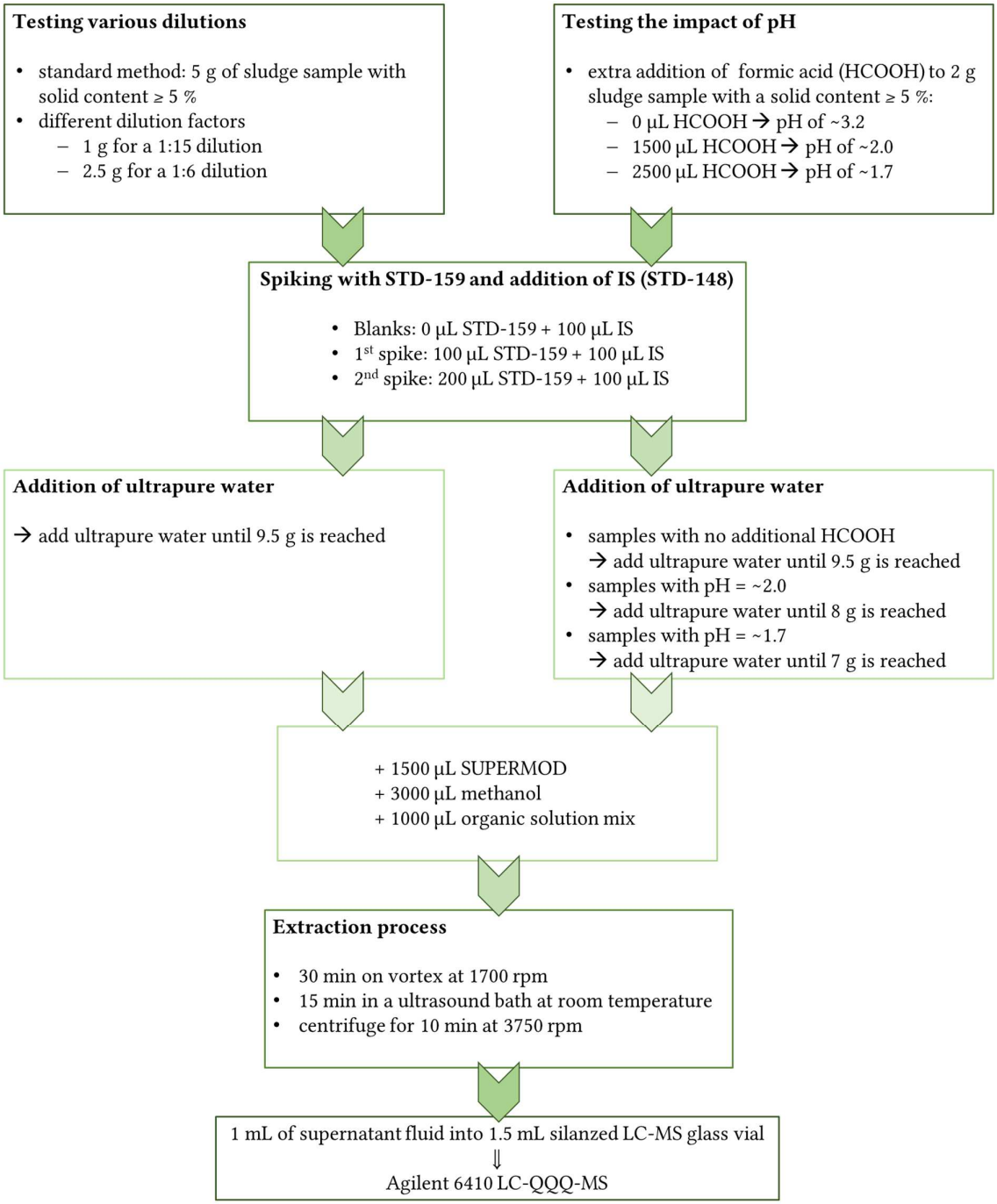


Figure 19: Flow diagram visualizing the experimental approach for handling sludge samples with a solid content of ≥ 5%

3.3.2. Reactive extraction

3.3.2.1. Materials

Citrate buffer (pH 3.99) consisting of 0.1 M citric acid monohydrate (99.5%, BOOM BV) and 0.1 M tri-sodium citrate dihydrate (≥99.9%, Normapur VWR), phosphate buffer (pH 8.06) consisting of 0.1 M sodium hydrogen phosphate (100%, GPR Rectapur VWR) and 0.1 M tri-sodium phosphate dodecahydrate (min. 96%, Technical VWR), as organic solution a mixture of 5.00 wt% Aliquat® 336 (tricaprylylmethylammonium chloride, Sigma Aldrich), 2.5 wt% 1-decanol (≥99%, for synthesis Merck KGaA) and 92.5 wt% n-butyl acetate (≥99%, EMPURA Merck KGaA), reconstruction solution (see section 3.2.1.), Wetsus standard-mix STD-155, STD-158 and internal standard-mix STD 148 with concentrations according to Table 17.

Table 17: AB standard for reactive extraction, with standard concentrations and dilution concentration in µg L^{-1}

Wetsus-STD		Compounds	Standard concentration [µg L^{-1}]	Dilution concentration [µg L^{-1}]
STD-MIX	STD-155	CHEX	5208	248.8
		ASULF	5213	249.1
		SUCRAL	5208	248.8
		TRIM	5203	248.6
		SMO	5211	249.0
		CFX	5218	249.3
		CLAR	5203	248.6
		BAC	5216	249.2
		TRC	5207	248.8
		GAPE	5211	249.0
		CM	5216	249.2
		DM	5211	249.0
		ERYT	5238	250.3
		AZI	5219	249.4
		OFX	5187	247.8
	STD-158	AMOX	10489	250.6
		AMP	10203	243.7
		CLAV	10671	254.9
		MEROP	10383	248.0
		GENTA	10558	252.2
		CFT	10490	250.6
	STD-148	TRIM-D_9	4559	379.9
		DIA	4557	379.8
		ATL-D_7	4557	379.8
		CFX-D_8	4555	379.6

3.3.2.2. Method

An array of experiments was performed modifying different parameters such as buffer pH, composition of organic solution and others. However, it would go beyond the scope to describe all trials in detail. Therefore, the focus of this chapter will lie on the preparation of a nursing home waste water sample.

In Figure 20 the scheme of a reactive extraction process on a nursing home waste water sample is shown. For each sample-set blank samples, samples spiked with 15 µL STD- MIX and with 125 µL STD were measured in triplicates. Prior to analysis with LC-MS, 190 µL stripping solution after re-extraction was mixed with 10 µL internal standard and transferred into a 1.5 mL silanized LC-MS vial.

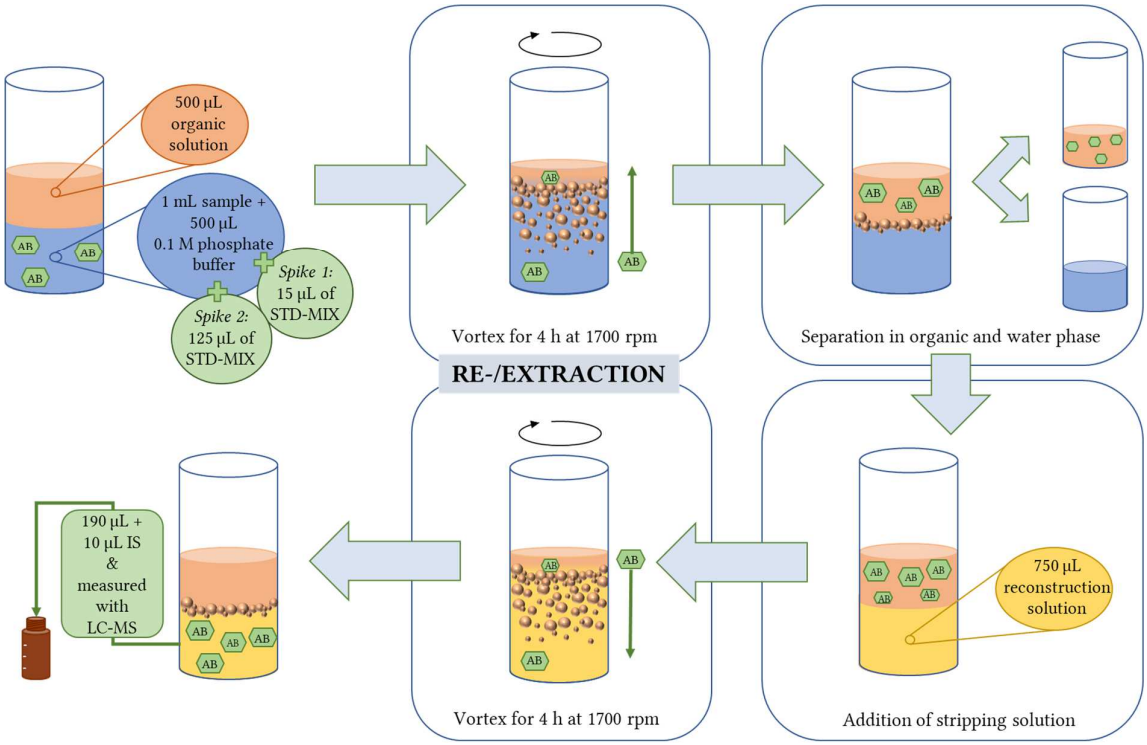


Figure 20: Process of a reactive extraction shown for the preparation treatment of a nursing home sample

3.3.3. Electromembrane extraction (EME)

3.3.3.1. Materials

18 mL Nalgene™ LDPE sample vials with closure (6250-0018), polypropylene hollow fibres with an inner diameter of 1.8 mm, a wall thickness of 450 µm and a nominal pore size of 0.2 µm (3 M), Consort bvba EV1450 electrophoresis power supply (400 V 50 Watts 500 mA, 4-channel), two platinum filaments reused from old pH electrodes for cathode and anode, "SUPERMOD" modifier prepared as mentioned in previous chapter. 1-octanol (EMPLURA®. Merck), formic acid (≥ 96% ACS reagent, Sigma Aldrich), N_2-stream, acetone (≥ 97%, Sigma Aldrich), pH 2 acetate buffer Wetsus standard-mix STD-155 and internal standard-mix STD-148 according to Table 16.

3.3.3.2. Method

The electromembrane extraction is carried out for various samples such as hospital, nursing and community wastewater as well as for sludge samples (≥ 5% and ≤ 5% solid content). Depending on the sample type 1.5 g (or 1.5 mL) of semi-solid sludge was diluted with ultrapure water to a final weight of 10 g or 10 mL of wastewater were used and transferred into an 18 mL LDPE sample vial. Afterwards 25 µL internal standard diluted STD-148 and 25 µL standard diluted STD-159 were added. To ensure that the ABs are more easily ionized 1 mL SUPERMOD were added and the samples were then treated with N_2 gas stream, to be sure that no soluble CO_2 is disturbing the measurement.

Afterwards 100 µL of formic acid was added to the sample solution (donor). It was slightly stirred and an aliquot of 1 mL of the donor solution was taken to measure the concentration prior to the extraction process. The hollow fibre was cut into 12-15 cm pieces, washed in an acetone bath and

kept under ultrasound for 15 minutes. After drying at room temperature, the fibre was immersed with 1-octanol for 8-10 seconds, then removed from the solution and the excess was removed with a clean tissue. Subsequently, the soaked hollow fibre was filled with a pH 2 acetate buffer and placed into the donor in the sample vial. In this case, the buffer acted as an acceptor, into which the negative electrode was placed, whereas the positive electrode was placed in the donor solution. In Figure 21, a general scheme of the developed and used EME cell is presented.

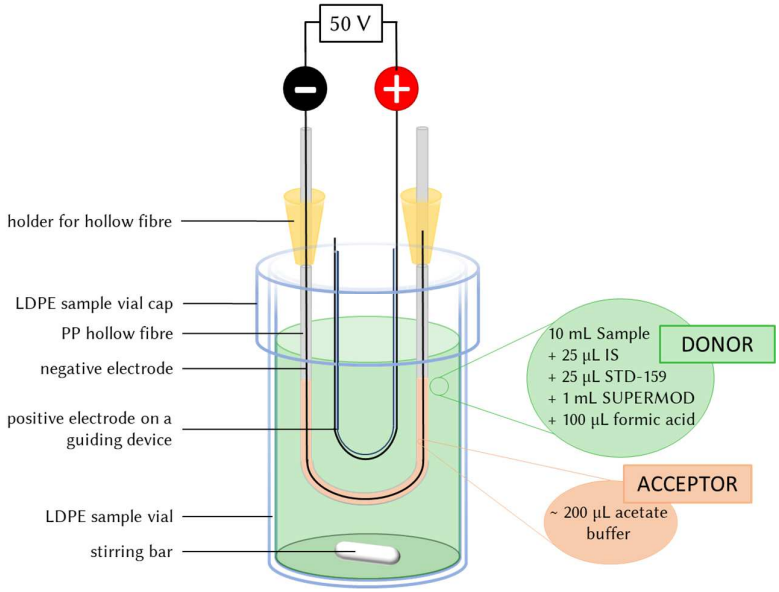


Figure 21: General scheme of an electromembrane extraction cell, used for extraction of samples with a volume of 10 mL

To determine the influence of time on the enrichment factor, extractions of a community waste water samples were performed for 30 minutes, as well as for 40 min at 50 V. In Figure 22, no significant difference in the enrichment factor was detectable and therefore 30 min as sufficient extraction time period was selected for further experiments.

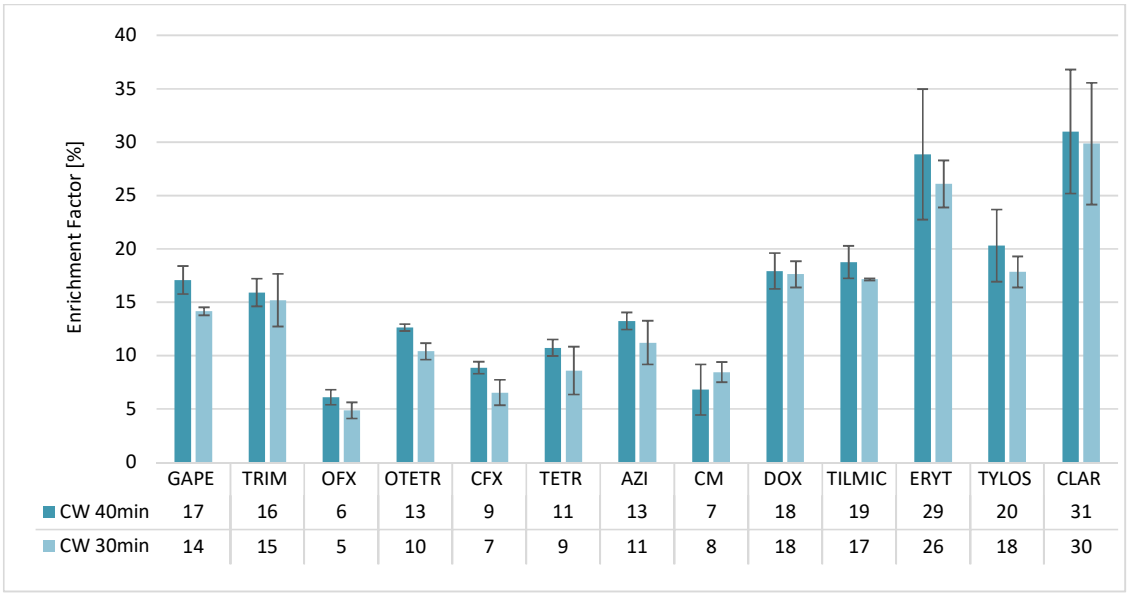


Figure 22:Time influence on the enrichment factor of EME: Triplicates with standard deviation at 30min and 40 min

After the extraction, the acceptor solution was transferred into a micro vial. In order to control the AB transfer 1 mL of the donor solution was measured for a second time. To get a better understanding of an EME, the whole extraction set-up is represented in Figure 23. The used LC-MS/MS method is described in section 3.1.

