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MASTER'S THESIS

Analysis of the Antibiotic Ampicillin in Activated Sludge:

Method Development and Adsorption Studies

Mari Wøien Håland

10th of June, 2019

ABSTRACT

The presence of antibiotics in wastewater treatment plants is a concern, both because of potential release to natural water systems, and connected to the spread of antibiotic resistance genes. In this thesis, the focus is on the β-lactam antibiotic ampicillin in activated sludge.

A method for analysing ampicillin in wastewater samples was found in literature, adapted, and tested using concentrations in the 0.050-1.0 mg/L range. Key steps in the method are sample preparation by solid phase extraction (SPE), and analysis by high performance liquid chromatography (HPLC) with UV-detection. Automated SPE by a RapidTrace® instrument was employed. β-lactam antibiotics cloxacillin and dicloxacillin were used as internal standards.

When applied, the method measured ampicillin concentrations down to 10 µg/L. Recovery from SPE on wastewater effluent samples was found to be 75% or greater, with high precision. A version of the method for larger sample sizes proved time-consuming.

The developed method was used to study the fate of ampicillin in activated sludge, with a focus on adsorption. This included measuring removal over time and an attempt at equilibrium modelling using Freundlich and Langmuir adsorption isotherms. The results from these studies were largely inconclusive. However, removal of ampicillin in the sludge was found to be high, for 1-3 g/L mixed liquor suspended solids (MLSS). Some of the removal was shown to be due to chemical degradation.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

BOD_5 = Biological Oxygen Demand (5 day assay)

COD = Chemical Oxygen Demand

DAD = Diode Array Detection

HRT = Hydraulic Retention Time

HPLC = High Performance Liquid Chromatography

IVAR = Interkommunalt Vann, Avløp og Renovasjon

MBR = Membrane Bioreactor

MLSS = Mixed Liquor Suspended Solids

MS = Mass Spectrometry

SBR = Sequencing Batch Reactor

SNJ = Sentralrenseanlegg Nord-Jæren

SPE = Solid Phase Extraction

SRT = Solids Retention Time

TBA = Tetra-*n*-butylammonium hydrogen sulphate

TFA = Trifluoroacetic acid

WWTP = Wastewater Treatment Plant

1 INTRODUCTION

"But I would like to sound one note of warning…"

With these words, spoken in his Nobel Lecture in 1945 [1], Sir Alexander Fleming began his caution to the world of the potential dangers of emerging antibiotic resistance. He had already observed development of resistance in bacteria exposed to non-lethal concentrations. Widespread use of antibiotics posed the risk of this happening on a large scale.

In many ways, the world did not heed Fleming's warning. Antibiotic resistance is one of many crises facing society today, killing an estimated 23 000 people each year in the USA alone [2].

Wastewater treatment plants are important in the context of antibiotic resistance. In biological treatment, dense bacterial communities are present. When sub-inhibitory concentrations of antibiotics are added to the mix, there is concern that this provides a favourable environment for the emergence and spread of antibiotic resistance genes [3]. In addition to the consequences of releasing resistance genes, emission of antibiotics from these plants may have detrimental effects on ecosystems and human health [4, 5].

This thesis is part of a research project seeking to model the spread of antibiotic resistance genes in wastewater treatment plants. However, genes and their spread are not the focus of this work. Rather, the attention is on the antibiotic ampicillin. This compound belongs to the β-lactam antibiotics, and more specifically to the penicillins [6, p. 838]. These antibiotics are the most commonly used in Europe [7].

As ampicillin and resistance genes to this antibiotic have been chosen as model compounds for the larger research project, a reliable analysis method for the antibiotic in wastewater was required. The development and testing of a method from literature [8] was the first part of the work described in this thesis.

Furthermore, as previous research [9-12] shows that ampicillin is largely removed in biological wastewater treatment, the fate of the antibiotic in activated sludge was of interest. Thus, the study of its removal became the second part of this thesis work. Focus was on adsorption to sludge. This is stated in research [9-11] as a major removal pathway for ampicillin in wastewater treatment.

2 BACKGROUND

In this chapter, theoretical background relevant for the thesis is presented. First, a brief introduction to antibiotics and antibiotic resistance is given, before an overview of biological wastewater treatment and a description of the Sequencing Batch Reactor (SBR). More details on the fate and analysis of β-lactam ampicillin in wastewater follows. The two key analysis methods used in this work, solid phase extraction (SPE) and high performance liquid chromatography (HPLC), are then described. Finally, the objectives of the thesis are stated.

2.1 Antibiotics and Antibiotic Resistance

The discovery of penicillin G by Alexander Fleming in the first half of the 20th century heralded the introduction of antibiotics for treatment of bacterial infection. Whether produced naturally by microbes or modified to become semisynthetic, these chemicals became key players in the treatment of bacterial disease, revolutionising this field of medicine [6, pp. 837-838].

The most commonly used antibiotics in all European countries are the penicillins [7]. These are a subgroup of the β-lactam antibiotics, which are characterised by the four-membered β-lactam ring in their chemical structure; they act by inhibiting the cell wall synthesis of bacteria [6, p. 838]. Hydrolysis cleaves the β-lactam ring, and this can be brought about by β-lactamase enzymes [13] produced by resistant bacteria [6, p. 838]. Cleaving the β-lactam ring inactivates the antibiotic [6, p. 843].

Unlike penicillin G, which is mainly effective towards gram-positive bacteria, the semisynthetic derivative ampicillin also works against certain gram-negative bacteria [6, p. 838]. Ampicillin is resistant to acid due to its electron-withdrawing amino (NH_2) substituent [13]. Figure 2.1 shows the chemical structure of ampicillin, with the β-lactam ring to the right.
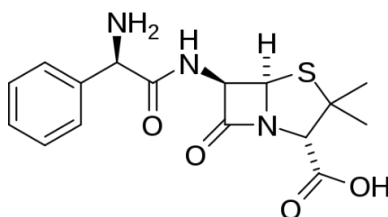
Figure 2.1: Chemical structure of ampicillin
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Three other β-lactams relevant for this thesis work are oxacillin and its chlorinated derivatives cloxacillin and dicloxacillin. Their structures are shown in Figure 2.2.

While antibiotics are currently still important in the treatment of bacterial diseases, natural selection is relentless. Discovery of antibiotic resistance follows in the wake of development of new antibiotics, and each year at least 23 000 people in the USA die due to infections by antibiotic-resistant bacteria [2]. Development of resistance in harmless bacteria is a cause for concern as many microorganisms have the ability to share genes with unrelated, and possibly pathogenic, species [15]. In biological wastewater treatment plants, the mixing of bacteria with sub-inhibitory levels of antibiotics might create a favourable environment for the emergence and spread of antibiotic resistance [3].
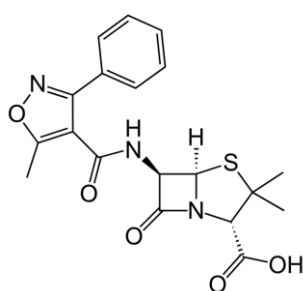
Oxacillin

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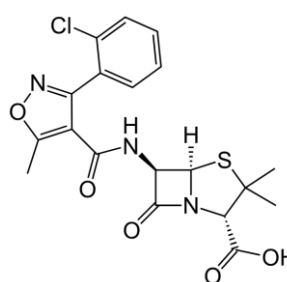
Cloxacillin

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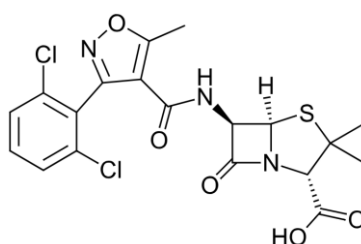
Dicloxacillin

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Figure 2.2: Chemical structures of oxacillin and derivatives

Release of both antibiotics and antibiotic resistant bacteria from wastewater treatment plants (WWTPs) is an issue due to the possible effects on ecosystems, the risk of spread of antibiotic resistance genes, and the effects on human health in the case of water reuse [4, 5].

The following section describes relevant aspects of wastewater treatment for this current work.

2.2 Wastewater Treatment

Wastewater is water that due to contamination (e.g. from domestic or industrial use) should not be reused or discharged into natural waters without some degree of treatment. Wastewater treatment consist of various engineered methods for removing this contamination; these can be physical, chemical or biological [19].

2.2.1 Biological Wastewater Treatment

A way to look at biological wastewater treatment is the act of moving natural processes, for instance aerobic microbial degradation of wastewater constituents, into a controlled environment where they will not have detrimental effects on the surrounding nature. This controlled environment may take numerous shapes, a common one being the activated sludge process. In the typical textbook example [19, 20], this process system consists of a completely mixed aerated bioreactor where suspended microorganisms (the activated sludge) degrade wastewater constituents. This is followed by a clarifier where solids settle to the bottom and clarified effluent decants from the top. A sludge recycling system, from the clarifier underflow to the bioreactor, enables a large concentration of active biomass in the latter. This increases the efficiency of the biological degradation. The system operates under continuous flow, and is typically assumed to be at steady state.

2.2.2 Sequencing Batch Reactors

The Sequencing Batch Reactor (SBR) is a variation of the conventional activated sludge process [19, pp. 701-702]. It is different in how the processes are ordered, in time rather than space [21]. Aeration, mixing and sedimentation occur in the same vessel, at different times in repeating cycles.
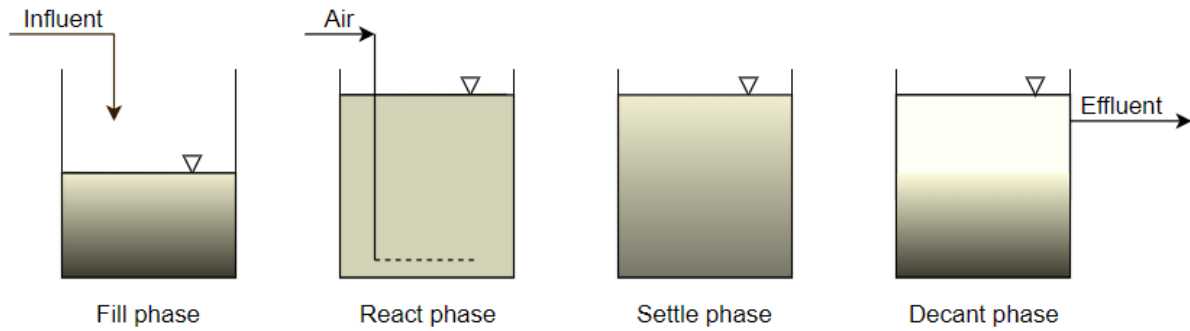
Figure 2.3: Operating phases for a sequencing batch reactor, adapted from [19, p. 702]

There are typically five process phases in a SBR system, as described in [19, p. 771, 22, pp. 2-3]. These are *fill, react, settle, decant,* and *idle*. *Fill* is the loading of wastewater into the reactor; *react* is the phase where required reactions are finalised; *settle* is when the SBR acts like a clarifier, separating solids from treated effluent; *decant* is when effluent is discharged through decanting; and idle is the phase between decant and fill. Together they make up one process cycle for the SBR. The first four operating phases are illustrated in Figure 2.3.

The volume taken out of the SBR during the decant phase equals the volume entering the reactor in the fill phase. The fraction of the total SBR volume used for fill/decant is called the volumetric exchange ratio (f_{exr}), and is related to the hydraulic retention time (HRT) in a single SBR by the following equation:

$$HRT = \frac{t_c}{f_{exr}} \quad (2.1)$$

Where t_c is the time for one cycle [21, 22, p. 3].

Sludge wasting from a SBR can be done at various intervals, and at different points in the cycle [22, p. 2]. If it is done during the react phase, it yields a "uniform discharge of solids" [19, p. 772]. The solids retention time (SRT) is given by the following equation [23]:

$$SRT = \frac{Sludge\ mass\ in\ reactor}{Sludge\ mass\ wasted\ per\ day} \quad (2.2)$$

From this, the SRT in a SBR may be written as:

$$SRT = \frac{\overline{X}_t \cdot V_r}{X_{tw} \cdot Q_w} \quad (2.3)$$

Where $\overline{X_t}$ is the average solids concentration in the reactor during the react phase, V_r is the volume contained in the SBR during the react phase, Q_w is the total volume taken out during the react phase over the course of a day (volume taken out per cycle, times the number of cycles per day), and X_{tw} is the solids concentration in this volume.