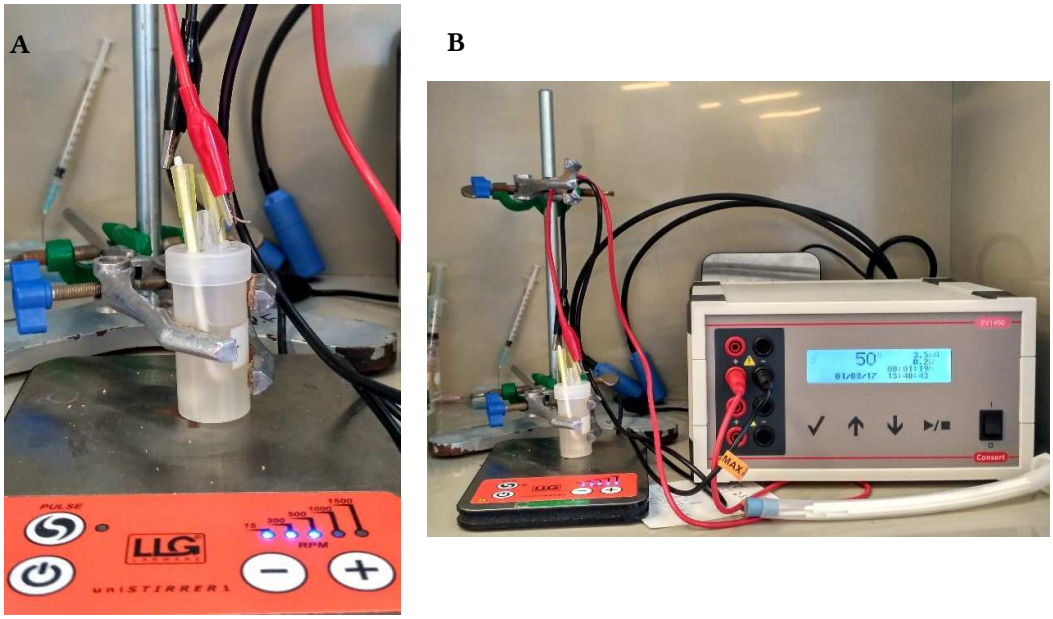


Figure 23: Extraction set-up: A) EME cell with (red) positive and (black) negative electrode; B) cell attached to Consort bvba EV1450 electrophoresis power supply

4. Results and discussion

In this chapter the findings of the various experiments are presented and discussed. As mentioned before, the goal of this thesis was to determine low antibiotics (AB) concentration in different complex matrixes and therefore new preparation techniques were tested to achieve better and lower limits of quantifications (LoQ). In order to get a first glimpse of the results, LoQ of some sample preparation techniques compared to the Wetsus standard method are presented in Table 18. The LoQ is derived from 10 times the standard deviation of blanks without standard spikes.

Table 18: Overview of limits of quantification (LoQ) of various sample preparation techniques for measured antibiotics (AB)

	Wetsus standard method for SW	**RP-SPE**	**Precipitation**	**Dilution method (≤ 5%)**	**EME**
Compound	*[ng L^{-1}]*	*[ng L^{-1}]*	*[ng L^{-1}]*	*[ng L^{-1}]*	*[ng L^{-1}]*
AMOX	2000	-	-	468	208
AMP	1000	-	-	90	14
AZI	5000	940	227	25	4274
CFT	1000		-	4	0
CFX	1572	78	6530	316	4265
CLAR	10	27	18	98	1594
CM	20	13	81	105	104
DM	381	24	170	1019	780
DOX	10	-	-	908	644
ERYT	610	394	12036	429	1707
FLUMEQ	-	-	-	119	55
LINCOM	-	-	-	54	11
OFX	5000	4	75	92	104
OTETR	-	-	-	123	127
PENG	-	-	-	193	35
PENV	-	-	-	423	84
SMO	23	31	35	235	5
SUCLOP	-	-	-	102	77
SUDOX	-	-	-	200	149
SULFAM	-	-	-	68	584
SULYPR	-	-	-	54	39
TETR	-	-	-	581	614
TILMIC	-	-	-	18	5332
TRIM	30	62	56	287	668
TYLOS	-		-	344	1589
GAPE	2.5	508	2256	194	124
SUCRAL	1276	607	104	33	1234
CHEX	2622	413	-	-	-
BaCl-12	23	-	-	-	332
BaCl-14	31	-	-	6123	3416

Table 18 clearly shows how difficult it is to detect quantitatively all AB, disinfectants and human markers in very low concentrations with just one ultimate preparation technique. Comparing the LoQ of ABs within the Wetsus standard method, it seems that for TRIM a very low LoQ of 30 ng L^{-}

[1] is achievable whereas for OFX with a LoQ of 5000 ng L^{-1} the same method is not as satisfying. For RP-SPE it turns out to be exactly the opposite case, OFX has a lower LoQ of 4 ng L^{-1} as TRIM with an LoQ of 62 ng L^{-1}. In this chapter the generated results are summarized and discussed in more detail.

4.1. Commonly used sample preparation techniques

4.1.1. RP-SPE

RP-SPE experiments were performed to assess the applicability of a RP-SPE sample preparation approach with a Strata™-X cartridge to ultimately measure AB compounds in wastewater and other matrixes such as effluent. Recoveries and LoQs were derived from LC-MS/MS peak areas. For comparison an effluent and a surface water sample are discussed in this section. Furthermore, the obtained antibiotic (AB) concentrations are adjusted according to their achieved recoveries. In Table 19, the results of the LC-MS measurements of AB in an effluent sample and a surface water sample are represented.

Table 19: Results of LC-MS measurements after RP-SPE based on an effluent sample and a surface water sample (Leeuwarden 20170404 and SW2 20170206, PNEC, limit of quantification, corrected concentration, standard deviation, relative standard deviation (RSD) and recovery blank subtracted)

			Leeuwarden 20170404				SW2 20170206			
Comp.	***PNEC***[11] ***[ng L^{-1}]***	***LoQ*** ***[ng L^{-1}]***	*Conc. [ng L^{-1}]**	*St.Dev [ng L^{-1}]*	*RSD [%]*	*Recovery [%]*	*Conc. [ng L^{-1}]**	*St.Dev. [ng L^{-1}]*	*RSD [%]*	*Recovery [%]*
GAPE	-	508	*624*[#]	±2640	14	3	288	±24	8	47
SUCRAL	-	606	*2264*[#]	±100	4	-	916	±122	13	17
CHEX	-	413	*515.5*[#]	±79	15	-	*9.7*[#]	±3.3	34	2
DM	-	23.6	30.4	±8	27	41	19.6	±2	11	60
OFX	500	3.8	19.8	±0	2	43	*1.9*[#]	±1	30	7
CFX	64	77.7	349	±52	15	25	*4.2*[#]	±3	60	3
AZI	250	939	392	±86	22	65	*542*[+]	±132	24	≫100 (150)
ERYT	1000	393	249	±74	29	32	802.	±750	93	77
CLAR	250	27.2	26.1	±4	16	37	0.2	±0	141	51
CM	1000	12.6	34.5	±5	14	42	6.4	±1	14	61
TRIM	500	62.2	224	±25	11	47	9.5	±1	8	77
SMO	16000	31.0	457	±42	9	10	27.7	±7	25	28

*concentrations are corrected via recovery
[#]no correction possible due to very low recovery (<10%)
[+]no correction possible due to very high recovery (>100%)

In effluent, gabapentin and sucralose as human markers show a very low recovery, a relative standard deviation (RSD) of only 14% and 4% and concentrations of *624* ng L^{-1} and *2264* ng L^{-1}, respectively. The high concentration of sucralose is not surprising due to its high usage in food and beverages. The very low recovery can be explained by the relatively high concentration of GAPE and SUCRAL, which are already existent in the sample. As a result, the added spikes are significantly smaller than the concentration in the sample, and therefore only a "drop in the ocean". In surface water, the human markers show higher recoveries with 47% and 17% and plausible smaller concentration of 288 ng L^{-1} GAPE and 916 ng L^{-1} SUCRAL than in the effluent. As the results are showing, the GAPE concentration is under the limit of quantification and therefore it is not considered as a reliable value. According to Robert Loos *et al.*, SUCRAL concentrations of EU waste water treatment plant effluents show average concentrations of 2600 ng L^{-1}, which are

comparable to the SUCRAL concentrations in the used effluent sample [79]. Whereas the SUCRAL concentration in the surface water is much higher than concentrations reported in literature [80].

For the macrolides (AZI, CLAR and ERYT) very different results could be observed. AZI for instant is measured with high recoveries in both cases (effluent 65% and SW much higher than 100%) compared to ERYT, which is found with a recovery of 32% in effluent and 77% in SW. The achieved concentration of ERYT in surface water with 802.5 ng L^{-1} should not be considered as a trustworthy value, because of its high RSD of 93%. Even though a recovery of 51% for CLAR is verifiable, the concentration in the surface water is very low and far below the LoQ of 27.2 ng L^{-1}, therefore the CLAR concentration with an RSD of 141% is not considered as an absolute value. Interestingly the recovery and the concentration of AZI is oddly high compared to literature and the concentration for both water types exceeds the PNEC value, which means it will have a negative impact on the environment. Christian *et al.* states in a study that in East-Westphalia an AZI concentration of 3 ng L^{-1} with a recovery of 100% is detectable [81]. The findings of this analysis support the assumption that an ion enhancement of AZI took place during measurement due to matrix effects or reactions with modifiers and adsorption material [82], [83]. For an explanation without assumptions, further investigation is necessary.

The results for CFX and OFX as fluoroquinolones reveal a completely different picture. Even though better LoQ (77.7 and 3.8 ng L^{-1}) are achieved with RP-SPE sample preparation than with the Wetsus standard procedure (see Table 18), the recoveries of both compounds with 7% and 3% in SW are hardly convincing. Although, the recoveries for both compounds are higher in effluent, the result are not as satisfying as expected. Better results with 88% recovery for CFX and 112% for OFX have been recorded from M.C. Campos-Mañas *et al.* with a less time consuming filter sample preparation technique [84].

The sulpha drug SMO and TRIM show better recoveries for RP-SPE sample preparation of surface water than for effluent. Although no lower LoQ could be achieved, the limits with 31.0 ng L^{-1} and 62.2 ng L^{-1} are still acceptable, due to the fact that the limits do not exceed the PNEC of SMO with 16000 ng L^{-1} and 500 ng L^{-1} [11]. The found TRIM concentration of 224 ng L^{-1} in effluent is comparable to the conducted study of Robert Loos *et al.* which says that in EU-wide waste water treatment plant effluents an average TRIM concentration of 229 ng L^{-1} is detectable [79]. Whereas the 457 ng L^{-1} of SMO is considered to be higher than the average of 280 ng L^{-1} but smaller than the maximum with 1691 ng L^{-1} [79].

As the results show, the RP-SPE is rather more applicable as a sample preparation technique for less polluted samples than for polluted ones. Especially for effluent it is assumed that the RP-SPE cartridge is clogged with particles or becomes saturated, what would lead to a loss of analyte during washing. Another reason could be that polluted waters like effluent include particles, which are very attractive for antibiotics to bind on and this effect causes a loss in detectability [47]. The major drawbacks of this technique are not only the high consumption of sample volume and chemicals but also the relatively high sample preparation time and production of waste.

Nevertheless, as a sample preparation for ABs the RP-SPE must be further optimized with respect to its applicability for less concentrated and less polluted samples as well as only measuring a few AB compounds at the time might help to reduce ion enhancement and suppression during MS detection.

4.1.2. Precipitation of higher contaminated samples with copper (II) sulphate and aluminium sulphate

Precipitation coupled with Strata™-X RP-SPE was performed to reduce the interference of particles and to remove natural organic matter (NOM). The sample treatment technique is based on literature and modified with copper(II) sulphate [85], [86]. In Table 20 the results of the LC-MS measurements after precipitations are presented.

Table 20: Results of LC-MS measurements after precipitation coupled with RP-SPE based on nursing home waste water sample (N20170228, PNEC, limit of quantification, corrected concentration, standard deviation, relative standard deviation (RSD) and recovery blank subtracted)

Comp.	PNEC [ng L^{-1}]	LoQ [ng L^{-1}]	Nursing home waste water N 20170228			
			*Conc.** [ng L^{-1}]	*St.Dev.* [ng L^{-1}]	*RSD* [%]	*Recovery* [%]
GAPE	-	2256	*202*[#]	±226	112	1
SUCRAL	-	104	37077	±1204	19	17
DM	-	170	*81.4*[#]	±17	21	2
OFX	500	75	*61.0*[#]	±8	12	1
CFX	64	6530	*995*[#]	±653	66	1
AZI	250	227	*93.5*[+]	±23	24	115
ERYT	1000	12036	*11.1*[#]	±8	73	1
CLAR	250	18	15.4	±6	82	44
CM	1000	81	38.5	±2	35	13
TRIM	500	56	68.1	±10	40	38
SMO	16000	35	*4.2*[#]	±4	83	0

*concentrations are corrected via recovery

[#]no correction possible due to very low recovery (<10%)

[+]no correction possible due to very high recovery (>100%)

The results in Table 20 show that only a few compounds are detectable with a recovery over 10% and the RSD% is in general much higher than without precipitation. Due to the high RSD values, the results are dismissed as not reliable and in the end this sample treatment was not modified further.

The reason for the negative effect of contaminations is already discussed in the previous chapter (section 4.1.1). In the case of precipitation, more binding sites are introduced to the sample through the precipitation solution ($CuSO_4$ and $Al(OH)_3$), which helped to reduce the NOM but hindered the detectability of the ABs drastically [87]. For example, some ABs like CFX sorb strongly to sediments and inorganic material and in some cases even metal complexes are formed, which explains the lack of the detection and the recovery of 1% [88], [89].