This equation assumes that the average mass of solids in the reactor during the react phase is representative for the average solids mass present in the SBR at all times, and that there are negligible solids leaving the SBR during the decant phase. Assuming $\overline{X_t} \approx X_{tw}$, SRT can be approximated by:

$$SRT = \frac{V_r}{Q_w} \quad (2.4)$$

This is similar to the expression for SRT in a continuous flow activated sludge system where wasting is done from the aerated bioreactor, see e.g. Tchobanoglous, et al. [19, p. 727].

In SBRs, having all processes in the same vessel yields advantages such as a minimal footprint and flexibility of operation [24].

2.3 B-lactam Ampicillin in Wastewater

The presence of antibiotics in wastewater and wastewater treatment plants is a cause for concern. In this section, central research on the fate of β-lactam ampicillin in wastewater, as well as on how to analyse it, is summarised.

2.3.1 Fate of Ampicillin in Wastewater Treatment

Both biodegradation and abiotic mechanisms may remove antibiotics in WWTPs [25]. Abiotic mechanisms include sorption, hydrolysis and photolysis. Sorption, the removal of chemical species by interaction with particles, is a key process [25]. Some antibiotics are susceptible to chemical hydrolysis, including the β-lactams [25, 26]. Hydrolysis rates depend on environmental factors, primarily pH and temperature.

Mitchell, et al. [27] found that ampicillin had a hydrolysis half-life, at 25 °C, of 27 days at pH 7 and 31 days at pH 4. At alkaline pH (9) the half-life was much shorter: 6.7 days.

From an aerated batch experiment, mixing antibiotics with activated sludge, Li and Zhang [9] found that all the tested antibiotics, including ampicillin, were stable towards hydrolysis over the course of the experiment (48 h). The pH values in the experiment were kept at around neutral range (6.9-7.3). Furthermore, the study found that removal of ampicillin by the activated sludge process was primarily by adsorption, and that it was complete. After 10 h, no presence of dissolved ampicillin was measured.

Shen, et al. [10] looked at the removal of ampicillin by an airlift biofilm reactor where granulated activated carbon was the biofilm carrier. No ampicillin was measured in the effluent during the study. For a mature biofilm, the article states that 60% of the ampicillin was removed by adsorption, whereas the rest was eliminated by biodegradation.

Interestingly, the 60:40 adsorption:biodegradation ratio appears in another study, by Jia, et al. [11]. By use of a membrane bioreactor (MBR) with both anoxic and aerobic zones, the effect of SRT and HRT on removal of antibiotics was examined. Here too, complete removal of ampicillin was observed for most experiment conditions (lowest removal efficiency 94%). The results from a batch test using sludge from the MBR showed an approximate 60% removal of ampicillin by adsorption, 40% by biodegradation.

As part of a study on the properties of intermediates produced by photocatalytic degradation of antibiotics, Adamek, et al. [28] did a 5-day biological oxygen demand (BOD_5) biodegradability test on the original antibiotics, ampicillin included. Barely any oxygen consumption was found, yielding the conclusion that the biodegradability of the antibiotics was negligible. Unlike the case in [11], where sludge from the antibiotics-exposed MBR was used as inoculum, the microorganisms tested by Adamek, et al. [28] were not adapted to these compounds.

In a study published in 1985, Richardson and Bowron [29] assessed the biodegradability of several pharmaceuticals using tests with high bacterial density. The listing for ampicillin states that it is 48% biodegradable. These tests were said to indicate which pharmaceuticals were likely to survive wastewater treatment, either partly or wholly.

Contrasting with the studies above, Islas-Garcia, et al. [12] found complete mineralisation of ampicillin by denitrifying sludge. The reaction time for complete removal was 12 h, and the study found minimal removal by abiotic processes, including adsorption.

In summary: with one exception, studies indicate that the main removal process of ampicillin in wastewater treatment is by adsorption; with the second most important process being biodegradation. The β-lactam also appears to have a certain stability towards hydrolysis in

mildly acidic or neutral solution. Finally, the removal of ampicillin is complete in most of the studies, for several different treatment setups.

2.3.2 Adsorption and Adsorption Isotherms

Adsorption, in a wide sense, is a compound's tendency to have a higher concentration at an interface than in the bulk phases that form it [30, p. 33]. In the following, the interface is the surface between a solid and a liquid.

The importance of adsorption as a removal mechanism for ampicillin might not be that surprising, considering its log K_{ow}: 1.35 [27, 31]. The following equation shows the relation between K_{oc} and K_{ow}:

$$\log K_{oc} = A \log K_{ow} + B \quad (2.5)$$

Where K_{oc} is the partitioning coefficient between the carbon fraction of sludge and the water, K_{ow} is the *n*-octanol/water partitioning coefficient, and A and B are empirical coefficients [19, p. 667]. Unless the empirical coefficients are small or negative, a high K_{ow} predicts a high K_{oc}, and thus more adsorption of the compound to the sludge. For ampicillin, the log K_{ow} value gives a K_{ow} of 22.4, equal to around 96% of the ampicillin present in a water solution partitioning into *n*-octanol when this is mixed with the water in equal amounts. For more information on K_{ow}, see e.g. [32, p. 35].

The theoretical capacity of an adsorbing material (adsorbent) for a given compound (adsorbate) may be expressed through adsorption isotherms [33, p. 1227]. These are fitted to experimental data, measured after equilibrium has been reached at a given temperature; they give the adsorption as a function of the amount of adsorbate left in solution [30, p. 37, 33, p. 1227].

Different isotherm equations have been set up to describe experimental adsorption data. Two common ones are the Freundlich (2.6) and the Langmuir (2.7) isotherms:

$$C_s = K_F \cdot C_e^{\frac{1}{n}} \quad (2.6)$$

$$C_s = C_{s,max} \cdot \frac{K_L \cdot C_e}{1 + K_L \cdot C_e} \quad (2.7)$$

Where C_s is the mass of adsorbed compound per unit mass of adsorbent (mg/g), C_e is the equilibrium concentration of adsorbate in solution (mg/L), K_f and n^{-1} are the Freundlich

capacity factor and the Freundlich intensity parameter, respectively, and $C_{s,max}$ and K_L are the theoretical maximum adsorption capacity and the Langmuir constant [33, pp. 1228-1231, 34].

While the Freundlich isotherm was developed empirically, the Langmuir isotherm was derived from theoretical considerations. These were based on modelling the adsorption as a reversible chemical phenomenon, with a limited number of accessible sites on the adsorbent that all have the same energy [33, pp. 1230-1231, 34].

Both the Freundlich and the Langmuir isotherms can be transformed to linear equations:

$$\log(C_s) = \log(K_F) + \frac{1}{n} \cdot \log(C_e) \quad (2.8)$$

$$\frac{1}{C_s} = \frac{1}{C_{s,max}} + \frac{1}{K_L \cdot C_{s,max}} \cdot \frac{1}{C_e} \quad (2.9)$$

Where equation 2.8 is the linear Freundlich and equation 2.9 is the linear Langmuir. With these forms, linear regression can be used to find the isotherm parameters from experimental data [33, pp. 1229-1232].

A literature search yielded several studies that present adsorption isotherms for ampicillin. Most are for relatively well-defined adsorbents such as activated carbon [35] or clay [36]. Only two studies were found that had prepared adsorption isotherms for ampicillin on activated sludge. Jia, et al. [11] present Freundlich and Langmuir isotherms, obtained through linear regression. Shen, et al. [37] give an additional four isotherms (Tóth, BET, Temkin and Redlich-Peterson), adding up to six, all obtained through non-linear regression. A comparison of the parameters found in both articles is shown in Table 2.1.

Table 2.1: Comparison of isotherm parameters

Isotherm	Parameter	Jia, et al. [11]	Shen, et al. [37]
Freundlich	K_F (mg/g)(L/mg)^{nF}	6.57	1.21
	n_F	0.693	0.468
Langmuir	$C_{s,max}$ (mg/g)	32.3	14.7
	K_L (L/mg)	0.564	0.023

In both articles, the n^{-1} exponent in the Freundlich equation has been replaced with n_F. For ease of comparison, that parameter is also used in this thesis. For a discussion on the differences between the isotherm parameters and the adsorption they predict, see 4.2.1.

Setting up adsorption isotherms for ampicillin requires a reliable method for detecting the compound in complex solution. Research on this is summarised next.

2.3.3 Analysing Ampicillin in Wastewater

Methods for analysing antibiotics in wastewater are reviewed in [38]. The common analysis is HPLC with detection being either spectrophotometric (e.g. UV) or mass spectrometry alone (MS) or in tandem (MS/MS). For sample extraction and concentration, SPE is usually employed. For the analysis of β-lactam antibiotics, Le-Minh, et al. [38] list the combination of Oasis® HLB cartridges (Waters) for sample preparation and LC-MS/MS for analysis, citing Cha, et al. [39].

Oasis® HLB cartridges are popular for upconcentrating water samples containing antibiotics. Shen, et al. [10] used them for this purpose, for samples with too low concentration for direct HPLC injection. Recently, Opriş, et al. [40] optimised a method for analysis of antibiotics combining SPE with HPLC, using both diode array (DAD) and MS detection. Of the three sorbent types tested for the SPE step, the study found Oasis® HLB to work best.

In this thesis work, wishing to test the use of a HPLC instrument with only UV detection led to discovery of the method developed by Benito-Peña, et al. [8]. There, SPE with Oasis® MAX cartridges (Waters) followed by HPLC-DAD was used for analysis of β-lactams in wastewater. The limit of detection for ampicillin in WWTP effluent was measured to 3.7 μg/L. Recovery was tested using concentrations of 25-75 μg/L in WWTP effluent. For ampicillin, the stated recovery is 90-95%. Such high recovery, combined with use of the desired HPLC detector, sparked interest in this method and the Oasis® MAX sorbent. Waters Norway [41] recommended using that sorbent for this thesis work due to it being more specific and the recovery cited in [8].

Thus, the decision was made to adapt the method described by Benito-Peña, et al. [8] for analysis of ampicillin in wastewater.

2.4 Analytical Methods

In this section, the analytical techniques used in this thesis work are introduced.

2.4.1 Solid Phase Extraction

Solid phase extraction is comprehensively introduced by Simpson and Wells [42], and the following summary of key points is extracted from there. The steps of the SPE process are shown in Figure 2.4.

Where liquid-liquid extraction utilises the varying partitioning of chemicals in different immiscible solvents to isolate a desired analyte, the partitioning in solid phase extraction is between a liquid sample and the solid sorbent in the SPE cartridge. Ideally, all of the analyte is retained on the sorbent, ready to eluted by a liquid (solvent D in the figure). This liquid is different from the sample solvent, and the analyte should have stronger affinity for it than for the SPE sorbent. An important step between applying sample to the SPE cartridge and eluting the analyte is the washing out of other compounds. This can be seen in the figure, where washing is with solvent C.
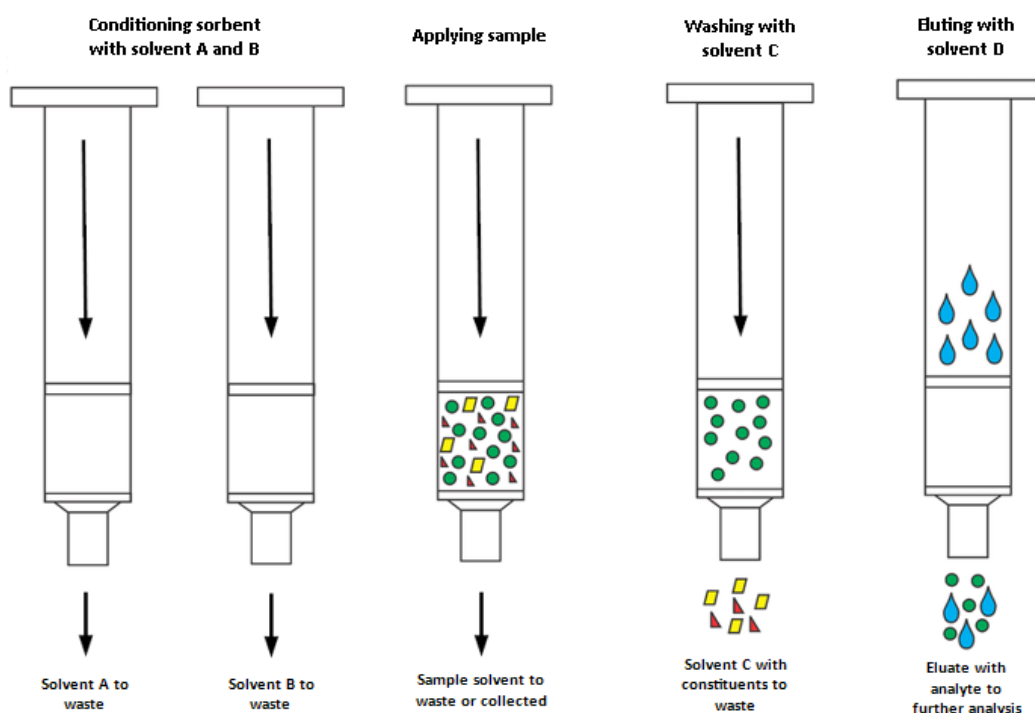
Figure 2.4: Steps in the solid phase extraction process
By Abo, R; Kummer, N.-A. and Merkel, B – "The steps of solid-phase extraction"
edited (arrows and captions), CC BY 3.0 [43]

As noted in [42], all the steps in the SPE process are controllable, leading to a flexible method for extracting and upconcentrating an analyte. Upconcentration is achieved by applying a larger sample volume to the SPE cartridge than the solvent volume used to elute the analyte.