4.2. Unconventional sample preparation techniques

4.2.1. Modified dilution method

4.2.1.1. Dilution experiments on sludge samples with a solid content of ≤ 5%

The developed method was derived from the Wetsus standard sample preparation for waste water samples (see Appendix I). Instead of a neutral modifier, SUPERMOD was used to break possible bonds of the ABs to sediments and particles in the matrix. Sludge samples with a solid content below 5% were treated as liquids. Due to higher solids content a sufficient preparation treatment of sludge samples is much more difficult. Adsorption effects are increased, thus complicated to control and detection with a recovery loss is the result. For development of the

dilution method, experiments with different dilutions and with different pH were carried out according to 3.3.1.1.2.

Figure 24 shows the recoveries (blank subtracted) with the corresponding standard deviation in % of the sample treatment of a Stage B 20170727 sample according to the changes in pH and dilutions for a few chosen ABs. The complete results can be found in Appendix III Table 21 gives a short overview of the differences in pH and dilution levels, which were tested.

Table 21: Overview of the tested pH differences and dilution levels in the sample preparation of sludge samples with a solid content ≤ 5%

	Average pH - values
pH1	2.94
pH2	1.87
pH3	1.64
	Sample [mL] diluted to 7.5 mL
1:2	3.75
1:4	1.5

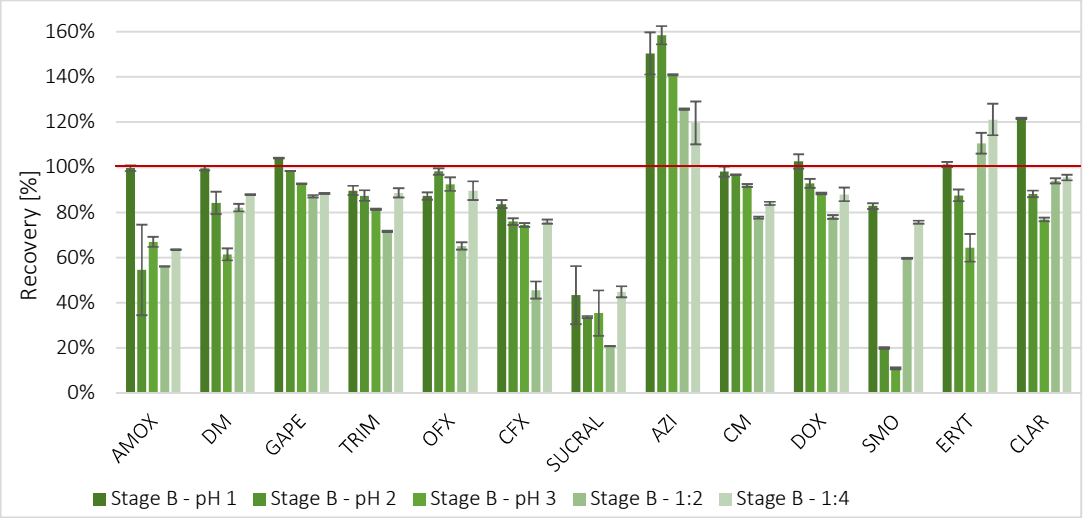


Figure 24: Recoveries of the dilution experiments of Stage B 20170727 with three different pH levels and two different dilutions with corresponding standard deviations in %

Generally, Figure 24 indicates that the change of pH has more impact on the recovery (except ERYT) than the dilution experiments. However, a positive tendency towards a higher dilution can be seen, i.e. dilutions with a 1:4 fraction show for the majority of compounds higher recoveries than a dilution fraction of 1:2. For completeness of the recovery results, the relative standard deviations (RSD) in % are shown in Table 22. Due to general low RSD% (except some exceptions like AMOX and SUCRAL) the recovery results are classified reliable and trustworthy.

Table 22: Relative standard deviations (RSD) of the recoveries in % for Stage B 20170727

	pH1	pH2	pH3	Dilution 1:2	Dilution 1:4
Compound	*RSD [%]*	*RSD [%]*	*RSD [%]*	*RSD [%]*	*RSD [%]*
GAPE	0	0	0	1	0
SUCRAL	30	1	28	0	5
AMOX	1	37	3	0	0
DM	1	6	4	2	0
OFX	2	1	3	2	5
CFX	2	2	1	8	1
CM	2	0	1	1	1
DOX	3	2	0	1	3
TRIM	2	3	0	0	2
SMO	2	2	3	0	1
AZI	6	3	0	0	8
ERYT	1	3	10	4	6
CLAR	0	2	1	1	1

Within the pH variation experiments, a decrease in pH from 2.94 to 1.64 has a negative effect on the recovery, especially in the case of SMO, where a drop in recovery of over 60% is detectable. SMO has a pKa of 5.6 and in acidic environment the molecule occurs to be neutral, which reduces the ability to be ionized through the electro spray ionization [90]. Another reason could be the higher concentration of oxalic acid in the sample preparation. Oxalic acid is used to achieve a better resolution in chromatography, because it is a strong chelate agent, which can combine with metal ions, but it also has its drawbacks. The acid is not eager to be volatilized and can pollute the ionisation source, which might cause a signal suppression [91].

Of all compounds in Figure 24 SUCRAL as a human marker shows by far the lowest recoveries between 22% and 45% with higher RSD in %. Sucralose is a molecule with only hydroxyl as functional groups and according to Loos *et al.* the molecule shows therefore a poor ionization efficiency and difficult fragmentation, which leads to a loss in sensitivity in the LC-MS analysis [92]. For further experiments it is advisable to measure in negative mode, probably better results can be achieved.

The macrolides show again the highest recoveries with AZI as precursor with a recovery up to 158%. ERYT and CLAR are more or less around 100%, which confirms the theory that usually the recovery of macrolides is high [93]. Referring to the results of this thesis, this effect is only observed for macrolides and it might occur because of matrix effects. Due to more pollution the risk of interferences during analysis increases and in the case of AZI a signal enhancement can be observed [94].

A comparison of the different types of dilution experiments according to the found concentrations is represented in Figure 25. The average and the standard deviation are calculated from all Stage B 20170727 without spikes (n=10) to compare the differences of the concentrations within the different dilution experiment types (changed pH and dilution levels). All concentrations are corrected with the corresponding recoveries. The LoQ of the Wetsus standard method is named *LoQ current* and the achieved LoQ is stated as *LoQ*. In case of CM, TRIM and ERYT no lower LoQ could be attained with the dilution method and four out of seven ABs show concentration beyond the achieved LoQ.

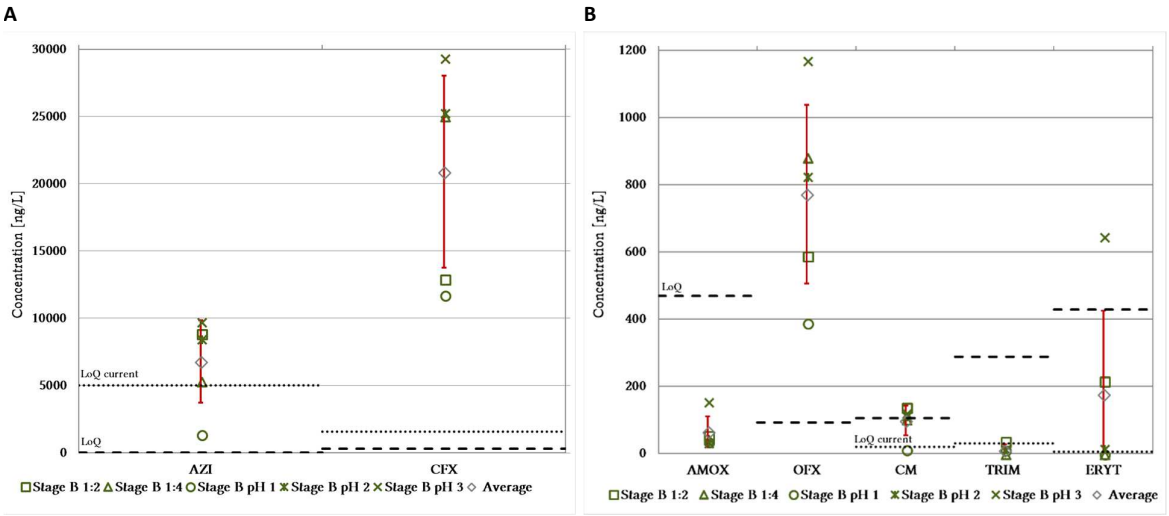


Figure 25: Examples for concentration profiles of chosen ABs in Stage B 20170727 according to the different types of dilution experiments. Separated into two diagrams due to different concentration scales in A with AZI and CFX in ng L^{-1} and B with AMOX, OFX, CM, TRIM and ERYT in ng L^{-1}

Figure 25 demonstrates clearly that there is a big scattering of antibiotic concentrations in the sample. The scattering of the concentration is no surprise because it is again a proof for unstable system, and how dilution and pH changes affect the analysis.

The results of experiments with a pH of 2.94 (pH1) are the lowest throughout the whole ABs and out of the standard deviation except for TRIM and AMOX. This effect is surprising because the recovery is at its highest for pH1 experiments (Figure 24). For pH3 experiments with the lowest pH of 1.64 it seems to extract the highest concentrations of ABs and the results are out of the standard deviation except for TRIM, CM and AZI. It seems that a higher admixture of acid increases the ability to separate a large proportion of compounds from the sludge, but obviously hinders the recovery measurements.

In comparison to literature, the found concentrations are still small. The fluoroquinolones CFX and OFX appear to be much higher in various conducted studies, for instance Ferhi *et al.* found a CFX concentration in a secondary sludge with 530 µg L^{-1} and OFX with 591 µg L^{-1} [95]. Since there is no knowledge about the residence time of the sample in the sludge reactor, about the usage profile of ABs in different states (e.g. Greece or France) or the season (winter, spring, summer, autumn) of the withdrawn sample in literature, a direct comparison would be questionable [96]. Nevertheless, the tendency of CFX to show the highest concentration in sludges is stated in different papers, which have been confirmed through the dilution experiments in this thesis [95], [97].

AMOX as a β-lactam antibiotic shows next to TRIM the lowest concentrations and both are under the LoQ and LoQ current. β-lactam ABs degrade after a period of time, because they consist of a highly reactive β-lactam amide bond, which breaks easily open especially in the presence of acid [19]. For this reason, the detection of AMOX in aqueous sample matrixes is very difficult and in the case of Stage B it can be assumed, that AMOX was already degraded before the sample even enters the column of the LC-MS.

Consequently, the results of the recovery measurements show, that the dilution method with a pH of 2.94 can be successfully adopted for higher diluted sludge samples, which can be treated as liquid. Nevertheless, further modifications (e.g. higher pH or less sample) need to be tested, especially regarding the analysis of SUCRAL and AZI to avoid interferences during analysis with LC-MS.

4.2.1.2. Dilution experiments on sludge samples with a solid content of ≥ 5%

In search of an easy, cheap and satisfying sample preparation technique for solid samples and sludge samples, which cannot be treated as liquids (solid content ≥ 5%), a method based on the Wetsus standard sample preparation for waste water samples (see Appendix I) was developed. The same SUPERMOD modifier as mentioned in section 4.2.1.1 is used. Due to higher solid content the sludge samples needed to be weight and diluted to 15 mL. Adsorption effects in sludge samples with a solid content ≥ 5% are even more increased than in sludges with less solid content, thus complicated to control and reproducible results are more difficult to achieve.

For development of the dilution method, experiments with different dilutions and with different pH were carried out according to 3.3.1.2.2. Table 23 gives a short overview of the differences in pH and dilution levels, which were tested.

Table 23: Overview of the tested pH differences and dilution levels in the sample preparation of sludge samples with a solid content ≥ 5%

	Average pH - values
pH1	3.22
pH2	2.03
pH3	1.72
	Sample [g] diluted to 15 mL
1:6	2.5
1:15	1

Figure 26 shows the recoveries (blank subtracted) with the corresponding standard deviation in % of the sample treatment of a Digest 20170727 sample according to the changes in pH and dilutions for a few selected ABs. The complete results can be found in Appendix III.

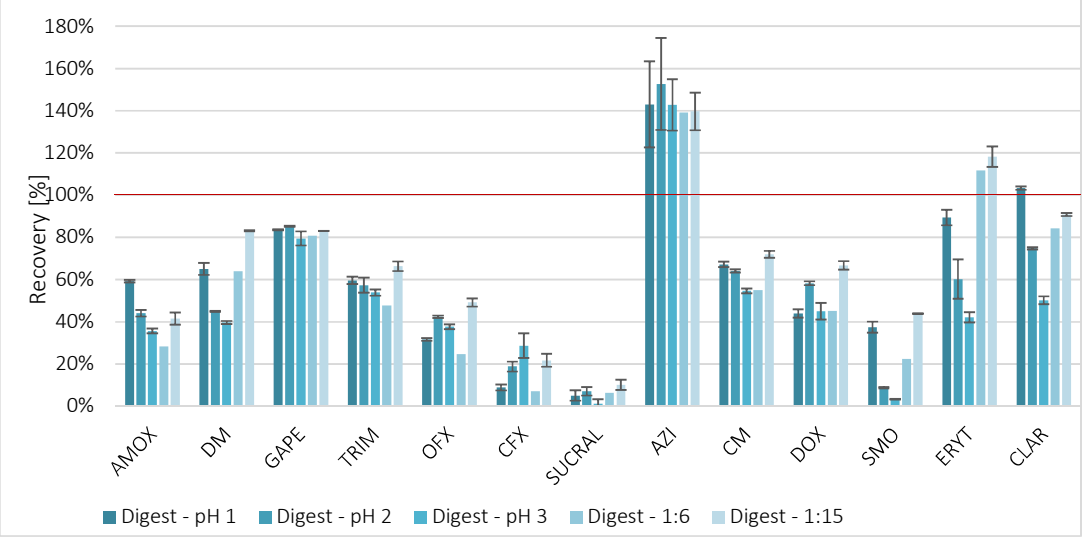


Figure 26: Recoveries for the dilution experiments of Digest 20170727 with three different pH levels and two different dilutions with corresponding standard deviations in %

At a first glance, recoveries seen in Figure 26 are lower than the recoveries for sludge samples with a solid content ≤ 5%. This outcome is not surprising due to higher contamination of the samples and bigger particles with more pores, hence larger surfaces are available to adsorb compounds. A tendency through the pH experiments is not clearly recognisable but it is obvious that a higher dilution has a positive impact on the recovery for nearly every compound. For completeness of the recovery results, the relative standard deviations (RSD) in % are shown in Table 24. Besides a SUCRAL at pH1 and pH3 and dilution 1:6, general low RSD are achieved, and therefore the recoveries are believed to be reliable and trustworthy.