The conditioning of the sorbent seen first in Figure 2.4 is usually required to achieve good recovery of analytes [44]. However, for some modern SPE sorbents, such as the Oasis® HLB and derivatives, this step is optional [45].

2.4.2 High Performance Liquid Chromatography

As in SPE, the partitioning of analytes between phases is also key in chromatography. This method of analysis is described in numerous analytical chemistry textbooks. The following summary on high performance liquid chromatography is extracted from "Fundamentals of Analytical Chemistry" by West, et al. [46, pp. 861 & 912-924].

Present in all chromatography methods are the mobile phase and the stationary phase. The mobile phase moves through the stationary phase, and the analytes it carries are separated from each other due to differing affinities for the two phases.

In HPLC, the stationary phase is often in the form of a liquid layer adsorbed or bonded to the surface of particles with diameter below 10 μm. Through this stationary phase the liquid mobile phase flows, driven by high pressure. The small particle size increases the efficiency of the separation (lower plate height/higher plate number). If the stationary phase is non-polar and the mobile phase more polar, the process is called reverse phase chromatography. In this type of chromatography, the most polar compounds will leave the column (elute) first.

If the composition of the mobile phase changes over the course of the process, it is called gradient elution. For instance, at the start of elution the mobile phase can be pure water, while over time another, less polar, solvent is mixed in. Gradient elution often gives better separation and can shorten the time it takes to elute all components in a sample.

As the chromatography column merely separates compounds, a detector is required for measuring when a compound elutes as well as how much of it is present. This measurement yields chromatograms, where detection in the form of peaks are plotted against time. The placement of the peak for a given compound gives its retention time in the column, and the peak area may be used for quantitative measurement. Common detectors for HPLC include UV/visible light absorption and mass spectrometry.

For light absorption, Beer's law states that the absorbance is proportional to the concentration of the compound and the path length through which the radiation passes [46, p. 660]. In the context of HPLC with UV/visible light detection, a linear relationship between peak area and compound amount for a range of concentrations means that Beer's law is obeyed there [47, p. 572].

The ratio of peak area to a known concentration for a compound is known as the response factor:

$$Response\ factor = \frac{A_{analyte}}{C_{analyte}} \quad (2.10)$$

Where $A_{analyte}$ is the peak area of the analyte in the chromatogram, and $C_{analyte}$ is the concentration of the analyte [48]. From the definition of a linear function, if Beer's law is obeyed, the response factor is constant.

For improving accuracy of HPLC analysis, as described in [47, pp. 90-91], internal standards can be employed. The internal standard, a compound different from the analyte, is added at known concentration to known concentrations of analyte. These solutions are then analysed. An internal standard calibration is prepared from the ratio of analyte peak area to standard peak area, plotted against the ratio of analyte concentration to standard concentration. By adding a known amount of internal standard to unknown samples, this calibration can be used to determine the amount of analyte. The equations used for internal standard calibration are shown in 3.1.4.3.

Internal standards can also be used to make up for loss of sample during preparation for analysis [47, p. 90]. The standard is then added prior to treatment of the sample. This type of internal standard is termed recovery standard in this thesis.

2.5 Objectives of Thesis

This thesis is part of a larger research project seeking to model the spread of antibiotic resistance genes in biological wastewater treatment plants. Ampicillin and ampicillin resistance genes have been chosen as model compounds for the experimental part of the research. In this context, a reliable method for analysing ampicillin in wastewater is required. Furthermore, prior to the introduction of resistance genes there was interest in studying the fate of ampicillin in activated sludge.

The work presented in the following consists of two parts, Method Development and Adsorption Studies. The objectives for the first part were to develop and test a method for analysing ampicillin in complex wastewater solution. Key questions addressed were the accuracy of the analysis, the recovery and precision of the sample preparation, how low concentrations the method could detect, how practical it was, and finally how it fared when used in an actual research context.

The objective for the second part of the thesis work was to study the fate of ampicillin in activated sludge, with a focus on adsorption. Key questions were how much removal was observed, when and how fast adsorption equilibrium was achieved, and if the results could be modelled by Freundlich and Langmuir isotherms.

3 MATERIALS AND METHODS

In this chapter, the materials and methods used in this thesis work are presented. First is a description of the materials used, as well as calculations done (3.1). Then follows the method that was developed in the first part of this thesis work (3.2). The steps taken in the method development are then detailed (3.3). Finally, the experimental work done in the adsorption study is described (3.4).

3.1 Materials and Calculations

The chemicals, instruments, SPE cartridges, and calculations used are presented here.

3.1.1 Chemicals

Water was purified using a Purelab Flex system from Elga.

Stock solutions (500-1000 mg/L) of ampicillin in ultrapure water were prepared using either ampicillin trihydrate (Sigma-Aldrich: prod.nr. A6140) or ampicillin analytical standard (Supelco, Sigma-Aldrich: prod.nr. 59349). In the former case the stock solutions were stored in the fridge and used within a week, while in the latter case aliquots of the stock solution were dispensed into autoclaved vials using sterile filtration, and frozen at -70 °C. Vials were then thawed when needed. The stock solutions using ampicillin trihydrate were only used at the start of the method testing, and when high accuracy of concentration was not needed.

Stock solutions (500 mg/L) of oxacillin, cloxacillin and dicloxacillin in ultrapure water were prepared using oxacillin sodium salt monohydrate (Alfa Aesar, VWR: art.nr. J66380), cloxacillin sodium salt monohydrate (Acros Organics, VWR: art.nr. ACRO455300010) and dicloxacillin sodium salt (Alfa Aesar, VWR: art.nr. J61581). Aliquots of the stock solutions were filtered into vials as described for ampicillin above, and kept frozen at -70 °C.

Tetra-*n*-butylammonium hydrogen sulphate (TBA) from Acros Organics (art.nr. ACRO394200250), trifluoroacetic acid (TFA) from Alfa Aesar (art.nr. L06374), sodium azide from Alfa Aesar (art.nr. 14314), HPLC-grade methanol (art.nr. 20864) and HPLC-grade acetonitrile (art.nr. 83639) were all supplied by VWR.

Bacteriological grade peptone, a protein digest, was from Amresco (code: J636), while soluble starch from potato was from Sigma-Aldrich (prod.nr. S2004).

Hydrochloric acid, nitric acid, sodium hydroxide, sodium dihydrogen phosphate monohydrate and sodium hydrogen phosphate were all from Merck.

3.1.2 Description of the Solid Phase Extraction Equipment

For automated SPE, a RapidTrace® instrument from Caliper (now Biotage) was used, together with Oasis® MAX cartridges.

Figure 3.1: RapidTrace® instrument

Clockwise from top: Instrument with solvent reservoirs in front; rotating SPE cartridge rack;
test tube rack accommodating up to 10 tubes of samples (right) and 10 tubes to receive fractions or eluates (left).

3.1.2.1 RapidTrace® Instrument

Figure 3.1 shows an overview and key features of the RapidTrace®.

The instrument accommodates 1 mL and 3 mL SPE cartridges and allows up to ten samples to be run in an automated sequence. The sample sizes appear at first glance to be limited by what the sample test tubes can contain, and the maximum amount of sample that can be applied to the cartridge per load step (5.8 mL). However, this can be overcome, as shown below. Each sample can be assigned a specific SPE procedure, or all samples may be run using the same procedure. The instrument allows for the use of up to eight different solvents for conditioning, washing and eluting compounds from the cartridge.

Procedures for SPE are entered into specific software where type of step, amount and flow rate are some of the input options. See 3.2.1 for examples of procedures in the RapidTrace® software. For more information on the RapidTrace® instrument, the reader is referred to the instrument manual [49] and the Biotage website [50]. Note that the RapidTrace® used for this thesis is the first version of the instrument.

3.1.2.2 Oasis® MAX Cartridges

The Oasis® MAX cartridges, produced and supplied by Waters, contain a mixed mode sorbent made up of the hydrophilic-lipophilic backbone of the Oasis® HLB sorbent together with a positively charged quaternary amine group. The Oasis® MAX sorbent thus supplies both reverse-phase retention (due to the HLB backbone) and anion-exchange (due to the cationic group) [41, 45]. Figure 3.2 shows the chemical structure of the Oasis® MAX sorbent along with the other Oasis® sorbents.

The decision to use the RapidTrace® instrument for automated SPE introduced some constraints on the cartridge size. As the instrument could only accommodate 1 and 3 mL SPE cartridges, the 6 mL cartridges employed by Benito-Peña, et al. [8] could not be used. Furthermore, the restrictions on sample size, although somewhat possible to overcome, led to the assumption that 60 mg sorbate (unlike the 500 mg in [8]) were enough for this thesis work. Thus, 3 mL, 60 mg Oasis® MAX cartridges were used.
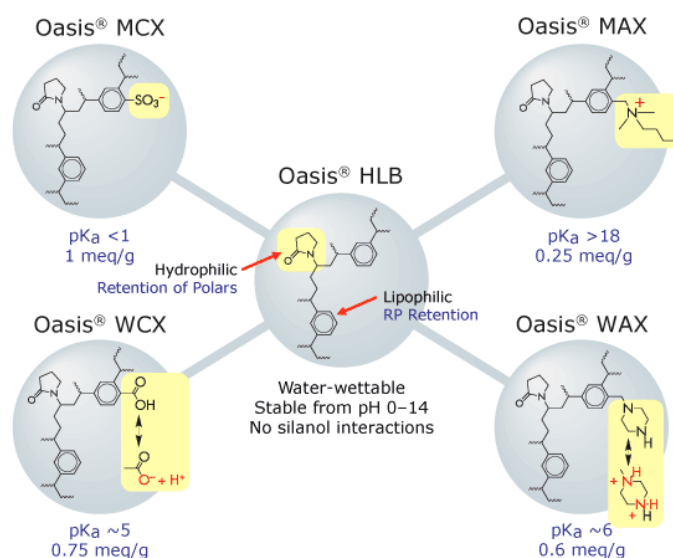
Figure 3.2: Chemical structures of the Oasis® sorbents [45]

Image © Waters, used with permission

3.1.3 Analytical Instruments

The HPLC instrument used was a Dionex UltiMate 3000 from Thermo Scientific equipped with an autoinjector and a Diode Array Detector. The column was an XBridge™ C18 (100 mm x 2.1 mm, 3.5 μm) from Waters.

The pH of samples was measured using an *inoLab pH 730* pH meter (WTW).

Weights were measured using either a Mettler Toledo Excellence Plus analytical balance (± 0.1 mg) or a Sartorius LE6202P (± 0.01 g) for larger amounts.

3.1.4 Calculations

Key equations used in this work and not already described above are detailed here.

3.1.4.1 Uncertainties

Confidence intervals (hereafter called uncertainties) have been calculated, when relevant, for prepared and measured values. If y is the prepared or measured quantity and s_y is the uncertainty (reported as $y \pm s_y$), s_y was calculated by one of the three following equations [46, pp. 110-111, 51]:

$$s_y = \sqrt{s_1^2 + s_2^2 + \ldots + s_n^2} \quad (3.1)$$

Where *y* is the result of addition and/or subtraction. *s₁, s₂*… are the uncertainties of each input value.

$$s_y = y \cdot \sqrt{\left(\frac{s_1}{y_1}\right)^2 + \left(\frac{s_2}{y_2}\right)^2 + \cdots + \left(\frac{s_n}{y_n}\right)^2} \quad (3.2)$$

Where *y* is the result of multiplication and/or division. *y₁, y₂*… are the input values.

$$s_y = \frac{SD}{\sqrt{n}} \cdot t_{\frac{\alpha}{2}, n-1} \quad (3.3)$$

Which was used for independent measured values with minimum two parallels. SD is the standard deviation for the measurements and n is the number of measurements. $\frac{\alpha}{2}$ was set to 0.025, corresponding to 95% confidence. $t_{\frac{\alpha}{2}, n-1}$ for each calculation was found from tables in [51].

3.1.4.2 Recovery

Recovery from sample preparation was calculated by the following formula [52]:

$$R_A = \frac{Q_m}{Q_o} \cdot 100\% \quad (3.4)$$

Where Q_m is the measured quantity after extraction and Q_o is the quantity that corresponds to 100% extraction (the original known amount). Input values for recovery calculations were obtained in different ways, as is detailed in 4.1.

3.1.4.3 Calibration

Single point calibration was done by calculating the slope of the straight line between the single data point and the origin.

The regression analysis tool in Microsoft Excel was used to prepare linear calibrations from multiple data points. The constant was set to zero and the level of confidence to 95%.

For internal standard calibration, the following three equations were used [48]:

$$Response\ ratio = \frac{A_{analyte}}{A_{IS}} \quad (3.5)$$

Where $A_{analyte}$ is the peak area of the analyte, and A_{IS} is the peak area of the internal standard in the same chromatogram.

$$Concentration\ ratio = \frac{C_{analyte}}{C_{IS}} \quad (3.6)$$

Where $C_{analyte}$ is the known concentration of the analyte and C_{IS} is the concentration of the internal standard. Calibrations were prepared between these two ratios (3.5 and 3.6).

$$C_{unknown} = \frac{\frac{A_{analyte}}{A_{IS}} - b}{a} \cdot C_{IS} \quad (3.7)$$

Where $C_{unknown}$ is an unknown analyte concentration, *a* is the slope of the calibration curve and *b* is the y-intercept (set to zero in all calibrations).

3.1.4.4 Adsorption

The amount of ampicillin per unit mass activated sludge was calculated according to mass-balance considerations [33, p. 1228]:

$$C_s = \frac{C_0 - C_e}{MLSS} \quad (3.8)$$

Where C_0 is the known start concentration of ampicillin, C_e is the measured equilibrium concentration of ampicillin in solution and MLSS is the concentration of mixed liquor suspended solids.

3.2 Method for Analysing Ampicillin in Solution

The method described in this section is an adaption of the one developed by Benito-Peña, et al. [8]. It consists of sample preparation by SPE, followed by analysis by HPLC. The steps taken in adapting the method are described in 3.3. The detailed procedure can be seen in Appendix B.

Table 3.1: Solid phase extraction procedure

Step	Solvent	Volume (mL)
Conditioning	Methanol	3.0
Conditioning	Ultrapure water	3.0
Conditioning	Phosphate buffer[1]	3.0
Loading sample	N/A	Variable
Wash	Phosphate buffer[1] with 5 % methanol	3.0
Elute	0.05 M TBA[2] in methanol	1.0 + 1.0

[1] 0.05 M, pH 7.5

[2] Tetra-*n*-butylammonium hydrogen sulphate

3.2.1 Solid Phase Extraction

At start-up of the RapidTrace®, as advised in its manual, all reagent lines were primed with fresh reagents. At the end of the day, the instrument was cleaned using a specific cleaning procedure, with 2 M sodium hydroxide and 2 M nitric acid as "samples".

Before performing SPE, a known concentration of dicloxacillin was added to unknown samples, before the pH of all samples was adjusted to 7.5 using dilute hydrochloric acid or sodium hydroxide solution. To account for dilution, the mass each solution was measured before and after this pH adjustment. The sample was then transferred to a test tube for SPE by the RapidTrace®.

The steps in the SPE procedure are shown in Table 3.1. The flow rate for all steps was 2.5 mL/min.

A short and a long SPE procedure were prepared using the RapidTrace® software. The former loaded maximum 12 mL sample and was for use on smaller size samples. The latter was for larger samples, and loaded maximum 56 mL. For all procedures additional steps were included, purging the cannula to avoid carryover between samples. This was advised in the manual [49].

The short procedure is shown in Figure 3.3. Running this procedure on six samples took approximately 2 h to complete.

No	Step	Source	Output	Vol	ml/min	Liquid Sense
1	Condition	MeOH	Gen1	3	2,5	No
2	Condition	H2O	Gen1	3	2,5	No
3	Condition	Buff	Gen1	3	2,5	No
4	Load	Sample	Gen1	4	2,5	No
5	Load	Sample	Gen1	4	2,5	No
6	Load	Sample	Gen1	4	2,5	No
7	Rinse	Buff_MeOH	Gen1	3	2,5	No
8	Purge-Cannula	Buff_MeOH	Cannula	3	30	No
9	Collect	MeOH_TBA	Fract1	1	2,5	No
10	Collect	MeOH_TBA	Fract1	1	2,5	No
11	Purge-Cannula	MeOH	Cannula	3	30	No
12	Purge-Cannula	H2O	Cannula	3	30	No
13						No

Procedure Name: AMP_AI1.SPE

Figure 3.3: Short SPE procedure, in RapidTrace® software

The long procedure consisted of three separate RapidTrace® procedures, run in order and, for one of them (AMP2_v2.SPE), three times. These are shown in Figure 3.4. Each procedure was run on all samples, before the test tubes were refilled with more of each sample, and the next procedure was run. Total run time for six samples was approximately 6.5 hours.

3.2.2 High Performance Liquid Chromatography

The 2 mL eluates from the SPE cartridges were prepared for HPLC by dilution 1:1 with either ultrapure water (for blank samples), ultrapure water containing a known concentration of the internal standard cloxacillin (for unknown samples), or a known concentration of ampicillin, dicloxacillin and cloxacillin (for matrices extracted for calibration). This final 4 mL sample meant that, in the case of 100% recovery, the short SPE procedure caused a threefold increase in concentration (12 mL to 4 mL), while the long procedure caused a fourteenfold increase (56 mL to 4 mL). This upconcentration was corrected for when calculating measured concentrations.

Each sample was filtered through 0.2 μm Acrodisc® GHP syringe filters (Pall Laboratory, VWR: art.nr. 514-4121), into a HPLC vial. The samples were then analysed by HPLC, with minimum three injections per sample.

Procedure Name AMP1_v2.SPE

No	Step	Source	Output	Vol	ml/min	Liquid Sense
1	Condition	MeOH	Gen1	3	2,5	No
2	Condition	H2O	Gen1	3	2,5	No
3	Condition	Buff	Gen1	3	2,5	No
4	Load	Sample	Gen1	5,6	2,5	No
5	Load	Sample	Gen1	5,6	2,5	No
6	Rinse	Buff_MeOH	Gen1	3	2,5	No
7	Purge-Cannula	MeOH	Cannula	3	30	No
8						No
9						No
10						No

Procedure Name AMP2_v2.SPE

No	Step	Source	Output	Vol	ml/min	Liquid Sense
1	Load	Sample	Gen1	5,6	2,5	No
2	Load	Sample	Gen1	5,6	2,5	No
3	Rinse	Buff_MeOH	Gen1	3	2,5	No
4	Purge-Cannula	MeOH	Cannula	3	30	No
5						No
6						No
7						No
8						No
9						No
10						No

Procedure Name AMP3_v2.SPE

No	Step	Source	Output	Vol	ml/min	Liquid Sense
1	Load	Sample	Gen1	5,6	2,5	No
2	Load	Sample	Gen1	5,6	2,5	No
3	Rinse	Buff_MeOH	Gen1	3	2,5	No
4	Purge-Cannula	Buff_MeOH	Cannula	3	30	No
5	Collect	MeOH_TBA	Fract1	1	2,5	No
6	Collect	MeOH_TBA	Fract1	1	2,5	No
7	Purge-Cannula	MeOH	Cannula	3	30	No
8	Purge-Cannula	H2O	Cannula	3	30	No
9						No
10						No

Figure 3.4: Long SPE procedure, in RapidTrace® software

Table 3.2: Gradient programme for high performance liquid chromatography

Time (min)	Amount of eluent A[1] (%)	Amount of eluent B[2] (%)
5	100	0
5	100-63	0-37
6	63	37
2	63-33	37-67
2	33	67
2	33-100	67-0
2	100	0

[1] Ultrapure water with 0.01% TFA

[2] Acetonitrile with 0.01% TFA

The flow rate for HPLC was 0.31 mL/min; the injection volume 20 μL. UV-detection was measured at four wavelengths: 205 nm, 220 nm, 254 nm and 280 nm. Unless otherwise stated, the chromatograms and peak areas discussed below are from the 220 nm measurements. The gradient programme used ultrapure water and acetonitrile, both containing 0.01% trifluoroacetic acid, as the mobile phases. The programme is shown in Table 3.2.

Samples were analysed in order of least to most concentrated to minimise carryover.

The resulting chromatograms were produced by Chromeleon™ 7 software from Thermo Scientific™. The automatic integration of peaks in this software was refined manually.

3.3 Method Development

In this section, the steps taken during adaption and testing of the method by Benito-Peña, et al. [8] are described. The results from the tests, as well as the rationale for the steps taken, are presented and discussed in 4.1.

3.3.1 Testing the HPLC Method

As the available HPLC column was smaller than the column used by Benito-Peña, et al. [8] (150 mm x 4.6 mm, 5 μm) the flow rate and injection volume were scaled down according to formulas given in the care and use manual for the column [53]. The injection volume was limited by the injection loop (20 μL) available, and was therefore smaller than the downscaled value (27.8 μL).

3.3.1.1 Initial Testing and Preparation of First Calibration Curve

To determine the retention time of ampicillin, several ultrapure water solutions containing ampicillin at varying concentrations were analysed by HPLC using the same gradient programme as described in [8]. The gradient programme was then trimmed at the end, yielding the programme shown in Table 3.2.

Four dilute ampicillin solutions were prepared in ultrapure water. An aliquot of each of these, along with a blank of ultrapure water, was pipetted out into HPLC vials. An equal amount of 0.05 M TBA in methanol was added to each vial. The final concentrations of the solutions were in the 0-1 mg/L range. All were analysed by HPLC immediately after preparation, using the gradient programme in Table 3.2. A calibration was prepared from the results.

3.3.1.2 Checking Background Peaks

Three aliquots of 2 mg/L ampicillin in ultrapure water were diluted with equal parts ultrapure water, pure methanol, and the 0.05 TBA in methanol solution, respectively. They were then analysed by HPLC. Each solution was injected once.

3.3.1.3 Validation of Analysis

After preparing the calibration, and parallel to the initial testing of the SPE method, solutions of known ampicillin concentration were diluted 1:1 with 0.05 M TBA in methanol and analysed by HPLC. Some of these solutions had a dual purpose, also being used to calculate recovery from the SPE procedure. The concentrations of these solutions are shown along with the analysis results in Table 4.1.

3.3.2 Testing the SPE Method on Ampicillin in Ultrapure Water

The SPE eluates in this subsection were prepared for HPLC and analysed as described in 3.2.2, except the dilution was with ultrapure water only.

3.3.2.1 First Short SPE Procedure

The first SPE procedure tested was one which loaded 8 mL sample onto each cartridge. In the RapidTrace® software the procedure was almost identical to the one shown in Figure 3.3, but with one less line for loading samples.

Three solutions, tagged T1-T3, were used to test this procedure. Their concentrations are shown along with the results in Table 4.2. Three parallels of each solution were extracted and upconcentrated together, along with 1-2 blanks to check for carryover.

3.3.2.2 First Long SPE Procedure

The second SPE procedure tested loaded 40 mL sample onto each cartridge. This procedure was similar to the one in Figure 3.4, but with each sample load step loading 4 mL instead of 5.6 mL.

To test this procedure, two solutions tagged T4 and T5 were used. Two parallels of T4, spaced by two blanks, were extracted and upconcentrated, while for T5 three parallels followed by a blank were run. Concentrations for these solutions are shown in Table 4.3.

3.3.3 Testing Internal Standards

The three compounds tested for use as internal standards were the β-lactam antibiotics oxacillin, cloxacillin and dicloxacillin.

To find retention times, three separate dilute solutions of the compounds were prepared. These were further diluted 1:1 with 0.05 M TBA in methanol solution, filtered through GHP syringe filters, and analysed by HPLC.

Furthermore, a mixed solution was made with each potential standard and the ampicillin stock solution. pH was adjusted to 7.5. For calculating recovery, an aliquot of this solution was diluted 1:1 with 0.05 M TBA in methanol solution, filtered and analysed directly by HPLC. The rest

was extracted and upconcentrated, in three parallels, by SPE using the long procedure (loading 40 mL sample), before analysis by HPLC.