Table 24: Relative standard deviations (RSD) of the recoveries in % for Digest 20170727

	pH1	**pH2**	**pH3**	**Dilution 1:6**	**Dilution 1:15**
Compound	*RSD [%]*	*RSD [%]*	*RSD [%]*	*RSD [%]*	*RSD [%]*
GAPE	0	0	4	1	0
SUCRAL	49	28	183	40	24
AMOX	1	4	3	4	7
DM	4	1	2	0	0
OFX	2	1	3	7	4
CFX	16	13	20	27	14
CM	2	1	2	0	2
DOX	5	2	9	13	3
TRIM	3	6	3	6	3
SMO	7	4	4	3	0
AZI	14	14	9	13	6
ERYT	4	15	6	6	4
CLAR	1	1	4	1	1

Similar to prior analysis, the highest recoveries are achieved by macrolides. Hence the recoveries for these compounds seem to be consistently high or over 100% with RSD below 15%, an enhancement error due to matrix effects as cause becomes more plausible (see previous discussion 4.2.1.1) [98], [99].

Interestingly the recoveries for CFX are between 5 and 25% (RSD between 13 and 27%) and compared to Stage B very low. Polesel *et al.* studied the sorption reaction of ciprofloxacin on digested sludge and the impact of pH on the adsorption [100]. As a result it is stated that the sorption capacity is strongly pH dependent and the greatest sorption occurs at a neutral pH [101]. Compared to the obtained recovery data it makes sense that the recovery is very small for a pH of 3.22 but higher for a lower pH at 1.72, due to the favourable effect of sorption hindrance between sludge and compound in an acidic environment.

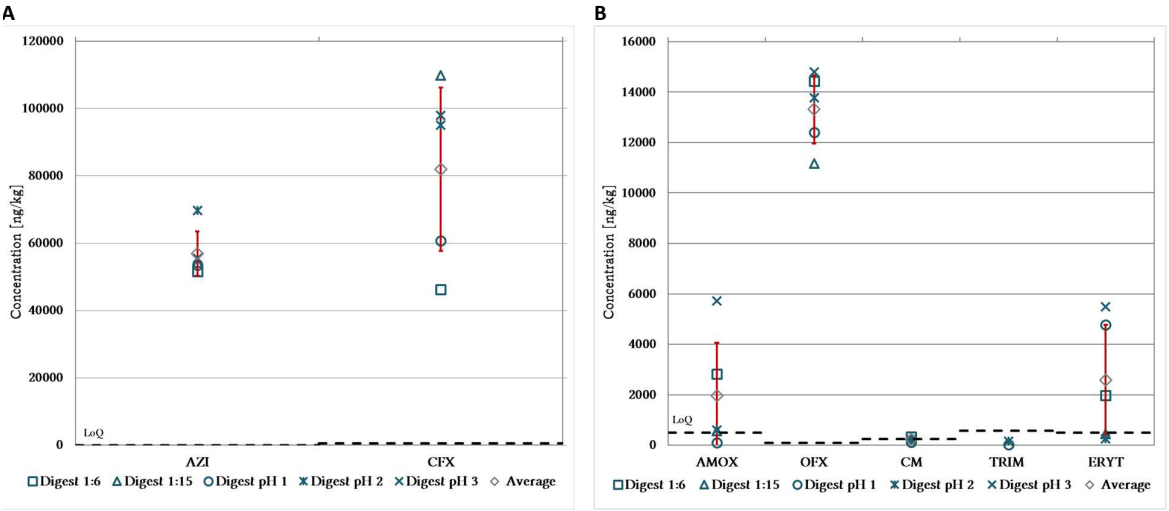
The highest obtained recoveries for ERYT in the range of 90 to 119% (RSD ≤15%) and for TRIM with 48-65% (RSD ≤ 6%) cannot be reached, according to the literature, with pressurized liquid extraction prior to LC-MS with 46.9% (RSD 6.6%) and 29.3% (RSD 10.6%) [93]. Consequently, the dilution method seems to be a better sample preparation technique regarding ERYT and TRIM.

As a human marker SUCRAL show very low recoveries with high RSD of up to 183%. According to Arbeláez *et al.* recoveries are lower because of the polar extraction solution, for a pH of 3 with water a recovery below 15% was obtained, which is comparable to recoveries found in this thesis. It is further stated that a change to a less polar extraction solution may increase the recovery of SUCRAL. SUCRAL is detected in Digest 20170727 with a concentration of 15927 ±4394 ng L^{-1} (recovery corrected, RSD 28%), but in literature higher values are found with a concentration

around 44.4 µg L^{-1} [102]. The concentration strongly depends on the location, where the sample is withdrawn, i.e. higher population densities lead to higher concentrations. GAPE is more likely to be detected through the dilution method and showed reproducible recoveries of over 80% with an RSD value lower than 5%. No significant differences within the various dilution method types are detectable.

In general, in literature higher recoveries are found for compounds in digested sludge than with the dilution method developed within this thesis, e.g. DOX shows with a ultrasound assisted extraction a higher recovery around 105% (RSD 10%) whereas in this thesis only 42-68% (RSD ≤ 13%) could be achieved [103], [104].

A comparison of the different types of dilution experiments according to the found concentrations is represented in Figure 27. The average and the standard deviation are calculated from all Digest 20170727 samples without spikes (n=10) to compare the differences of the concentrations within the different dilution experiment types (changed pH and dilution levels). All concentrations are corrected with the corresponding recoveries. The calculated concentrations are presented in ng/kg wet weight (ww), due to high water content. Until the time of evaluation of the data, Wetsus have not yet obtained LoQs for solids or semisolids, therefore no "current LoQ" is presented in Figure 27.



*ng/kg: ng/kg wet weight (ww)

Figure 27: Examples for concentration profiles of chosen antibiotics in Digest 20170727 according to the different types of dilution experiments. Separated into two diagrams due to different concentration scales in A with AZI and CFX in ng/kg (ww) and B with AMOX, OFX, CM TRIM and ERYT in ng/kg (ww)

Figure 27 represents, similar as in the previous discussed chapter, that there is a big scattering of found antibiotic concentrations in the sample. The scattering of the concentration is lower than in Stage B, but no real tendency towards pH or dilution levels is recognizable. Obtained LoQ are low enough to quantitate the majority of the represented compounds in *Figure 27*, except CM and TRIM.

CM as lincosamide and TRIM show the lowest concentration in digest sludge over the whole dilution experiments whereas CFX again shows the highest. This tendency is also shown in various studies, such as in Marx *et al.*, where a CFX with a concentration of 643.5 ± 427.5 µg/kg was by far the highest found antibiotic in Spanish digested sludge [105]. Through the gained results, it becomes clear that CFX is pH sensitive and results are strongly dependent on the dilution. Highest

difference within the experiments is found between the dilution fraction of 1:6 and 1:15, which displays repeatedly the high sorption capacity of CFX on particles. TRIM and SMO are below the LoQ and these results indicate that especially TRIM and SMO are unstable in anaerobic, mesophilic environments, such as digested sludge [106].

For the sample preparation from AMOX, OFX and ERYT at pH of 1.72 higher concentrations are detectable, which are out of the overall standard deviation. Changes in pH and dilution fraction has less impact on the behaviour of AZI than on AMOX, CFX or ERYT. In average AZI shows a concentration of 57 ± 7 µg/kg and is comparable to the found concentration range in literature [93], [105].

In summary the dilution method as sample preparation technique for sludge samples with a solid content over 5% seems to be more difficult. Even though, mostly low LoQ are achievable through this method, satisfying recoveries are not detected. More modifications must be tested, such as higher pH, higher dilutions and different detection modes (e.g. negative) to avoid matrix interferences. Overall it is very difficult to measure the whole spectrum of compounds in sludge samples with this method in a satisfactory manner, due to different tendency of the compounds to adsorb on surfaces. Furthermore, it is necessary to study the binding reactions of ABs, disinfectants and human markers on various surfaces or particles, to gain more knowledge for developing better sample preparation techniques and detection methods. Unfortunately, the latter was not possible to be investigated, as it would be beyond the scope of this thesis.

4.2.2. Reactive extraction

On the basis of the dissertation “Supported liquid membranes with strip dispersion for recovery of cephalexin” written by Michael Edward Vilt in 2015, a sample method known as reactive extraction was developed [67]. It is known to be a successful treatment for recovering bioproducts such as penicillin G [66]. Due to lack of time, a non-ideal design was chosen to test the applicability of reactive extraction for various ABs in quick and simplified experiments. For depiction of the transfer of compounds from feed to stripping solution, the results of a surface sample (SW2 20170117) with a spike of 100 µL STD- MIX (see 3.3.2.1) are displayed in Table 25. The results reported in the literature could not be reproduced [63], [64], [66].

Table 25: Reactive extraction results of a surface water sample (SW220170117) spiked with 100 µL STD-MIX according to the found concetration in ng L^{-1} with standard deviation (St.Dev. in ng L^{-1}) and relative standard deviation (RSD) in %

	Feed solution			**Reconstruction sol. as stripping solution**			**Citrate buffer as stripping solution**		
Compound	*Conc. [ng L^{-1}]*	*St.Dev. [ng L^{-1}]*	*RSD [%]*	*Conc. [ng L^{-1}]*	*St.Dev. [ng L^{-1}]*	*RSD [%]*	*Conc. [ng L^{-1}]*	*St.Dev. [ng L^{-1}]*	*RSD [%]*
TRIM	99.2	±3.1	3	3.9	±3.5	91	0.6	±0.004	0.6
OFX	89.1	±4.8	5	11.3	±11.3	100	3.0	±0.003	0.1
CFX	67.0	±2.5	4	2.7	±1.8	68	2.6	±0.002	0.1
AZI	84.5	±15.4	18	18.9	±18.9	100	6.6	±0.007	0.1
SUCRAL	3.4	±3.4	100	41.8	±19.8	47	2.5	±0.002	0.1
CM	49.5	±0.9	2	8.0	±7.9	99	3.5	±0.003	0.1
ERYT	102.6	±40.1	39	0.0	±0.0	-	7.7	±0.009	0.1
CLAR	21.9	±6.2	28	18.7	±9.6	51	8.9	±0.003	0.0

The results of the “quick and dirty” experiments show clearly that the complete transfer of compounds is not possible. Especially the transfer between feed and citrate buffer through the organic phase seems to be difficult. Even if compounds such as SUCRAL are transferable into the

reconstructed stripping solution, the RSD values are very high, and it can be interpreted that there must be a strong scattering of the data, which makes the results not trustworthy. On the one hand the low surface in the vial could be the reason, because only a relatively small part of the solution surface is available for reaction and transfer and on the other hand it could be assumed that some compounds are not even able to get extracted by the organic phase. It may also be possible that a few compounds get lost in the organic solution and cannot be reextracted in an aqueous solution with a lower pH. A hollow fibre setup may help to increase the surface for reaction and transfer [64].

Since the results of the "quick and dirty" experiments showed no satisfying success and a more advanced experimental setup would be necessary, further investigation of the reactive extraction as sample preparation technique for surface water samples and wastewater samples was discarded. At the same time the reactive extraction system has too much variables like pH in both aqueous phases or composition of organic solution (amount of butyl acetate or 1-decanol) that need to be changed and to be tested, which would be beyond the scope of this thesis.

4.2.3. Electromembrane extraction (EME)

Electromembrane extraction is an alternative sample preparation method, which is still in development. The developed type of EME set-up is based on Ramos-Payán *et al*. and Pedersen-Bjergaard *et al.* [73] [71]. Instead of a sample solution at pH of 5, the pH of the sample solution was reduced to a pH of 2 and SUPERMOD was added to reduce bonding of the compounds to pollutions in the waste water sample in this project. Another difference to the method described in literature is the usage of a standard with more compounds, such as other ABs, human markers and disinfectants. Like the RP-SPE, the EME procedure is only applicable for some compounds, especially charged compounds are more likely to be extracted through the organic phase. For various compounds the achieved enrichment factors of preliminary tests are shown in Figure 28. The enrichment factor is calculated as the ratio between the detected concentration in the acceptor solution and its initial concentration in the donor solution in %, blank corrected.

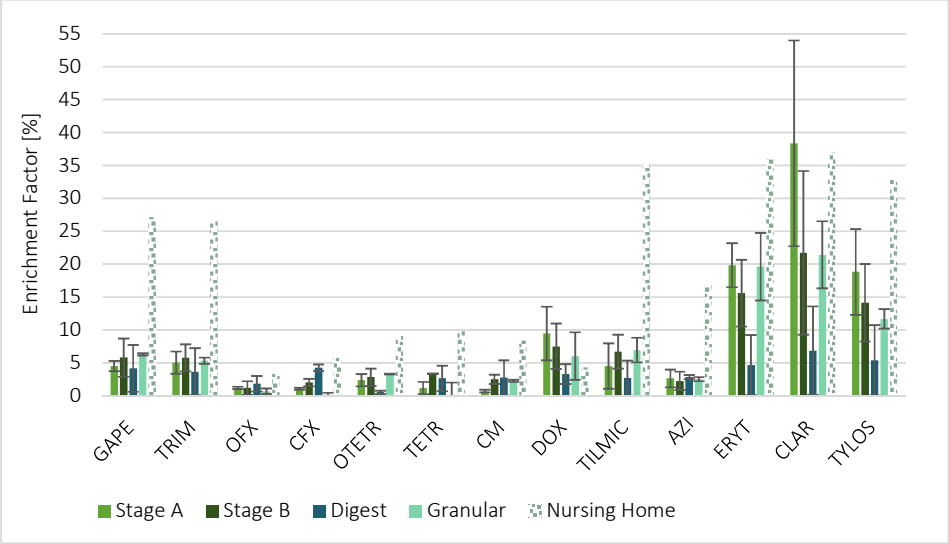


Figure 28: Obtained enrichment factors for various compounds via EME experiments with their corresponding standard deviation in %. Stage A/ Stage B/ Digest/ Granular 20170727 and Nursing home waste water sample N 20170228

Due to one-time measurement of the nursing home waste water sample no standard deviation is available. Nevertheless, the EME results for N 20170228 are still represented in Figure 28 because the method is assumed to work well for the extraction of compounds in lighter polluted samples. Also, the trend of the enrichment factor to be higher for Stage A with lower solids content to digested sludge with higher solids content confirms this hypothesis. To get an overview about the reliability of the results, the RSD in % are listed in Table 26.