3.3.4 Testing the SPE Method on Ampicillin in Wastewater Effluent

For testing the developed SPE method on samples of ampicillin in wastewater effluent, a supply of such effluent was required. To achieve this, a simple SBR was set up using a 2000 mL conical flask aerated by an Eheim 200 air pump with an Eheim air diffuser attached. The bioreactor was inoculated with sludge from one of the aeration tanks at IVAR Sentralrenseanlegg Nord-Jæren (SNJ). Maximum once a day, aeration was turned off and the sludge was allowed to settle. Settling times varied between 10 minutes to 1.5 hours. The supernatant was decanted down to between the 200- and 800-mL mark. Then the flask was refilled up to the 2000-mL mark with tap water, aeration was re-started and the bioreactor was fed a small spoon of either peptone, starch from potato, or a combination. Figure 3.5 shows the simple SBR set-up.

As in [8], the wastewater effluent (the decanted supernatant) was filtered prior to addition of β-lactams, using Whatman™ GF/C filters (Whatman, VWR: art.nr 513-5227).

Figure 3.5: Simple SBR set-up

3.3.4.1 Observing the Matrix

The day after the bioreactor was inoculated, five parallels of filtered wastewater effluent were extracted by SPE, using the long procedure loading 40 mL onto each cartridge. Three solutions of varying ampicillin concentration and constant cloxacillin concentration were prepared in ultrapure water. Each of these were added 1:1 to the SPE eluates. The two remaining SPE eluates were used as blanks.

3.3.4.2 Extracting B-lactams Using Long SPE Procedure

Extracting and upconcentrating β-lactams from filtered wastewater effluent was first done using the long SPE procedure that loaded 40 mL sample. The solutions extracted contained around 0.05 mg/L ampicillin and 0.2 mg/L dicloxacillin. The SPE eluate was diluted 1:1 with a solution of approximately 2 mg/L cloxacillin.

Before the second extraction test, the SPE procedure was edited so that it now loaded a total of 56 mL sample (the final long procedure, Figure 3.4). This procedure was tested twice, both times using solutions containing around 0.05 mg/L ampicillin, and varying the amount of dicloxacillin. Three parallels of each of these solutions were extracted, along with two samples containing only filtered wastewater effluent (blanks).

For calculating recovery in both these tests, a solution of ultrapure water was prepared containing 28 times the amount of ampicillin and dicloxacillin used for testing SPE. Prior to HPLC, this was added 1:1 to one of the blank SPE eluates. A solution with a known concentration of cloxacillin was added to the eluates containing ampicillin and dicloxacillin. In the last test, this same concentration of cloxacillin was present in the recovery solution as well.

3.3.4.3 Extracting B-lactams Using Short SPE Procedure

The final short SPE procedure, shown in Figure 3.3, was tested on both a relatively concentrated solution (around 1 mg/L ampicillin and dicloxacillin) and a dilute solution (around 0.05 mg/L ampicillin and dicloxacillin). Three parallels of each solution were extracted, along with two blanks. Before HPLC, one of the blank eluates was diluted 1:1 by a recovery solution containing six times the amount of ampicillin and dicloxacillin used for testing. The sample eluates were diluted 1:1 with a solution containing a known concentration of cloxacillin. This same concentration was also present in the recovery solution.

3.4 Adsorption Studies

In this section, the experimental work done on the adsorption studies is described. The experiments were modelled on the method used by Jia, et al. [11].

Prior to laboratory experiments, the adsorption isotherms for ampicillin in [11, 37] were studied numerically in Microsoft Excel. Different equilibrium concentrations of ampicillin (0.1-1.0 mg/L) were entered, and the corresponding adsorptions calculated. By setting a constant MLSS, the predicted start concentrations of ampicillin were also calculated, using the relationships in equation 3.8. Finally, the predicted percent ampicillin remaining in solution was found.

3.4.1 Preliminary Adsorption Experiment

The MLSS of the simple SBR was measured with GF/C filters, using an adapted version of the total suspended solids method in [54]. A portion of the sludge was diluted down to a MLSS of 0.201 ± 0.007 g/L. Sodium azide was added for bacterial growth inactivation, at a concentration of around 1 g/L.

Parts of the diluted sludge mixture were placed in three pre-weighed 15 mL centrifuge tubes (Corning Life Science, VWR: art.nr 734-0451). Ampicillin stock solution was added to yield a concentration of 1 mg/L in each tube. The tubes were capped, covered with aluminium foil, and put on a shake table for seven hours at 153 rpm.

The remaining diluted sludge mixture was allowed to settle, and the supernatant decanted and filtered through GF/C filters. The filtrate's pH was measured, before it was divided into four test tubes and extracted by a variation of the short SPE procedure, loading 10 mL of sample onto each cartridge.

Four calibration solutions (0-1 mg/L range) containing ampicillin, cloxacillin and dicloxacillin were added 1:1 to the SPE eluates. These were then analysed by HPLC and the resulting chromatograms used for internal standard calibration.

After seven hours, the centrifuge tubes were removed from the shake table and centrifuged at 5000 rpm for three minutes. The supernatant was filtered using CHROMAFIL® GF/PET-45/25 0.45 μm syringe filters (Macherey Nagel, REF 729033), into pre-weighed test tubes. The samples were extracted and analysed as described in 3.2. pH adjustment was not done due to the small size of the samples.
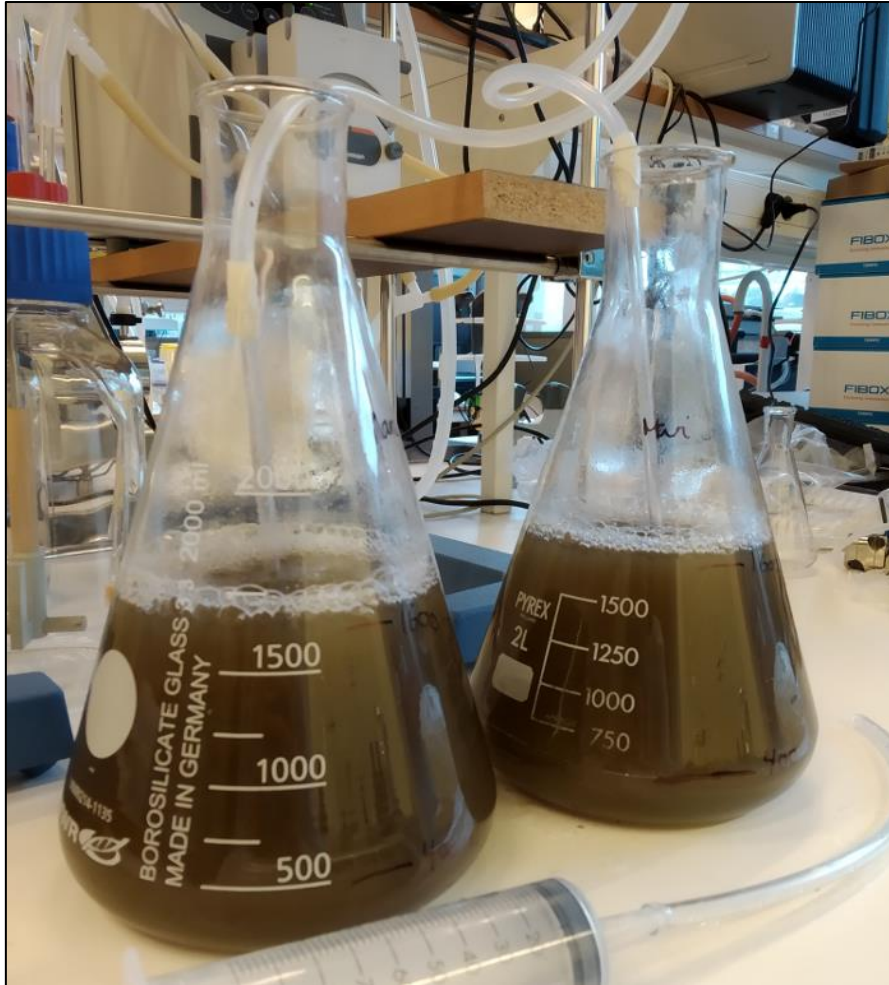
Figure 3.6: SBR set-up

3.4.2 New Sequencing Batch Reactors

Seeking to achieve a repeatable activated sludge composition for both of the last two adsorption experiments, new SBRs were set up and run for each of them. Two parallels were prepared each time, using 2000 mL conical flasks containing 1600 mL activated sludge. Aeration was by an Eheim 200 air pump with fine bubble diffusors attached. The reactor set-up is shown in Figure 3.6.

These SBRs were run for 9-10 days prior to each experiment. A HRT of 16 hours was achieved by employing a volumetric exchange ratio of 0.75 and a cycle time of 12 hours (equation 2.1). By wasting 100 mL well-mixed reactor contents every cycle, the SRT was kept at eight days (equation 2.4).

The settle time for each cycle was 30 minutes, after which the supernatant was decanted and the reactor re-filled with 1200 mL synthetic wastewater. This was prepared from 100 mL of a

20 g_{COD}/L substrate solution, 7 mL of seawater to adjust salinity, and tap water. The COD load was thus 2 g/cycle. The substrate solution was prepared from 10 g peptone and 4.2 g starch in 1 L distilled water, and stored in the fridge between use. Conversion factors for amount of COD per gram of each substrate were supplied by Kommedal [55].

Both sets of SBRs were inoculated using activated sludge from SNJ. Due to maintenance shutdown, there had been no wastewater inflow to the aeration basins for a week prior to collecting inoculum for the first run. Thus, this sludge was considered to be free from biodegradable substrate when transferred to the SBRs, and feeding with synthetic wastewater was started immediately. For the second run, the sludge was aerated for two days before commencing feeding, in order to remove as much of the remaining substrate as possible.

3.4.3 First Study of Adsorption Kinetics

For reasons described in 4.2.3, all peak areas used for quantification in this and the following experiment were determined using the chromatograms from the 205 nm wavelength measurements.

The MLSS of one of the SBRs was measured, and a portion of the reactor contents diluted down to around 3.0 g/L using tap water. Sodium azide was added to achieve a concentration of approximately 2 g/L. 200 mL of this mixture was taken out, and the rest left overnight on a shake table at 153 rpm.

The 200 mL sludge mixture was allowed to settle, after which the supernatant was filtered through 0.45 μm syringe filters. The pH of the filtrate was adjusted to 7.5, before it was divided into six test tubes and extracted by the RapidTrace® using the short SPE procedure. The resulting eluates were used to prepare a calibration, similarly to what is described in 3.4.1.

The following day, the sludge mixture was removed from the shake table and divided into five 500 mL conical flasks, 250 mL in each. To four of these, ampicillin stock solution was added to achieve concentrations of 1 mg/L. The fifth flask served as the experiment blank. All five flasks were then covered with foil and placed on the shake table at 153 rpm for 8.5 hours.

At set sampling times (5 min, 30 min, 1 h, 2 h, 4 h and 8.5 h) around 30 mL well mixed sludge from each flask was transferred to 50 mL centrifuge tubes (VWR: art.nr. 525-0402) and centrifuged at 5000 rpm for 10 minutes. The supernatants were filtered into new, pre-weighed

50 mL centrifuge tubes using 0.45 μm syringe filters. The tubes were then weighed to determine the mass of sample, and frozen at -20 °C.

The samples were thawed, and analysed as described in 3.2.

3.4.4 Second Kinetics Study and Adsorption Isotherm Study

The MLSS of one SBR was determined and a portion of it diluted down to 0.99 ± 0.04 g/L. 2.0 g/L sodium azide was added.

Four 50 mL centrifuge tubes filled with this sludge mixture were centrifuged, and the supernatant filtered into new, pre-weighed tubes using 0.45 μm syringe filters. Ampicillin stock solution was added to three of these, achieving a concentration of 1 mg/L, the fourth being the blank. The tubes were covered with foil and left standing on the bench for the rest of the experiment, with mixing by inversion every 2 hours. This was the negative control for the experiment.

Five 500 mL conical flasks were prepared and placed on the shake table identically to the experiment described in 3.4.3, but with sampling at 5 min, 30 min, 1 h, 2 h and 5 h only.

Additionally, two solutions each of the ampicillin concentrations 2.6 mg/L (A1), 0.5 mg/L (A2) and 0.2 mg/L (A3) (see Table 4.9 for exact concentrations) were prepared from the sludge mixture and ampicillin stock solution in 50 mL flasks. These were also wrapped in foil and placed on the shake table at 153 rpm. This is referred to as the adsorption isotherm experiment.

The sampling and treatment of samples was also identical to what is described in 3.4.3. For the adsorption isotherm experiment, the 50 mL flasks were only sampled at 5 h. The negative control tubes were frozen after the 5 h sampling.