Table 26: Relative standard deviation of the electromembrane extractions (EME) in %

Compound	Stage A	Stage B	Digest	Granular
		RSD [%]		
GAPE	18	50	86	3
TRIM	34	36	98	9
OFX	15	86	65	58
CFX	15	30	11	126
OTETR	40	46	44	1
TETR	79	0	73	-
CM	28	29	98	7
DOX	43	46	46	60
TILMIC	77	39	100	27
AZI	51	61	11	13
ERYT	17	33	98	26
CLAR	41	57	98	24
TYLOS	35	42	99	13

Measurements in digested sludge with this kind of EME set-up lead to results with higher scattering within the dataset (relatively high RSD). Whereas the RSD values for Stage A, B and Granular are more acceptable. As this EME sample treatment set-up uses standards with a high compound number, is still in development and mostly experimental, high RSD are not surprising.

Interestingly, higher enrichment factors are achieved in granular sludge than in digested sludge. Probably the chemical interaction between the compounds and the granular surface is weaker at a pH of 2 and therefore easier to break through SUPERMOD addition and the application of current in the cell [107].

Concerning the achieved enrichment factors, Figure 28 shows that EME as sample treatment is more successful for macrolides (especially CLAR and ERYT) with factors up to 38%. The highest influence on the enrichment is assumed to be the sample pH and therefore knowledge about the pKa of the compounds is really important (see chapter 2.1) [108]. Compounds with a secondary amine group, such as ERYT, CLAR, TYLOS and TILMIC seem to be better extractable than others. Subsequently it can be assumed that these compounds are highly protonated on the amine and therefore a transport from the donor to the acceptor equipped with the negative electrode is slightly more effective. Whereas compounds with carboxyl group are neutral charged at a very low pH, no transport from donor to acceptor occurs.

Enrichment factors for CFX mentioned in literature could not be reproduced by the EME set-up developed in this thesis. According to Ramos-Payán *et al.* for instance a change in pH to 5 probably enhances the extraction efficiency for CFX [73]. Due to small acceptor to donor solution ratio, it could have happened that the high number of compounds was too much for the set-up to handle, i.e. the acceptor was already saturated with compounds, which were easily charged and extracted through the membrane and could have back-extracted [109]. Another possible reason might be the heat development during the extraction. At higher temperatures over 40 °C some ABs and

similar compound start to degrade, which causes a loss in detection of these compounds [110], [111].

In summary, the tendency of the preliminary experiments shows clearly that an extraction takes place, but not yet with satisfying outputs. EME as sample preparation technique is a very good alternative to solid phase extraction, because it not only reduces the cost for preparation of each sample but also reduces the material and chemical consumption. Nevertheless, several factors are known to affect the extraction performance, which have to be studied in order to implement this sample treatment technique as a thriving method for treating waste water samples in the future. First of all, different pH steps have to be tested particularly interesting would be a pH range between 3 and 9 to understand the impact of pH on every tested compound. Secondly, the amount of standard spikes and standards with fewer compounds need to be adjusted to tackle the issue of interferences due to overloading of the acceptor. As final criterion heat production during extraction should be monitored to rule out degradation due to temperature interferences and if necessary, an appropriate cooling device needs to be installed to ensure constant temperature. Additional to the mentioned necessary research steps may be reuse testing of the hollow fibre to reduce the cost and consumption of material.

4.3. Comparing different preparation techniques for nursing home waste water

In order to get a direct comparison about the performance of the various sample preparation techniques, each chosen technique is tested on a nursing home waste water sample (N 20170228). Especially the modified dilution method and the original Wetsus standard method depicted from Appendix I, in which a neutral matrix modifier was applied instead of SUPERMOD are tested and compared. For each technique blank samples and samples with 15 µL and 125 µL spikes of STD-155 (Table 14, section 3.2.1.1) are prepared. All other preparation steps were performed as mentioned in chapter 3.

In Figure 29 recoveries of some ABs achieved by the different preparation techniques are represented. As only a single measurement of the nursing home waste water samples was done with EME, no standard deviation could be calculated. The results of EME are only evinced for comparison, but are not considered reliable, for dependable data further measurements are required. For better evaluation of the data the RSD in % are listed in Table 27.

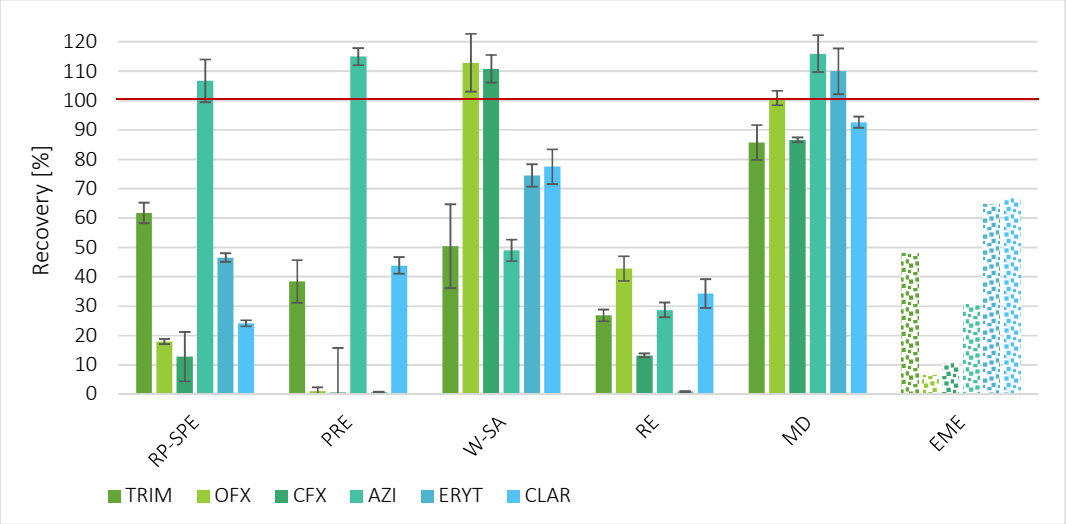


Figure 29: Comparison of the different sample treatment techniques according to their achieved recoveries showed on nursing home waste water sample N 20170228 (RP-SPE: reversed phase solid phase extraction, PRE: precipitation coupled with RP-SPE, W-SA: Wetsus standard analysis sample preparation; simple dilution preparation, RE: reactive extraction, MD: modified dilution method, EME: electromembrane extraction)

Regarding the recoveries of all presented ABs in Figure 29, modified dilution preparation method (MD) developed within this thesis next to the Wetsus standard preparation technique showed the best results with recoveries up to 116%. For AZI and TRIM it seems that solid phase extraction as sample preparation technique (RP-SPE of PRE) is successfully applicable as well. Whereas for fluoroquinolones (CFX, OFX) W-SA and MD are a good deal more effective with very low RSD (≤ 9%) and compared to literature with recoveries for fluoroquinolones in waste waters of 92 ± 5% even higher recoveries are achievable (113 ± 10% and 116 ± 6%) [112].

Table 27: Relative standard deviation (RSD) of the recoveries of the comparison experiments on N 20170228 in %

Compound	RP-SPE	PRE	W-SA	RE	MD
			RSD [%]		
TRIM	6	19	28	7	7
OFX	5	109	9	10	2
CFX	66	2656	4	5	1
AZI	7	3	7	9	5
ERYT	3	30	5	20	7
CLAR	4	6	8	14	2

Even though higher recoveries for AZI (115 ± 3), CLAR (44 ± 3%) and TRIM (38 ± 7%) are detectable with PRE, the lack of satisfying results especially for fluoroquinolones (~ 1%), ERYT (1 ± 0.2%) and other compounds (see section 4.1.2) as well as the high RSD seen in Table 27 lead to the result that PRE is not considered as a feasible sample preparation technique for waste water samples.

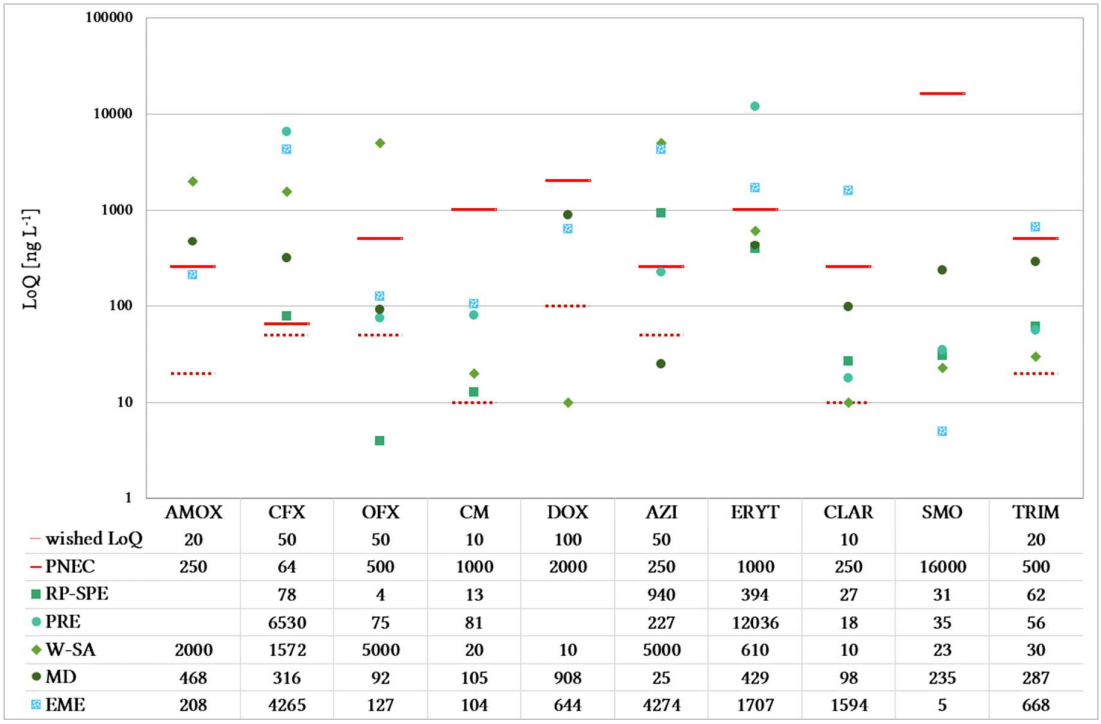
The reactive extraction (RE) of N 20170228 accomplished the highest recovery for OFX with 43 ± 4% and even lower recoveries for all compounds (see 4.2.2). RE is therefore also dismissed as an appropriate sample preparation technique. Despite low recoveries for OFX and CFX with respectively 7% and 11%, the developed electromembrane extraction technique as sample treatment is believed to be a potential method to treat sample with higher pollution. EME shows higher recoveries for CLAR (65%) and ERYT (67%) with the highest RSD of 20% and is therefore a more successful and less cumbersome technique than RE.

The outcome of the comparison experiments showed repeatedly the good applicability of the MD. The method presented by far the highest recoveries between 87 and 116% for the chosen ABs with low standard deviations in N 20170228. Even though some modifications are necessary it seems that MD is a quite decent, simple method to prepare higher polluted waste water samples.

5. Conclusion

The aim of the present thesis was to reduce the limits of quantification for LC-MS/MS analysis of various antibiotic residues in surface water, diverse waste water, and sludge samples. Within this thesis, different sample preparation techniques were developed, tested and compared to commonly used techniques such as RP-SPE and standard sample preparation technique used in Wetsus laboratory.

Despite the exploratory nature of this thesis, interesting and reliable data was obtained throughout a whole variety of samples and target analytes. To provide a better understanding of the results, a final overview about the general achieved LoQs of each different sample preparation technique compared to the expected LoQs by Wetsus laboratory as well as their relations to the PNEC is given in Figure 30. Overall the obtained data within this thesis showed clearly that it is nearly impossible to measure all ABs at once.



	AMOX	CFX	OFX	CM	DOX	AZI	ERYT	CLAR	SMO	TRIM
wished LoQ	20	50	50	10	100	50		10		20
PNEC	250	64	500	1000	2000	250	1000	250	16000	500
RP-SPE		78	4	13		940	394	27	31	62
PRE		6530	75	81		227	12036	18	35	56
W-SA	2000	1572	5000	20	10	5000	610	10	23	30
MD	468	316	92	105	908	25	429	98	235	287
EME	208	4265	127	104	644	4274	1707	1594	5	668

Figure 30: Final overview of the by Wetsus wished LoQs, the general achieved LoQs of the tested sample preparations and their relations to the PNEC quoted from [11]

In the early stages of this thesis, the RP-SPE with Strata™-X cartridges as commonly used sample preparation technique was tested on less contaminated samples, such as surface water and effluent. The expected LoQs could be only achieved for OFX with 4 ng L^{-1} and the achieved LoQ of CM is quite near to the expected one. As seen in Figure 30 the LoQs for the majority of the ABs are still below their PNEC, which is favourable. Through the recovery results of the RP-SPE it became clear that this sample preparation technique is applicable for surface water but not for effluents. Effluents appear to be much more polluted causing the ABs to fix rather on the particles than on the sorbent and therefore decrease the concentration of ABs in the extract. To summarize, the results reported in literature could not be reproduced for all ABs, but nevertheless the tested RP-SPE with Strata™-X cartridges can be applied for purification of surface water, especially for concentration of macrolides and sulphonamides.

To avoid interferences of pollutants in a sample with ABs, precipitation with copper (II) sulphate and aluminium sulphate prior to RP-SPE was tested. It was introduced especially for higher polluted waste water samples, such as hospital waste water to reduce the number of particles and natural organic matter (NOM) within the sample. Unfortunately, the results showed the opposite effect. Although the NOM was reduced, more binding sites were introduced through adding charged ions and the achieved recoveries were lower than without precipitation. For example, the achieved LoQs for ERYT and CFX were drastically high and above the PNEC, i.e. these compounds are only measurable at concentrations which already have a negative impact on their environment. Due to the undesired outcome no further investigations regarding precipitation in sample preparation for ABs are recommendable.

The second sample technique tested within this thesis is a modification of the standard sample preparation technique used in Wetsus laboratory, as shown in Appendix I, called modified dilution method (MD). Modifications regarding the concentration of the chelating agent, pH and dilution were tested on sludge samples with a solids content of ≤ 5 (treated as liquid) and ≥ 5% (treated as solid). Compared to the standard Wetsus sample preparation technique better LoQs regarding CFX are achievable but nevertheless above the PNEC. Despite of the LoQ of CFX, it was possible to develop a satisfactory and simple method to measure the majority of ABs with good recoveries in sludge samples with a solids content of less than 5%. Analysis of ABs in denser sludge samples were more difficult and more modifications are necessary to implement this kind of technique as an appropriate sample preparation method.

Finally, a more experimental approach was tested, namely electromembrane extraction. Even though this sample preparation technique showed higher LoQs compared to the others mentioned above, it has the tendency to be a noteworthy approach in the future. In spite of its limitations, the EME consumes much less chemicals, less preparation steps and less time than commonly used techniques, such as RP-SPE or dilution method. Nevertheless, the method is still in development and better understanding of the extraction process through the membrane of the various compounds is necessary.

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A. List of figures

B. List of tables

C. Appendix I

Analysis of Antibiotics in (waste) water using LC-QQQ-MS analysis

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Objective

This procedure describes the analysis of some Antibiotics in wastewater using LC-QQQ-MS analysis.

Area of Application

This method analyses for Amoxicillin, Ampicillin, Azithromycin, Cefotaxime, Ciprofloxacin, Clarithromycin, Clavulanate, Clindamycin, Doxycycline, Meropenem, Ofloxacin and Trimethoprim.

Introduction

Antibiotics are analysed directly in wastewater samples. The matrix of the wastewater samples is modified with the addition of EDTA in Ammonium Formate and vigorous shaking to break possible bonds of the antibiotics to compounds in the matrix. The samples are then centrifuged, and the supernatant is analysed directly for the dissolved antibiotics using LC-QQQ-MS. The modified samples are injected into the LC and separated on a Phenomenex Gemini C6 - Phenyl LC column (Length: 15 cm; ID: 3 mm; particle size: 5 µm) using a solvent gradient of 6 min. The antibiotics are ionized using positive electrospray and detected using compound specific QQQ transitions (appendix 2) with a triple quad mass analyser. The peak area of a specific QQQ transition signal corresponds with the amount of the compound injected in the instrument. The amounts of the compounds are calculated using internal standard calibration. For stabilization EDTA in Ammonium Formate buffer is also added to the calibration standard solutions. For good chromatography. a small amount of oxalic acid (0.2 mM) is added to the aquatic mobile phase.

Materials

Reagents

- ⇒ Disodium EDTA (TitriplexIII)
- ⇒ Milli-Q water (MQ)
- ⇒ Methanol (MeOH)
- ⇒ Ammonium Formate
- ⇒ Ammonia solution 30% in water
- ⇒ Neutral Matrix Modifier solution (NM): Milli-Q water with 0.1 M Na2EDTA and 1.0 M Ammonium Formate
- ⇒ Formic Acid 99%
- ⇒ Acetonitrile (MeCN)
- ⇒ Acetonitrile with 0.1% Formic Acid
- ⇒ Calibration standard (ABIO-STD) solution in MeOH containing the antibiotics to be analysed at a concentration of circa 200 mg/l (for actual concentrations see appendix 1). This solution should be made fresh every month and stored in the freezer at -20°C; especially Meropenem and Clavulanate are not stable for longer periods, even in the freezer. Most β-Lactam antibiotics are not very stable in aqueous solutions.

⇒ Internal standard (ABIO-IS) solution in MeOH containing Atenolol-D7, Ciprofloxacin-D8, Trimethoprim-D9 and Dihydrocarbamazepine at a concentration of circa 5 mg/l (for actual concentrations see appendix 1).

Apparatus

⇒ LC-MS Instrument consisting of
- Agilent Binary pump with degasser and solvent tray
- Agilent Automatic Sampler with cooled tray
- Agilent 6410 QQQ Mass Analyzer with Electrospray ion source
- Workstation with MassHunter LC-MS software and Excel

⇒ Milli-Q apparatus.
⇒ Heidolph Multi Reax vortex shaker for 15 ml tubes
⇒ Beckman Coulter Allegra X-12R centrifuge with inserts for 15 ml tubes.

Accessories

⇒ Amber 1.8 ml LC vials with caps.
⇒ Tray for 1.8 ml LC vials.
⇒ 15 ml centrifuge tubes.
⇒ Rack for 15 ml centrifuge tubes.
⇒ Eppendorf Pipettes with disposable tips of 5ml, 1ml and 200µl
⇒ 2 bottles of 2.5 litres for LC eluent.
⇒ Phenomenex Gemini C6 - Phenyl column 150x3 mm, particle size 5 µm (part.nr: 00F-4444-Y0), equipped with appropriate guard column.

Safety

⇒ Discard waste chemicals in appropriate waste containers.

Procedure

Preparation

⇒ Prepare neutral modifier (NM) solution: dissolve 1.88 gr Na2EDTA.2H2O (Titriplex III) and 3.18 gr AmmoniumFormate in 50 ml Milli-Q water. If necessary use ultrasound to dissolve.
⇒ Prepare HPLC mobile phases:
⇒ Mobile phase A : 2.5 liter Milli-Q water + 2.5 gr AmmoniumFormate + 64 mg Oxalic acid dihydrate + 100 µl Ammonia (30%)
⇒ Mobile phase B : 2.5 liter Acetonitrile with 0.1% Formic Acid
⇒ Prepare blank sample solution : 950 µl Milli-Q water + 50 µl Neutral modifier solution
⇒ Prepare diluted standard ABIO-STD-DIL from stock standard ABIO-STD (circa 200 mg/l) by dilution (20 x) with MeOH/MQ (1:1) to obtain a sufficient amount (for calibration curve and additions) of diluted standard ABIO-STD-DIL with component concentrations of circa 10 mg/l.
⇒ Prepare two 15 ml centrifuge tubes for each sample and two for the blank. Label the tubes and prepare them according to the following schema :

Table 28: Scheme for sample preparation

		MQ	SMPL	NM	ABIO-IS	ABIO-STD-DIL
TUBE	SAMPLE	ML	ML	µl	µl	µl
datecode-01	sample-1	0.0	1.0	50	50	0
datecode-01-a	sample-1	0.0	1.0	50	50	50
datecode-02	sample-2	0.0	1.0	50	50	0
datecode-02-a	sample-2	0.0	1.0	50	50	50
.	.	0.0	1.0	50	50	0
.	.	0.0	1.0	50	50	50
.	.	0.0	1.0	50	50	0
.	.	0.0	1.0	50	50	50
datecode-xx	sample-xx	0.0	1.0	50	50	0
datecode-xx-a	sample-xx	0.0	1.0	50	50	50
datecode-bl	blank	1.0	0.0	50	50	0
datecode-bl-a	blank	1.0	0.0	50	50	50

⇒ Close the tubes carefully and place them in vortex shaker. Set the vortex shaking speed to 8 (=circa 1750 rpm) shake the tubes vigorously for 10 min.

⇒ Take the tubes from the shaker and place them in the centrifuge, the samples should be centrifuged for 10 min at maximum speed.

⇒ Prepare an LC vial for each tube and label the vials, transfer 0.5 ml of the extract from each tube to the corresponding vial.

⇒ Prepare the calibration standards according to the following schema:

Table 29: Preparation scheme of the calibration standards

calibration standard	MQ	NM	ABIO-IS	ABIO-STD-DIL
	µl	µl	µl	µl
STD000	900	50	50	0
STD020	880	50	50	20
STD040	860	50	50	40
STD060	840	50	50	60
STD080	820	50	50	80
STD100	800	50	50	100

Instrumental analysis

⇒ Put the mobile phases in the solvent tray of the HPLC system and connect mobile phase A to degasser channel A1 (=A) and mobile phase B to channel B1 (=C).

⇒ Flush HPLC tubing, pump and injection system with these mobile phases A/B ratio= 1/1 with a flow of 5 ml/min for 5 minutes before connecting the column.

⇒ Load HPLC-MS method: ABIO-N.m, notice the flow speed of the method, after 1 minute set the flow to 0 ml/min (do not save the method when asked)

⇒ Connect the Phenomenex Gemini column with guard column to the HPLC ALS and the 6410 Mass analyzer.

⇒ Slowly increase (steps of 0.1 ml/min) the flow to the flow required in the method.

⇒ Use of mobile phase with oxalic acid should only be done when necessary and only with low amounts of oxalic acid to limit the pollution of the ion source.

⇒ Put a vial with acetonitrile or methanol in position 99 for needle washing.

⇒ Put a vial with blank sample solution in position 100.

⇒ Create a data directory containing the current date in the name.

⇒ Run the method several times (>3) injecting the blank sample solution (vial100) to reduce the background levels.
⇒ Setup MassHunter worklist: containing 2 * the blank sample solution (vial 100), the calibration standards, again a blank sample solution (vial 100) and the samples, followed by another blank sample solution, the calibration standards and again a blank. Fill the worklist with appropriate sample names, method file and data file names. If the worklist contains many samples repeat a calibration standard regularly. Be sure to run the blank sample solution (vial 100) before a low standard and after the high standard.
⇒ Save the worklist with a name containing the current date and the abbreviation of the researcher.
⇒ Run the worklist
⇒ When finished, decrease the flow to 0 and put the instrument in standby mode.

Data analysis

⇒ The Agilent MassHunter Quant software is used to integrate and quantitate the peaks in the data files.
⇒ Create a new Quant batch file in the data directory and add the calibration and sample datafiles to this batch.
⇒ Import a method from a previous batch with the same method, check and if necessary correct the retention times and standard concentration in this method. Set the sample types and level identification of the batch and apply the method to the batch. Check and correct calibration and integration.
⇒ Quantifiers, Qualifiers and Internal standard settings should be:

Table 30: List of compounds with according quantifiers, qualifiers and internal standards

Compound	Quantifier	Qualifier	Internal Standard
Amoxicillin	AMOX-349	AMOX-208	TRIM-D9
Ampicillin	AMP-106	AMP-160	TRIM-D9
Azithromycin	AZI-158	AZI-591	TRIM-D9
Cefotaxime	CFT-324	CFT-396	TRIM-D9
Ciprofloxacin	CFX-314		CFX-D8
Clarithromycin	CLAR-158	CLAR-116	TRIM-D9
Clavulanate	CLAV-156	CLAV-112	AT-D7
Clindamycin	CM-126		TRIM-D9
Doxycycline	DOX-428	DOX-154	TRIM-D9
Meropenem	MEROP-141	MEROP-254	AT-D7
Ofloxacin	OFX-318	OFX-261	TRIM-D9
Trimethoprim	TRIM-261	TRIM-275	TRIM-D9

⇒ Select the whole result table and export the results to excel.
⇒ If necessary, correct the results for blank levels and applied dilution to obtain the final results.
⇒ Calculate the recovery from the standard addition for each sample.
⇒ Calculate the Quantitation limits (10 * standard deviation) from the found concentrations in the blank sample matrix without additions.

Appendix 1: Standard solutions

Table 31: Compound standard solutions

Standard	compound	code	Concentration	
ABIO-STD	Amoxicillin	AMOX	188	µg/ml
	Ampicillin	AMP	164	µg/ml
	Azithromycin	AZI	226	µg/ml
	Cefotaxime	CFT	184	µg/ml
	Ciprofloxacin	CFX	192	µg/ml
	Clarithromycin	CLAR	195	µg/ml
	Clavulanate	CLAV	187	µg/ml
	Clindamycin	CM	199	µg/ml
	Doxycycline	DOX	266	µg/ml
	Meropenem	MEROP	199	µg/ml
	Ofloxacin	OFX	209	µg/ml
	Trimethoprim	TRIM	279	µg/ml
ABIO-IS	Trimethoprim-D9	TRIM-D9	6.6	µg/ml
	Atenolol-D7	ATL-D7	4.3	µg/ml
	Ciprofloxacin-D8	CFX-D8	7.9	µg/ml
	Dihydrocarbamazepine	DHCBZ	25.0	µg/ml

Table 32: Final compound concentrations in calibration set

calibration	AMOX	AMP	AZI	CFT	CFX	CLAR	CLAV	CM	DOX	MEROP	OFX	TRIM
standards	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l
STD000	0	0	0	0	0	0	0	0	0	0	0	0
STD020	188	164	226	184	192	195	187	199	266	199	209	279
STD040	375	328	453	367	384	390	374	398	532	397	419	559
STD060	563	492	679	551	576	585	561	597	798	596	628	838
STD080	751	655	905	735	768	779	748	796	1063	794	838	1117
STD100	938	819	1131	918	960	974	935	995	1329	993	1047	1397

Table 33:Final internal standard compound concentration.

IS concentration	TRIM-D9	ATL-D7	CFX-D8	DHCBZ
	µg/l	µg/l	µg/l	µg/l
STD	331	215	393	1250

Appendix 2: LC-MS method details

Table 34:Automatic Liquid Sampler (ALS) settings

Stoptime	As Pump
Posttime	Off
Drawspeed	400
Ejectspeed	400
InjVolume	5 µl
Injectmode	Needle Wash
Washvial	99
Tray temp (°C)	6
OverlapTime	Overlap when flushed

Table 35:Binary Pump (BinPump) settings

SolventA1	MQ with AmmoniumFormate buffer and Oxalic Acid
SolventB1	MeCN + 0.1% Formic Acid
Stoptime	10 min
Posttime	Off
Flow	0.6 ml/min
SolvRatioA	95%
PressureMax	300 bar

BinPump		Timetable	
Time	Flow	MaxP	B%
0.0	0.6	300	5
1.0	0.6	300	5
3.0	0.6	300	15
6.0	0.6	300	98
6.6	0.6	300	98
6.8	0.6	300	5
10.0	0.6	300	5

Table 36: Mass Analyser (MS) settings

sourceParameter	
DGasHeater	320
DGasFlow	10
NebulizerPressure	50
VCap	4000

segment	Time	ionMode	ionPolarity	scanType	Valve	Save Data	deltaEMV
1	0.0	ESI	Positive	MRM	ToWaste	No	0
2	0.8	ESI	Positive	MRM	ToMS	Yes	500
3	8.0	ESI	Positive	MRM	ToWaste	No	0

Table 36: continued

segment	Name	ms1-Mz	ms1-Res	ms2-Mz	ms2-Res	dwell	FV	CE
1	Compound1	350.0	Unit	200.0	Unit	200	135	0
2	AZI-749-591	749.5	Unit	591.4	Unit	20	100	30
2	AZI-749-158	749.5	Unit	158.1	Unit	20	100	40
2	CLAR-748-158	748.5	Unit	158.1	Unit	20	150	28
2	CLAR-748-116	748.5	Unit	116.1	Unit	20	150	45
2	CFT-456-396	456.2	Unit	396.2	Unit	20	110	4
2	CFT-456-324	456.2	Unit	324.2	Unit	20	110	8
2	CFT-456-167	456.2	Unit	167.1	Unit	20	110	15
2	DOX-445-428	445.2	Unit	428	Unit	20	150	13
2	DOX-445-154	445.2	Unit	154.1	Unit	20	150	25
2	CM-425-126	425.3	Unit	126	Unit	20	110	30
2	MEROP-384-254	384.2	Unit	254	Unit	20	110	13
2	MEROP-384-141	384.2	Unit	141	Unit	20	110	13
2	AMOX-366-349	366.1	Unit	349.1	Unit	20	100	3
2	AMOX-366-208	366.1	Unit	208	Unit	20	100	8
2	OFX-362-318	362.3	Unit	318.2	Unit	20	120	16
2	OFX-362-261	362.3	Unit	261.2	Unit	20	120	28
2	AMP-350-160	350.1	Unit	160.1	Unit	20	100	10
2	AMP-350-106	350.1	Unit	106.1	Unit	20	100	22
2	CFX-D8-340-322	340	Unit	322	Unit	20	130	17
2	CFX-332-314	332.1	Unit	314.1	Unit	20	160	20
2	TRIM-D9-300-264	300	Unit	264	Unit	20	145	26
2	TRIM-291-275	291.1	Unit	275.1	Unit	20	140	24
2	TRIM-291-261	291.1	Unit	261.1	Unit	20	140	24
2	AT-D7-274-145	274	Unit	145	Unit	20	125	19
2	DIA-245	261.2	Unit	245.2	Unit	10	155	16
2	DHCBZ-194	239.2	Unit	194.2	Unit	10	160	22
2	CLAV-200-156	200	Unit	156	Unit	50	70	2
2	CLAV-200-112	200	Unit	112	Unit	50	70	6
3	Compound1	350.0	Unit	200.0	Unit	200	135	0