During analysis, the blank sample from each sampling time was divided into two test tubes prior to SPE. One was kept as the blank while the other provided matrix for a single point calibration. A calibration solution, containing 1 mg/L ampicillin and dicloxacillin and 0.5 mg/L cloxacillin, was added 1:1 to this eluate prior to HPLC.

The pH of samples prior to pH-adjustment was registered for the 5 min, 30 min and 5 h samples, as well as for the negative control.

Before determining peak areas in Chromeleon™, the blank chromatogram was subtracted from the other chromatograms from that sampling time, including the calibration.

The data from the adsorption isotherm experiment were transformed to fit the linear forms of the Langmuir and Freundlich isotherms (equations 2.8 and 2.9). A linear regression was performed using the regression analysis tool in Microsoft Excel. The level of confidence was set to 95%

4 RESULTS AND DISCUSSION

In this chapter, the results from both main parts of the thesis work are presented and discussed. The results from method development are described in 4.1, while the results from the adsorption studies can be found in 4.2. Finally, 4.3 gives suggestions for further work.

4.1 Method Development

In addition to presenting and discussing the results of the method development, the rationale for the steps taken are given in this section.

4.1.1 Testing the HPLC Method

Dissolving the ampicillin in 1:1 water and 0.05 M TBA in methanol for this testing and calibration was done to have a sample matrix for HPLC as similar as possible to what it would be after performing the complete method.

4.1.1.1 Initial Testing and Preparation of First Calibration Curve

Ampicillin emerged from the column at 11 minutes for all solutions. For an example, see Figure 4.2.

Figure 4.1 shows the peak areas of the calibration solutions, plotted against concentration. The horizontal error bars in the plot reflect the uncertainties of the calibration solution concentrations. These are calculated from uncertainties in input values (listed on the equipment) using equations 3.1 and 3.2. The uncertainties shown for the added trendline are calculated by the regression analysis tool in Microsoft Excel. The uncertainty for the slope is a 95% confidence value while the value for the R^2 is the standard error.

As can be seen from both the added trendline and the R^2 value, there is a clear linearity between concentration and area in this concentration range.
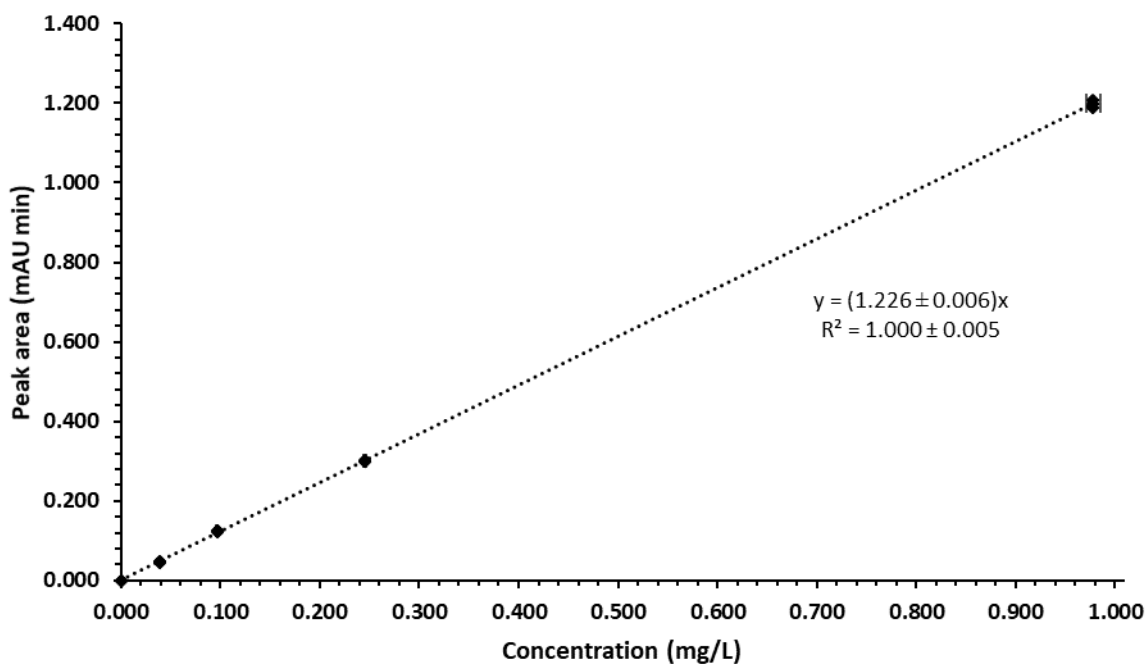
Figure 4.1: First calibration curve for ampicillin

It should be noted that the uncertainties listed for slope and R^2 do not take into account the uncertainties associated with the concentrations. As can be seen from the error bars however, these uncertainties are very small, and so would likely not have had a large effect if incorporated.

4.1.1.2 Checking Background Peaks

In the chromatograms for the calibration, there were consistent peaks at 10.6 min and 12.5 min, whose sizes did not change with ampicillin concentration. Due to suspicion that these peaks came from the added 0.05 M TBA in methanol solution, a check of these peaks was performed.

The relevant parts of each chromatogram are shown in Figure 4.2. The symmetric shape of the peaks in the top and bottom panel are typical for what was observed in the analysis of ampicillin at concentrations ≤ 1.001 mg/L.

As can be seen in the figure, the two peaks do not appear for the solution diluted with only ultrapure water or pure methanol. When diluted with 0.05 M TBA in methanol solution, the peaks appear, clearly showing them to be an effect of this addition.
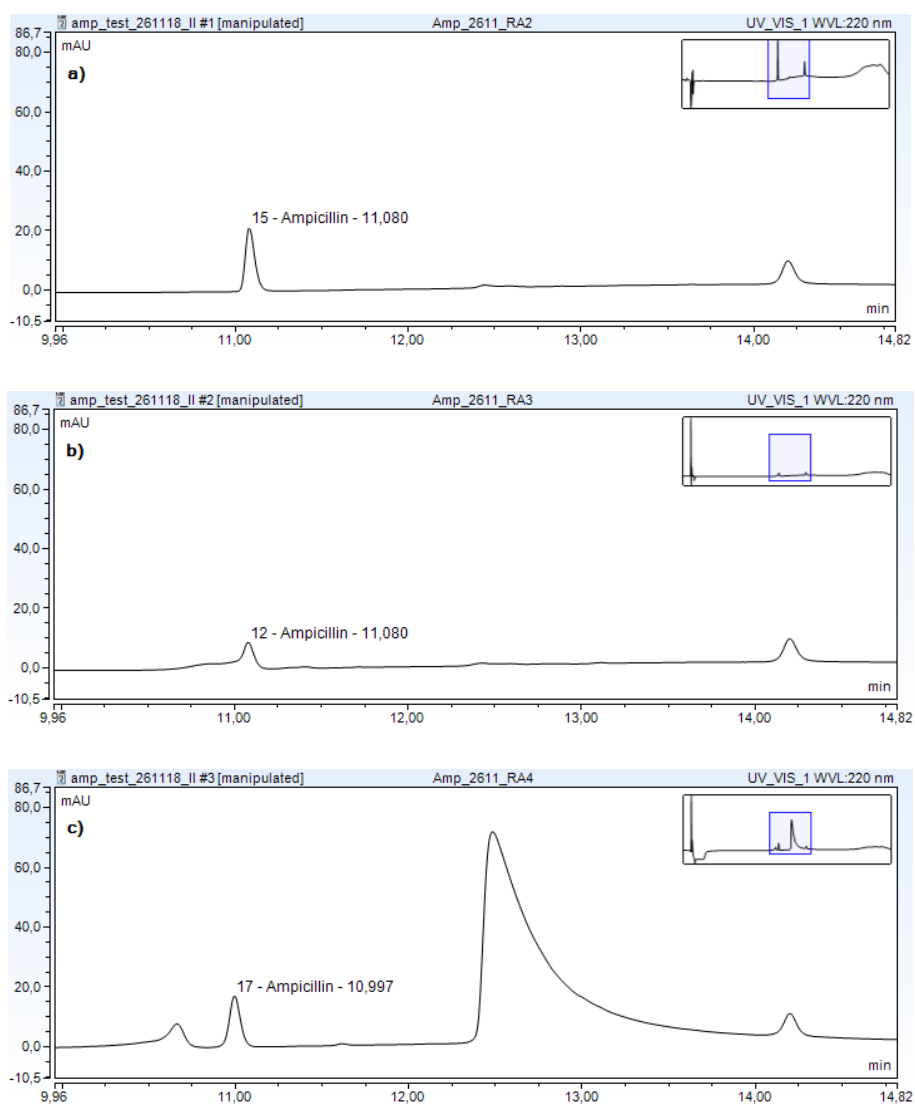
Figure 4.2: Results from background check (final ampicillin concentration 1 mg/L)

a) diluted with ultrapure water   b) diluted with pure methanol   c) diluted with 0.05 M TBA in methanol

The purpose of using TBA in the full method was, according to Benito-Peña, et al. [8], to enhance the elution of the β-lactams from the mixed mode sorbent in the Oasis® MAX cartridge. This is explained to be due to TBA's similarity to the sorbent in both charge and structure, and its high affinity for mixed mode polymeric sorbents.

4.1.1.3 Validation of Analysis

As variability in the HPLC instrument's performance was suspected, several solutions were prepared and analysed, over time. The results from the validations are presented in Table 4.1, in chronological order. The solutions marked by letters in addition to numbers (V1a and V1b)

were analysed on the same day. The measured concentrations are all calculated from the calibration shown in Figure 4.1.

As can be seen in the table, for most of the analyses there is a larger than ± 5 % discrepancy between measured and prepared concentrations. There also appears to be a shift over time from the measurements underestimating to them overshooting the target value. The differences are larger than what can be accounted for by the uncertainties listed (except for V6).

Certainly, some of the discrepancies in Table 4.1 can be explained by human error. In that case, increasing experience should have caused less discrepancies for the later analyses (V4-V7). This cannot be seen clearly in the table. Some of this might be due to the calibration being prepared first, which calls the calibration itself into question. However, another explanation is that there is some day-to-day variability in the HPLC instrument, for instance in injection volume or detector response or both.

Table 4.1: Results from validation of analysis

Sol.	Prepared concentration (mg/L)[1]	Average peak area (mAU min)[2]	Measured concentration (mg/L)[3]	Difference (mg/L)[3]	Relative difference (%)[3]
V1a	0.207 ± 0.001	0.245 ± 0.003	0.200 ± 0.002	-0.007 ± 0.003	-4 ± 1
V1b	0.0519 ± 0.0002	0.058 ± 0.002	0.047 ± 0.001	-0.005 ± 0.001	-10 ± 3
V2	0.245 ± 0.002	0.282 ± 0.003	0.230 ± 0.003	-0.015 ± 0.003	-6 ± 1
V3	0.488 ± 0.005	0.68 ± 0.04	0.55 ± 0.03	0.07 ± 0.03	13 ± 7
V4	0.048 ± 0.001	0.063 ± 0.001	0.052 ± 0.001	0.004 ± 0.001	8 ± 3
V5	0.488 ± 0.003	0.63 ± 0.01	0.514 ± 0.009	0.03 ± 0.01	5 ± 2
V6	0.038 ± 0.002	0.046 ± 0.002	0.038 ± 0.001	0.000 ± 0.002	0 ± 6
V7	0.486 ± 0.003	0.628 ± 0.009	0.512 ± 0.008	0.026 ± 0.009	5 ± 2

[1] Uncertainties calculated from uncertainties of the equipment used for preparation (equations 3.1 & 3.2)

[2] Uncertainty calculated using equation 3.3. n = 3 for V1a-V4 and V6; and n = 4 for V5 and V7

[3] Uncertainty calculated from uncertainties in input values (equations 3.1 & 3.2)

At the time, the conclusions drawn from testing the HPLC method was that it worked well for detecting ampicillin, but with variable results in the quantitative analyses. To mitigate this, it was decided to use internal standard calibration to achieve higher accuracy. The testing of internal standards is described in 4.1.3.

As noted, validation of the HPLC analysis was performed in parallel with the initial testing of the SPE method, which is described in the following. As they were done in parallel, the experiences and conclusions drawn from the validation were not completely implemented during the first SPE testing.

4.1.2 Testing the SPE Method on Ampicillin in Ultrapure Water

In order to avoid possible matrix interference in this initial testing, ultrapure water was used as solvent for the ampicillin.