Figure 31: Standard chromatogram

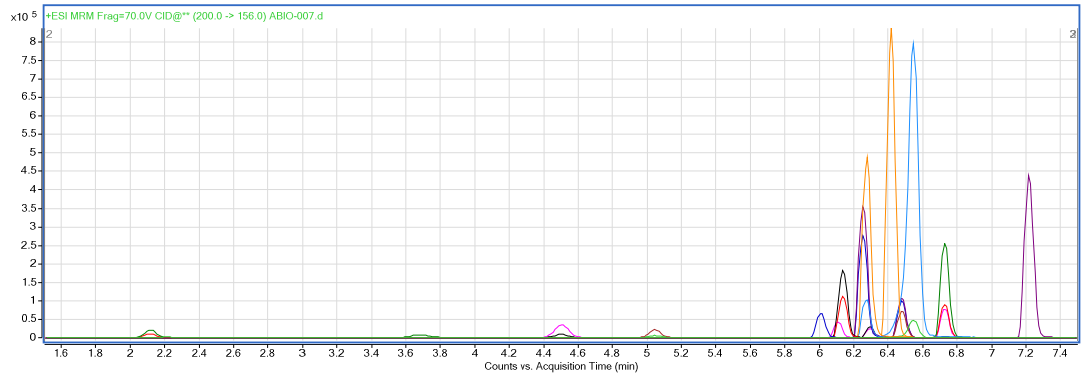

Table 37: Compounds with according retention times

RT	Compound
2.13	Amoxicillin
2.26	Clavulanate
3.51	Atenolol-D7
4.44	Meropenem
5.04	Ampicillin
6.07	Trimethoprim-D9
6.10	Trimethoprim
6.24	Ofloxacin
6.26	Ciprofloxacin-D8
6.26	Ciprofloxacin
6.28	Azithromycin
6.41	Clindamycin
6.52	Cefotaxime
6.55	Doxycycline
6.72	Clarithromycin
7.23	Dihydrocarbamazepine

D. Appendix II

Table 38: CAS numbers of used chemicals

Chemical	Additional information	Purity	CAS	Distributer
1-Decanol	for synthesis	≥99%	112-30-1	Merck KGaA
1-Dodecanol	for synthesis	≥98%	112-53-8	Merck KGaA
Acetonitril	HiPerSolv Chromanorm for HPLC/LCMS grade	≥99.9%	75-05-8	VWR
Aluminium sulphate 14 hydrate	GPR RECTAPUR		16828-12-9	VWR
Citric acid monohydrate		99.5%	5949-29-1	BOOM BV
Copper(II) sulphate pentahydrate	AnalaR NORMAPUR		7758-99-8	VWR
di-Sodium hydrogen phophat	GPR Rectapur	100%	7558-79-4	VWR
Formic acid	ACS reagent	≥96%	64-18-6	Sigma Aldrich
Methanol	HiPerSolv Chromanorm for HPLC/LCMS grade	≥99.9%	67-56-1	VWR
Na_2EDTA		≥97%	6381-92-6	Sigma Aldrich
n-Buthyl acetate	EMPURA	≥99%	123-86-4	Merck KGaA
n-Hexane	AnalR Normapur	≥97%	110-54-3	Merck KGaA
Oxalic acid dihydrate		98%	6153-56-6	Alfa Aesar
Sodium hydrogen carbonate	AnalR Normapur	100%	144-55-8	VWR
Sodiumcarbonate anhydrous	AnalR Normapur	100%	497-19-8	VWR
Tricaprylylmethylammoniumchloride	Aliquat 126		63393-96-4	Sigma Aldrich
tri-Sodium citrate dihydrate	Normapur	≥99.9%	6132-04-3	VWR

E. Appendix III

Results of modified dilution method

Table 39: Results for Digest 20170727 (MD for ≥ 5% solid content)

AB	Conc. [ng g^{-1} ww]	St. Dev. [ng g^{-1} ww]	RSD [%, ww]	Conc. [ng g^{-1} dw]	St. Dev. [ng g^{-1} dw]	RSD [%, dw]	Recovery [%]	corrected conc. [ng kg^{-1} ww]
AMOX	0	0	-217	-1.4	3.1	-217	38	0
AMP	0	0	115	0.5	0.6	115	36	76
AZI	53	4	7	989.3	69.3	7	150	53425
BaC12	398	31	8	7361.7	577.2	8	-160	0
BaC14	180	21	12	3334.1	389.1	12	-270	0
CFT	0	0	-1100	0.0	0.0	-1100	32	0
CFX	61	2	3	1128.2	35.6	3	8	60921
CLAR	0	0	23	6.0	1.4	23	97	333
CM	0	0	16	2.4	0.4	16	78	165
DM	0	0	0	0.0	0.0	0	66	0
DOX	53	2	4	989.4	35.5	4	55	97170
ERYT	4	1	15	79.0	11.8	15	92	4617
FLUMEQ	1	0	2	11.2	0.2	2	82	739
GAPE	1	0	17	12.1	2.0	17	91	716
LINCOM	0	0	103	1.2	1.2	103	80	79
OFX	4	0	4	72.9	3.1	4	33	11926
OTETR	3	0	8	59.1	4.4	8	56	5674
PENG	0	0	11	0.8	0.1	11	68	61
PENV	0	0	80	0.3	0.3	80	70	25
SMO	0	0	0	-0.7	0.0	0	33	-110
SUCLOP	0	0	112	0.5	0.5	112	24	106
SUCRAL	2	1	36	35.4	12.8	36	7	29393
SUDOX	0	0	386	0.2	0.6	386	63	13
SULFAM	0	0	112	0.4	0.4	112	50	40
SULPYR	6	1	13	116.4	15.2	13	83	7594
TETR	30	1	2	552.5	11.3	2	38	78338
TILMIC	0	0	121	0.1	0.1	121	173	3
TRIM	0	0	11	1.7	0.2	11	64	142
TYLOS	0	0	37	6.4	2.3	37	98	350

Table 40: Results for Granular 20170727 (MD for ≥ 5% solid content)

AB	Conc. [ng g^{-1} ww]	St. Dev. [ng g^{-1} ww]	RSD [%, ww]	Conc. [ng g^{-1} dw]	St. Dev. [ng g^{-1} dw]	RSD [%, dw]	Recovery [%]	corrected conc. [ng kg^{-1} ww]
AMOX	0.4	0.0	3	8.7	0.3	3	53	764
AMP	0.0	0.0	-532	0.0	0.1	-532	60	-1
AZI	10.8	0.6	6	232.2	13.4	6	139	10819
BaC12	12.8	0.8	6	275.5	17.7	6	30	42562
BaC14	4.1	0.1	3	87.2	2.9	3	16	25716
CFT	0.0	0.0	235	0.0	0.1	235	59	2
CFX	99.6	3.6	4	2136.4	77.5	4	53	187965
CLAR	0.6	0.1	8	13.7	1.1	8	101	638
CM	0.6	0.0	8	11.9	1.0	8	94	593
DM	0.0	0.0	0	0.0	0.0	0	88	0
DOX	27.4	0.8	3	587.2	17.5	3	87	31601
ERYT	1.5	1.5	100	32.7	32.8	100	93	1634
FLUMEQ	0.0	0.0	-53	-0.9	0.5	-53	89	-48
GAPE	1.6	0.3	17	34.4	6.0	17	93	1715
LINCOM	0.0	0.0	153	0.6	0.9	153	90	30
OFX	4.0	0.9	23	84.9	19.3	23	70	5636
OTETR	0.3	0.1	29	5.7	1.7	29	81	327
PENG	0.0	0.0	260	0.1	0.2	260	85	5
PENV	0.0	0.0	52	0.7	0.4	52	83	39
SMO	0.0	0.0	-95	-0.4	0.3	-95	63	-27
SUCLOP	0.0	0.0	-37	-0.3	0.1	-37	55	-23
SUCRAL	2.0	0.9	45	42.4	19.1	45	26	7653
SUDOX	0.0	0.0	-111	-0.2	0.3	-111	78	-14
SULFAM	0.0	0.0	113	0.4	0.4	113	67	27
SULPYR	2.0	0.1	3	43.9	1.5	3	79	2586
TETR	24.9	2.8	11	533.9	60.7	11	49	50512
TILMIC	0.0	0.0	103	0.4	0.5	103	175	21
TRIM	0.0	0.0	-4	-0.4	0.0	-4	82	-23
TYLOS	0.1	0.1	95	1.7	1.6	95	100	79

Table 41: Results for Stage A 20170727 (MD for ≤ 5% solid content)

AB	Conc. [ng mL^{-1} ww]	St. Dev. [ng mL^{-1} ww]	RSD [%, ww]	Conc. [ng g^{-1} dw]	St. Dev. [ng g^{-1} dw]	RSD [%, dw]	Recovery [%]	corrected conc. [ng L^{-1}]
AMOX	0.0	0.0	373	1.3	4.9	373	67	13
AMP	0.0	0.0	135	0.2	0.2	135	73	2
AZI	18.6	8.4	45	1861.7	842.9	45	150	18617
BaC12	48.8	58.1	119	4876.4	5812.9	119	72	67737
BaC14	0.0	3.7	0	0.0	366.3	0	67	0
CFT	0.0	0.0	100	1.6	1.6	100	63	25
CFX	21.7	1.4	6	2167.1	138.9	6	38	56564
CLAR	0.2	0.0	13	23.5	3.0	13	139	235
CM	0.2	0.1	43	17.3	7.4	43	87	199
DM	0.0	0.0	0	0.0	0.6	0	87	0
DOX	6.5	1.2	19	650.5	124.9	19	78	8327
ERYT	0.3	0.2	80	30.3	24.4	80	107	303
FLUMEQ	0.0	0.0	35	0.3	0.1	35	87	3
GAPE	4.2	0.7	17	422.0	73.2	17	96	4377
LINCOM	0.0	0.0	90	4.2	3.8	90	91	47
OFX	1.0	0.5	52	96.2	49.9	52	67	1433
OTETR	0.1	0.0	42	9.6	4.0	42	72	133
PENG	0.0	0.0	52	1.9	1.0	52	99	19
PENV	0.0	0.0	14	2.5	0.3	14	179	25
SMO	0.4	0.1	35	38.6	13.6	0	69	557
SUCLOP	0.0	0.0	0	0.0	0.4	0	70	0
SUCRAL	7.7	3.1	40	767.0	306.9	40	18	42874
SUDOX	0.0	0.0	188	0.3	0.5	188	83	3
SULFAM	0.0	0.0	0	0.0	0.5	0	71	0
SULPYR	1.0	0.3	27	97.2	26.0	27	99	981
TETR	4.0	1.4	34	403.3	135.6	34	59	6829
TILMIC	0.4	0.1	30	37.0	11.1	30	170	370
TRIM	0.3	0.1	28	32.6	9.1	28	75	432
TYLOS	0.0	0.1	0	0.0	7.0	0	163	0

Table 42: Results for Stage B 20170727 (MD for ≤ 5% solid content)

AB	Conc. [ng mL^{-1} ww]	St. Dev. [ng mL^{-1} ww]	RSD [%, ww]	Conc. [ng g^{-1} dw]	St. Dev. [ng g^{-1} dw]	RSD [%, dw]	Recovery [%]	corrected conc. [ng L^{-1}]
AMOX	0.5	0.0	4%	53.04	1.87	4%	64%	830
AMP	0.0	0.0	27%	0.56	0.15	27%	77%	7
AZI	17.2	9.9	57%	1721.97	985.85	57%	172%	17220
BaC12	49.5	57.9	117%	4945.38	5792.68	117%	99%	49917
BaC14	0.0	3.5	0%	0.00	349.31	0%	24%	0
CFT	0.0	0.0	72%	0.04	0.03	72%	68%	1
CFX	18.8	2.6	14%	1883.94	259.89	14%	47%	39850
CLAR	0.3	0.0	8%	25.44	1.97	8%	156%	254
CM	0.2	0.1	29%	21.62	6.18	29%	100%	216
DM	0.4	0.5	132%	37.47	49.37	132%	95%	393
DOX	6.7	0.8	12%	673.13	81.86	12%	87%	7729
ERYT	0.3	0.3	110%	31.04	34.15	110%	123%	310
FLUMEQ	0.0	0.0	0%	0.00	0.42	0%	101%	0
GAPE	4.1	0.7	16%	413.35	66.79	16%	107%	4134
LINCOM	0.0	0.0	93%	3.57	3.33	93%	100%	36
OFX	1.3	0.2	17%	126.78	21.50	17%	74%	1713
OTETR	0.1	0.1	78%	9.09	7.11	78%	81%	112
PENG	0.0	0.0	0%	0.00	0.44	0%	112%	0
PENV	0.1	0.1	175%	7.89	13.83	175%	140%	79
SMO	0.6	0.2	26%	57.02	15.01	26%	78%	733
SUCLOP	0.0	0.0	102%	0.51	0.52	102%	77%	7
SUCRAL	7.6	2.2	29%	758.26	222.44	29%	20%	38142
SUDOX	0.0	0.0	129%	0.24	0.31	129%	97%	2
SULFAM	0.0	0.0	64%	1.94	1.25	64%	78%	25
SULPYR	1.0	0.2	19%	95.04	18.10	19%	111%	950
TETR	4.0	1.0	25%	402.43	101.23	25%	92%	4351
TILMIC	0.1	0.1	102%	8.50	8.66	102%	160%	85
TRIM	0.4	0.3	96%	36.43	34.94	96%	88%	416
TYLOS	0.7	0.8	107%	71.95	76.77	107%	181%	719

Table 43:Results for Digest 20170727 (MD for ≥ 5% solid content, after modification)