4.1.2.1 First Short SPE Procedure

In the chromatograms from these tests, peaks are present at the retention time of ampicillin. As evidenced by this, the SPE procedure was qualitatively successful in extracting and upconcentrating the β-lactam. The analyses of the blanks show no peaks, so no carryover in the SPE process occurred.

Table 4.2 gives the results from the analysis. The average measured concentration for each solution is found from the measured concentrations of the three parallels extracted by SPE. These are again calculated by taking the average of the peak areas from the three injections and applying the calibration shown in Figure 4.1.

Recovery is calculated according to equation 3.4, in two ways: concentration and peak area comparison. This latter is either with a solution that was made to have the same theoretical concentration as the test solution would have after upconcentration; or the comparison is with an aliquot of the same solution analysed directly by HPLC. In this last case, the peak area for the recovery solution has been multiplied by twice the upconcentration from SPE before comparison.

Table 4.2: Results from test of first short SPE procedure

Solution	Prepared concentration (mg/L)[1]	Average measured concentration (mg/L)[2]	Recovery, concentration (%)[3]	Recovery, peak area (%)[3]
T1	0.244 ± 0.001	0.23 ± 0.02	95 ± 10	83 ± 10
T2	0.087 ± 0.003	0.09 ± 0.03	109 ± 37	N/A
T3	0.095 ± 0.002	0.08 ± 0.05	89 ± 55	82 ± 50

[1] Uncertainties calculated from uncertainties of the equipment used for preparation (equations 3.1 & 3.2)

[2] Uncertainties calculated using equation 3.3, n = 3

[3] Uncertainties calculated using input values (equation 3.2)

The two most notable observations from Table 4.2 are the high recovery for all solutions and the high uncertainties for the recovery of the more dilute solutions (T2 and T3). The former is promising, as it shows the SPE procedure works and not much compound is lost in the process. The latter may show that the SPE method is less precise for lower concentrations, and it casts doubt on how high the recovery actually is for these solutions. For T2 and T3, it should be noted that the precision within the measured peak areas for each parallel was much higher than between parallels. This points to the cause of low precision being the SPE method, not the HPLC analysis.

The recoveries calculated by peak area comparison are lower than the recoveries calculated by concentration comparison. This may have been caused by the suspected variation in the HPLC instrument, discussed above. Quite some time had passed since the calibration curve was made before the SPE procedure was tested. Thus, the recoveries from peak area comparison should carry more weight than the ones from concentration comparison. It should however be noted that T1 and its recovery solution were not analysed the same day and so this variation might have affected the peak area comparison for this solution as well.

As the precision appeared higher for the more concentrated T1, and also due to concern that in wastewater samples the matrix could prevent quantification of smaller peaks, a new SPE procedure was prepared and tested. This would yield a tenfold increase in ampicillin concentration. The results from testing this procedure are presented in the following.

4.1.2.2 First Long SPE Procedure

In the resulting chromatograms from these tests, peaks are again present at the retention time of ampicillin (11 min). Thus, this procedure also works as expected. The blanks showed no or negligible (≈2 µg/L) carryover in the process.

Table 4.3 shows the results from the analyses. The recoveries shown in the table are calculated similarly to those in Table 4.2.

As can be seen, the precision is better than was the case for T1-T3 even with one less parallel for T4. This could point to better precision if more compound is loaded onto the SPE cartridge. The recovery for T4, however, is less than for T1-T3. A possible explanation for this is that the new procedure includes refilling of samples, more loading of small sample volumes onto cartridges, rinse and purge steps to avoid carryover after sample loading, and simply more time where the ampicillin was left standing in room temperature (in solution or on SPE cartridge). Each of these could cause loss of compound, and thus lower recovery.

To evaluate if standing in room temperature caused much degradation of the ampicillin, an aliquot of T5 was left standing on the bench throughout the SPE procedure before analysis by HPLC. This was compared to an aliquot analysed immediately after preparation. Negligible reduction of ampicillin was observed (-1.3 % reduction in average peak area).

Table 4.3: Results from test of first long SPE procedure

Solution	Prepared concentration (mg/L)[1]	Average[2] measured concentration (mg/L)[3]	Recovery, concentration (%)[4]	Recovery, peak area (%)[4]
T4	0.095 ± 0.001	0.070 ± 0.001	74 ± 2	N/A
T5	0.076 ± 0.004	0.066 ± 0.002	87 ± 5	87 ± 4

[1] Uncertainties calculated from uncertainties of the equipment used for preparation (equations 3.1 & 3.2)

[2] n = 2 for T4, and n = 3 for T5

[3] Uncertainty calculated using equation 3.3

[4] Uncertainties calculated using input values (equation 3.2)

The higher recovery for T5 might be attributed to more experience with the method, or it is possible that there was some gross error when testing the method on T4. There could also be a degree of variability in recovery from day to day. For comparison, Benito-Peña, et al. [8] lists a relative standard deviation of 2% for the 91% recovery found from three replicates analysed on three different days. This corresponds to $\pm 4.5\%$ with 95% confidence, i.e. a better precision than observed in this present method test.

A tentative conclusion from the peak area comparisons in Table 4.2 and Table 4.3 is that recovery of pure water ampicillin solutions after SPE should be between 80-90%. This is a little lower than what was shown by Benito-Peña, et al. [8] for wastewater effluent (90-95%). Some of this discrepancy might be an artefact of the RapidTrace®-based procedure. As noted above, there are many places where compound might be lost. It could also be due to the use of smaller SPE cartridges with less sorbent than in the article.

This testing of the SPE method using ultrapure water as solvent showed it to be a promising method for extracting and upconcentrating ampicillin in water samples. The next step of the method development was testing the SPE method on ampicillin in wastewater effluent. However, as 4.1.1 shows, there was first a need to find and test compounds to use as internal standards. These were both an internal standard to compensate for variability in HPLC instrument and UV detection performance (internal standard calibration), and an internal standard to compensate for loss of compound during sample preparation (recovery standard).

4.1.3 Testing Internal Standards

The choice to test oxacillin, cloxacillin and dicloxacillin as potential internal standards was due to their structural similarity to ampicillin. Furthermore, they were among the other β-lactams analysed by Benito-Peña, et al. [8]. Thus, they should respond like ampicillin to both the SPE and HPLC methods. The HPLC peaks shown for them in the article are well spaced from ampicillin, promising good separation.

The chromatograms of the separate β-lactam solutions yield the retention times of each compound. Oxacillin elutes at approximately 15 minutes, cloxacillin at 16 minutes and dicloxacillin at 18.5 minutes, as illustrated in Figure 4.3.
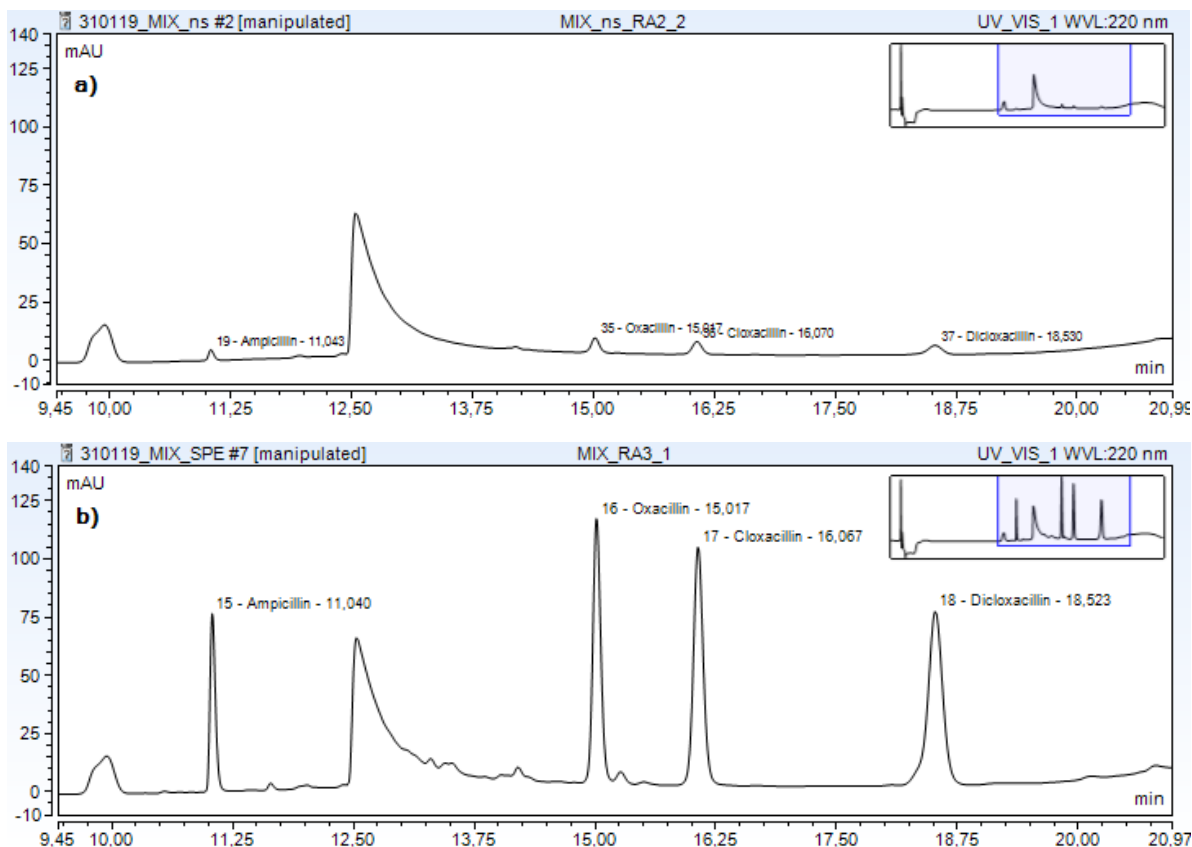
Figure 4.3: Chromatograms from mixed β-lactam solution
a) without SPE   b) with SPE

This figure shows two chromatograms: a) for the mixed solution that was only diluted with 0.05 M TBA in methanol solution prior to HPLC, and b) for the mixed solution that was extracted and upconcentrated by SPE first. The three other peaks are well separated from the ampicillin peak, appearing on the other side of the 12.5 min peak that is due to TBA. The peaks for oxacillin and cloxacillin are quite close, which could possibly pose a problem if used together in a more complex solution. Thus, using one of them along with dicloxacillin appeared to be the best choice for internal standards.

When finding the best candidate for recovery standard, it was important that the compound was properly extracted and upconcentrated by the SPE method. As can be seen in Figure 4.3b, all the potential internal standards respond well to the SPE method. Recovery for each β-lactam has been calculated by peak area comparison. The results are presented in Table 4.4.

Table 4.4: Recovery for ampicillin and potential internal standards

	Peak areas (mAU min)[1]					Recovery, (%)[2]
	SPE parallel 1	SPE parallel 2	SPE parallel 3	Average	Non-SPE (1/20)	
Ampicillin	4.94 ± 0.05	4.80 ± 0.08	4.83 ± 0.07	4.9 ± 0.2	0.302 ± 0.004	80 ± 3
Oxacillin	10.5 ± 0.2	10.1 ± 0.4	9.8 ± 0.2	10.2 ± 0.8	0.64 ± 0.02	80 ± 7
Cloxacillin	12.0 ± 0.2	11.8 ± 0.2	11.6 ± 0.2	11.8 ± 0.5	0.68 ± 0.01	87 ± 4
Dicloxacillin	13.5 ± 0.2	13.1 ± 0.3	12.9 ± 0.3	13.2 ± 0.8	0.73 ± 0.02	90 ± 6

[1] Uncertainties calculated using equation 3.3. n=3 for SPE, n=4 for non-SPE

[2] Uncertainties calculated using input values (equation 3.2)

Recovery is good for all compounds, for ampicillin it is comparable with previous results. For the other β-lactams, the recovery appears to increase with hydrophobicity. Dicloxacillin is the last compound to elute from the reverse phase HPLC column, and thus it is the least polar of the four β-lactams. That it has the highest recovery might be due to larger affinity for the hydrophobic part of the mixed-mode sorbent in the Oasis® MAX cartridges. Another interesting observation is the larger response factor for the other three β-lactams compared to ampicillin. While the concentrations of all four are approximately the same, the non-SPE peak area of ampicillin is half that of the three others. This is likely due the substituted isoxazole group present in oxacillin and its two chlorinated derivatives (see Figure 2.2), as unsaturated bonds in organic molecules absorb UV radiation [46, p. 723].

As can be seen in Figure 4.3b, there are some small peaks appearing right after the oxacillin peak. As these could cause inconsistent placement of the baseline and thus variable resulting areas, this compound was disqualified as internal standard. In deciding on cloxacillin or dicloxacillin as the recovery standard, the choice fell on dicloxacillin as it shows the highest recovery (90%). Thus, by elimination, cloxacillin would be used for internal standard calibration in the HPLC method.