AB	Conc. [ng g^{-1} ww]	St. Dev. [ng g^{-1} ww]	RSD [%, ww]	Conc. [ng g^{-1} dw]	St. Dev. [ng g^{-1} dw]	RSD [%, dw]	Recovery [%]	corrected conc. [ng kg^{-1}]
AMOX	2.0	1.6	79	37.8	30.0	79	36	5717
AMP	0.3	0.1	35	5.2	1.8	35	29	962
AZI	55.5	1.6	3	1028.3	29.0	3	143	55528
BaC12	725.6	15.2	2	13437.9	282.3	2	49	1466029
BaC14	443.1	14.8	3	8205.2	274.4	3	239	185210
CFT	0.0	0.0	97	0.2	0.2	97	25	44
CFX	98.0	0.3	0	1814.9	5.5	0	29	98006
CLAR	0.2	0.0	5	3.1	0.2	5	50	330
CM	0.1	0.0	14	2.2	0.3	14	55	222
DM	0.1	0.0	31	2.2	0.7	31	40	304
DOX	44.8	0.1	0	830.4	2.1	0	45	99593
ERYT	2.3	0.6	25	42.8	10.8	25	42	5497
FLUMEQ	0.5	0.1	12	8.6	1.0	12	70	665
GAPE	0.8	0.1	9	15.6	1.4	9	80	1058
LINCOM	0.1	0.0	2	2.4	0.1	2	59	218
OFX	5.6	0.0	0	103.3	0.3	0	38	14806
OTETR	3.0	0.3	10	55.8	5.5	10	48	6255
PENG	0.0	0.0	38	0.7	0.3	38	44	87
PENV	0.0	0.0	17	0.7	0.1	17	83	47
SMO	-0.1	0.0	-11	-1.5	0.2	-11	3	-2464
SUCLOP	0.0	0.0	-30	-0.8	0.2	-30	2	-1892
SUCRAL	3.3	0.2	8	61.2	4.6	8	1	278741
SUDOX	0.0	0.0	-65	-0.2	0.2	-65	5	-248
SULFAM	0.0	0.0	-37	-0.3	0.1	-37	5	-346
SULPYR	0.6	0.0	1	10.5	0.1	1	9	6481
TETR	25.9	0.7	3	479.2	12.5	3	24	107637
TILMIC	0.3	0.3	101	5.3	5.3	101	182	156
TRIM	-0.1	0.0	-7	-1.5	0.1	-7	54	-153
TYLOS	0.7	0.3	47	13.5	6.4	47	23	3232

Table 44: Results for Stage B 20170727 (MD for ≤ 5% solid content, after modification)

AB	Conc. [ng g^{-1} ww]	St. Dev. [ng g^{-1} ww]	RSD [%, ww]	Conc. [ng g^{-1} dw]	St. Dev. [ng g^{-1} dw]	RSD [%, dw]	Recovery [%]	corrected conc. [ng kg^{-1}]
AMOX	0.1	0.1	81	10.4	8.4	81	67	155
AMP	0.0	0.0	-6	-0.5	0.0	-6	82	-6
AZI	9.8	0.1	1	976.4	5.7	1	141	9764
BaC12	9.0	0.7	8	897.5	73.2	8	137	6543
BaC14	12.8	0.9	7	1281.3	92.1	7	245	5234
CFT	0.0	0.0	-7	0.0	0.0	-7	76	0
CFX	21.9	0.5	2	2187.7	54.4	2	74	29378
CLAR	0.0	0.0	9	4.1	0.4	9	77	53
CM	0.1	0.0	2	11.2	0.2	2	92	122
DM	-0.2	0.0	-17	-15.5	2.7	-17	61	-252
DOX	4.2	0.3	8	424.1	33.4	8	88	4795
ERYT	0.4	0.4	96	41.6	39.8	96	64	646
FLUMEQ	0.0	0.0	-32	-1.7	0.6	-32	99	-18
GAPE	1.2	0.1	9	123.7	10.8	9	93	1333
LINCOM	0.1	0.1	101	13.0	13.2	101	93	141
OFX	1.1	0.2	18	108.3	19.5	18	93	1170
OTETR	0.1	0.0	29	8.7	2.5	29	89	98
PENG	0.0	0.0	-151	-0.8	1.2	-151	95	-8
PENV	0.0	0.0	52	0.5	0.3	52	135	4
SMO	0.0	0.0	-52	-2.4	1.3	-52	11	-220
SUCLOP	0.0	0.0	-10	-2.0	0.2	-10	8	-234
SUCRAL	4.6	1.0	23	455.4	104.9	23	35	12842
SUDOX	0.0	0.0	-17	-1.9	0.3	-17	11	-169
SULFAM	0.0	0.0	962	0.1	0.8	962	9	9
SULPYR	0.0	0.0	-45	-1.1	0.5	-45	11	-98
TETR	2.0	0.2	10	202.3	20.3	10	85	2372
TILMIC	0.0	0.0	114	0.4	0.5	114	226	2
TRIM	0.0	0.0	-147	-1.1	1.7	-147	81	-14
TYLOS	0.2	0.1	67	16.4	11.0	67	33	501

Table 45: Results for Delft activated sludge 20170808 (MD for ≥ 5% solid content)

AB	Conc. [ng g^{-}w]	St. Dev. [ng mL^{-1}]	RSD [%, ww]	Conc. [ng $^{-1}$ dw]	St. Dev. [ng g^{-1} dw]	RSD [%, dw]	Recovery [%]	corrected conc. [ng L^{-1}]
AMOX	0.1	0.0	72	12.9	9.4	72	81	80
AMP	0.0	0.0	-17	-1.9	0.3	-17	108	-9
AZI	0.8	0.2	25	169.4	41.6	25	144	847
BaC12	1.7	0.1	9	332.1	29.0	9	87	1904
BaC14	1.6	0.1	4	324.4	13.7	4	102	1622
CFT	0.0	0.0	99	0.1	0.1	99	82	1
CFX	14.5	0.3	2	2892.7	50.1	2	101	14463
CLAR	0.2	0.0	8	39.2	3.3	8	112	196
CM	0.1	0.0	9	10.6	0.9	9	91	58
DM	0.1	0.0	74	12.9	9.5	74	91	71
DOX	2.4	0.1	5	473.3	22.2	5	98	2403
ERYT	0.2	0.2	100	31.1	31.1	100	169	155
FLUMEQ	0.0	0.0	290	0.4	1.0	290	96	2
GAPE	2.1	0.0	2	419.2	7.0	2	93	2259
LINCOM	0.0	0.0	27	1.1	0.3	27	91	6
OFX	1.8	0.3	16	351.8	56.1	16	111	1759
OTETR	0.1	0.0	2	19.3	0.3	2	90	108
PENG	0.0	0.0	55	2.2	1.2	55	98	11
PENV	0.0	0.0	31	4.5	1.4	31	99	23
SMO	0.1	0.0	3	14.7	0.5	3	80	92
SUCLOP	0.0	0.0	337	0.8	2.6	337	81	5
SUCRAL	10.9	0.6	5	2178.6	112.8	5	61	17946
SUDOX	0.0	0.0	-66	-2.5	1.7	-66	87	-15
SULFAM	0.0	0.0	155	1.4	2.2	155	83	8
SULPYR	0.3	0.0	1	55.6	0.5	1	91	305
TETR	2.0	0.1	7	392.6	27.4	7	84	2344
TILMIC	0.0	0.0	124	1.6	1.9	124	169	8
TRIM	0.0	0.0	101	9.0	9.1	101	88	51
TYLOS	0.0	0.0	0	-2.2	0.0	0	120	-11

Table 46: Results for Delft digested sludge 20170808 (MD for ≥ 5% solid content)

AB	Conc. [ng g^{-}w]	St. Dev. [ng mL^{-1}]	RSD [%, ww]	Conc. [ng $^{-1}$ dw]	St. Dev. [ng g^{-1} dw]	RSD [%, dw]	Recovery [%]	corrected conc. [ng L^{-1}]
AMOX	0.3	0.1	51	7.4	3.8	51	78	333
AMP	0.0	0.0	-18	-1.3	0.2	-18	102	-46
AZI	17.4	7.1	41	495.9	204.2	41	146	17358
BaC12	388.4	4.8	1	11098.2	137.9	1	41	954718
BaC14	181.7	9.6	5	5190.9	275.0	5	43	420763
CFT	0.0	0.0	502	0.0	0.0	502	70	0
CFX	140.3	0.9	1	4009.9	26.7	1	65	217360
CLAR	0.6	0.0	3	17.2	0.5	3	110	601
CM	0.1	0.0	3	1.7	0.1	3	83	72
DM	0.0	0.2	-855	-0.8	6.5	-855	84	-32
DOX	36.7	1.4	4	1048.8	38.9	4	84	43884
ERYT	0.2	0.2	105	6.2	6.5	105	150	217
FLUMEQ	0.0	0.0	244	0.1	0.3	244	90	5
GAPE	1.9	0.0	1	54.7	0.7	1	88	2165
LINCOM	0.0	0.0	28	1.2	0.3	28	87	49
OFX	13.9	1.2	9	397.9	34.7	9	85	16411
OTETR	0.8	0.4	46	22.8	10.4	46	79	1011
PENG	0.1	0.0	47	1.4	0.7	47	94	53
PENV	0.0	0.0	-282	-0.1	0.2	-282	98	-3
SMO	-0.1	0.0	-4	-2.3	0.1	-4	65	-127
SUCLOP	0.0	0.0	-129	-0.6	0.8	-129	65	-35
SUCRAL	7.2	1.2	16	204.4	33.3	16	36	19857
SUDOX	0.0	0.0	-94	-0.4	0.3	-94	81	-16
SULFAM	0.1	0.0	24	1.8	0.4	24	80	80
SULPYR	3.1	0.0	1	89.0	1.0	1	91	3431
TETR	24.6	1.9	8	702.0	55.1	8	83	29612
TILMIC	0.0	0.0	515	0.1	0.4	515	160	2
TRIM	0.0	0.0	117	0.3	0.3	117	75	13
TYLOS	0.2	0.2	135	4.7	6.4	135	113	165

Table 47: Results for Leeuwarden activated sludge 20170808 (MD for ≥ 5% solid content)

AB	Conc. [ng g^{-}w]	St. Dev. [ng mL^{-1}]	RSD [%, ww]	Conc. [ng $^{-1}$ dw]	St. Dev. [ng g^{-1} dw]	RSD [%, dw]	Recovery [%]	corrected conc. [ng L^{-1}]
AMOX	0.0	0.0	17	5.2	0.9	17	85	31
AMP	0.0	0.0	0	-2.2	0.0	0	91	-12
AZI	2.5	0.2	10	491.1	47.6	10	133	2455
BaC12	5.6	0.2	4	1119.1	48.1	4	73	7702
BaC14	2.9	0.1	3	584.2	15.5	3	97	3009
CFT	0.0	0.0	538	0.0	0.0	538	78	0
CFX	11.2	0.3	3	2233.6	63.5	3	94	11944
CLAR	0.0	0.0	16	5.7	0.9	16	109	28
CM	0.0	0.0	7	5.4	0.4	7	90	30
DM	0.1	0.1	124	17.1	21.3	124	92	94
DOX	1.9	0.0	1	374.1	4.8	1	100	1870
ERYT	0.0	0.0	106	5.5	5.8	106	148	27
FLUMEQ	0.0	0.0	112	2.0	2.2	112	94	11
GAPE	0.5	0.1	11	109.9	12.3	11	89	618
LINCOM	0.0	0.0	62	1.2	0.7	62	87	7
OFX	0.4	0.0	4	89.0	3.8	4	106	445
OTETR	0.0	0.0	44	3.5	1.5	44	91	19
PENG	0.0	0.0	-1151	0.0	0.2	-1151	98	0
PENV	0.0	0.0	-194	-0.1	0.3	-194	99	-1
SMO	0.1	0.0	10	10.5	1.1	10	82	64
SUCLOP	0.0	0.0	-117	-1.0	1.1	-117	84	-6
SUCRAL	3.7	0.1	3	739.2	25.0	3	48	7699
SUDOX	0.0	0.0	-38	-3.0	1.1	-38	89	-17
SULFAM	0.0	0.0	-205	-0.3	0.5	-205	83	-2
SULPYR	0.2	0.0	7	37.9	2.6	7	88	214
TETR	1.1	0.0	1	221.0	1.8	1	95	1163
TILMIC	0.0	0.0	-28	-0.2	0.1	-28	151	-1
TRIM	0.0	0.0	58	6.9	4.0	58	84	41
TYLOS	0.1	0.1	108	12.9	13.9	108	121	64

Table 48: Results for Leeuwarden digested sludge 20170808 (MD for ≥ 5% solid content)

AB	Conc. [ng g^{-}w]	St. Dev. [ng mL^{-1}]	RSD [%, ww]	Conc. [ng $^{-1}$ dw]	St. Dev. [ng g^{-1} dw]	RSD [%, dw]	Recovery [%]	corrected conc. [ng L^{-1}]
AMOX	0.3	0.1	25	6.9	1.7	25	75	402
AMP	0.0	0.0	-586	-0.2	1.0	-586	88	-9
AZI	2.1	0.7	33	47.4	15.6	33	143	2085
BaC12	171.5	0.9	1	3897.0	21.3	1	48	358562
BaC14	71.2	1.3	2	1618.9	28.6	2	59	120408
CFT	0.1	0.1	100	2.6	2.6	100	67	173
CFX	77.7	0.5	1	1765.5	12.2	1	46	167351
CLAR	0.0	0.0	-2	-0.4	0.0	-2	111	-18
CM	0.0	0.0	-17	-0.2	0.0	-17	81	-11
DM	0.7	0.1	22	15.2	3.3	22	80	839
DOX	27.8	2.0	7	632.8	46.3	7	85	32843
ERYT	2.9	2.9	100	66.2	66.4	100	150	2913
FLUMEQ	0.0	0.0	-238	-0.2	0.5	-238	89	-10
GAPE	0.6	0.1	17	14.4	2.5	17	88	717
LINCOM	0.0	0.0	50	0.4	0.2	50	81	21
OFX	6.5	0.9	13	148.7	19.4	13	74	8810
OTETR	0.6	0.3	42	14.0	5.8	42	71	862
PENG	0.0	0.0	62	0.7	0.4	62	95	33
PENV	0.0	0.0	233	0.2	0.5	233	99	9
SMO	0.0	0.0	-118	-0.9	1.1	-118	66	-61
SUCLOP	0.0	0.0	-199	-0.4	0.8	-199	67	-28
SUCRAL	5.2	3.2	62	118.2	73.7	62	33	15854
SUDOX	0.0	0.0	-50	-0.8	0.4	-50	84	-40
SULFAM	0.0	0.1	133	1.0	1.4	133	77	59
SULPYR	3.5	0.0	0	79.8	0.1	0	89	3955
TETR	6.5	0.7	11	148.8	15.7	11	66	9987
TILMIC	0.5	0.5	101	10.3	10.4	101	163	278
TRIM	0.0	0.0	-184	0.0	0.0	-184	77	0
TYLOS	0.1	0.1	127	2.1	2.7	127	113	95