Further testing of the analysis method, as well as implementation of the internal standards, was done using wastewater effluent as solvent.
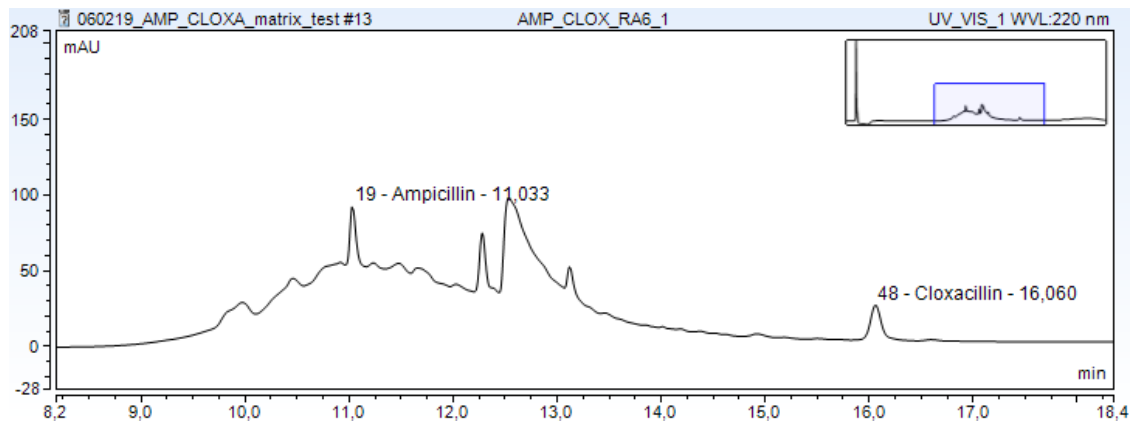
Figure 4.4: Ampicillin and cloxacillin in wastewater effluent extract (1.99 and 1.01 mg/L)

4.1.4 Testing the SPE Method on Ampicillin in Wastewater Effluent

As the main purpose of the simple SBR was to supply a wastewater effluent matrix for the ampicillin, a consistent schedule for settling, decanting and feeding was not set. Short settling times were applied when floating sludge was observed, to apply a selection pressure for good settling. Varying the substrate was done to see the effect on the matrix.

Filtering the effluent before SPE was proven necessary when an attempt to do SPE on non-filtered ampicillin solution immediately clogged the SPE cartridge.

4.1.4.1 Observing the Matrix

This test, where ampicillin and cloxacillin were added to the extracted wastewater effluent, was done to see the appearance of the chromatograms with this more complex matrix, as well as to view the ampicillin peaks at varying concentrations. Figure 4.4 shows the resulting chromatogram from the highest ampicillin concentration, including also the peak for cloxacillin. Figure 4.5 shows the peaks for all three ampicillin concentrations as well as for one of the blanks.

Comparing the chromatogram in Figure 4.4 with e.g. the one shown in Figure 4.3, it is clear that the sample matrix is much more complex, and that the SPE procedure extracts many components from the wastewater effluent. The appearance of the ampicillin peak in the midst of the noisiest part of the chromatogram is consistent with what is shown in the article by Benito-Peña, et al. [8]. As can be seen most clearly in Figure 4.5a, the baseline around the ampicillin peak is fairly free from other peaks, which is promising for quantification. It also makes even the lowest ampicillin concentration (Figure 4.5b) discernible from the noise.
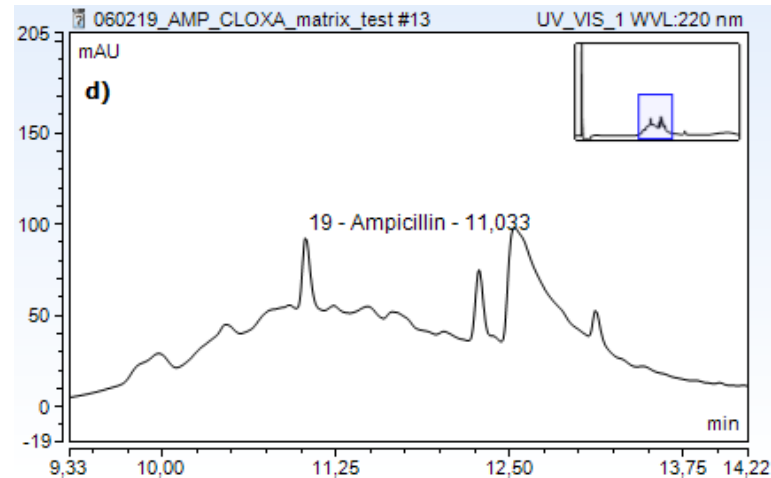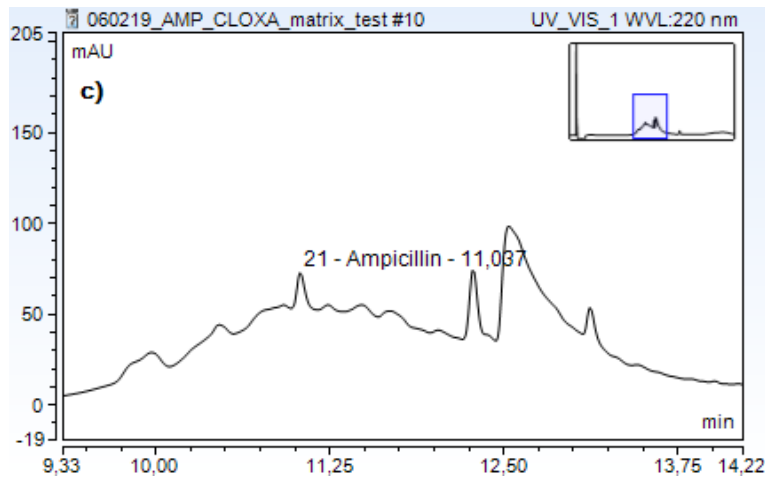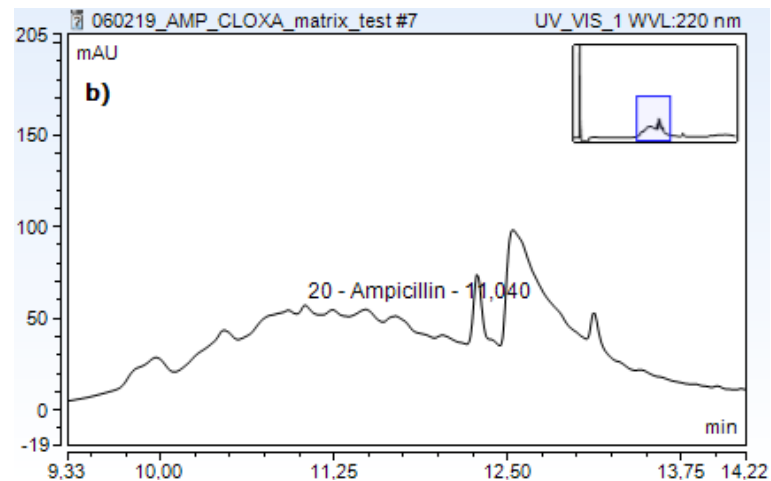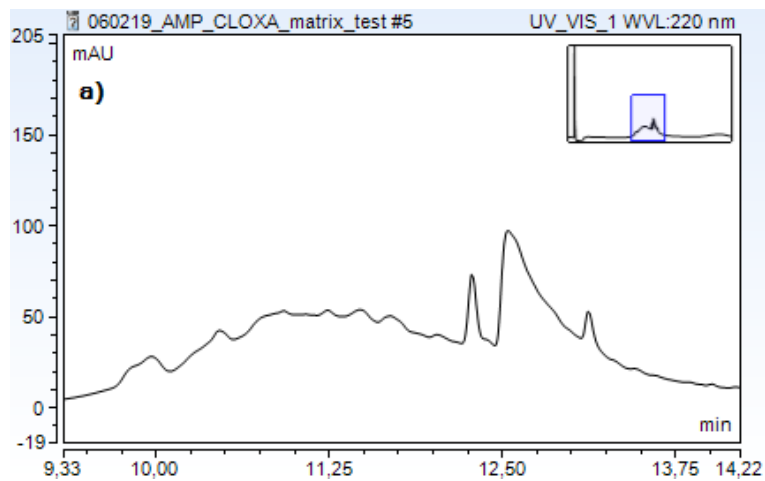
Figure 4.5: Peaks for ampicillin in wastewater effluent extract

a) 0 mg/L,   b) 0.245 mg/L,   c) 0.985 mg/L,   d) 1.99 mg/L

However, this peak is very small, casting doubt on whether this low concentration can be reliably measured with this degree of noise.

The placement of the ampicillin peak in the midst of the noisiest part of the chromatogram highlights the importance of preparing calibrations using solutions with the same matrix as the unknown samples. This because the placement of the baseline for integrating the peaks will be affected by the surrounding noise.

Because the baseline was expected to vary, the measurements of recovery in this part of the method testing were done using peak area comparisons only (no calibration).

4.1.4.2 Extracting B-lactams Using Long SPE Procedure

The first test was qualitative, to see whether the ampicillin and dicloxacillin would be extracted from the wastewater effluent at the given concentrations. Figure 4.6a shows one of the resulting chromatograms, representative of the rest and clearly showing peaks for both, as well as for the cloxacillin added after SPE.

Table 4.5 presents the results from the two subsequent, quantitative, tests. For the first of these, the areas listed are calculated from absolute values. For the second test, all peak areas used for calculation are relative to the peak area for cloxacillin present in the same chromatogram.

While recovery for both tests are very similar, the precision for the second test is better. A possible explanation for this follows.

The parallels were analysed in order. As can be seen in the results from test 1, the measured areas decrease going from one parallel to the next. A similar pattern can also be seen (with one exception) in Table 4.4. This is observed not only between parallels, but also within each parallel. The measured area for each injection gradually decreases, the reduction appearing to be proportional to the area of the peak itself. This could be due to decomposition of the β-lactams or heating of the samples over time, or a combination of the two. Both will cause less analyte to be injected and thus smaller peaks as time goes on. Decomposition as a possibility is based on Benito-Peña, et al. [8] stating that the tested β-lactams were only stable in TBA in methanol for up to 18 hours. However, as the sample solvent for HPLC was 1:1 this and ultrapure water, decomposition is not considered to have had a big effect.

Table 4.5: Recovery for ampicillin and dicloxacillin, long SPE procedure

| Test and compound | | Average (n = 3) peak areas (mAU min)[1] | | | | | Recovery (%)[2] |
		Parallel 1	Parallel 2	Parallel 3	Average	Recovery solution	
1[3]	**Ampicillin**	0.71 ± 0.02	0.69 ± 0.02	0.67 ± 0.02	0.69 ± 0.04	0.90 ± 0.01	77 ± 5
	Dicloxacillin	7.2 ± 0.3	6.9 ± 0.3	6.6 ± 0.3	6.9 ± 0.7	8.1 ± 0.2	86 ± 7
2[4]	**Ampicillin**	0.279 ± 0.004	0.279 ± 0.005	0.282 ± 0.003	0.280 ± 0.004	0.359 ± 0.005	78 ± 1
	Dicloxacillin	0.696 ± 0.007	0.682 ± 0.006	0.682 ± 0.006	0.69 ± 0.02	0.799 ± 0.008	86 ± 3

[1] Uncertainties calculated using equation 3.3

[2] Uncertainties calculated using input values (equation 3.2)

[3] Absolute areas

[4] Relative areas

The same pattern in reduction is seen for the peak areas of cloxacillin. Thus, calculating with relative peak areas rather than absolute values will cancel out some of the variation caused by this gradual reduction in peak sizes. And so, the precision in recovery for the second test in Table 4.5 is better than in the first. In this way, the internal standard cloxacillin should improve the precision of analysis as well as the accuracy.

The recovery precision is comparable to, or better than, what is listed in [8]. There, the relative standard deviation for recovery is between 1 and 7% for extraction of ampicillin from wastewater effluent.

Figure 4.6 shows representative chromatograms from the three extractions and analyses done using the long SPE procedure. The analyses represented by chromatograms a and b were done on consecutive days. In that period, the SBR was fed starch. The analysis illustrated by chromatogram c was done the week after, when the SBR was fed peptone. As can be seen, there is less noise around the ampicillin peak in the first two chromatograms. Peptone is a more complex substrate than starch, and appears to cause more complex soluble waste products, or simply more waste products that are extracted by SPE and absorb UV light.
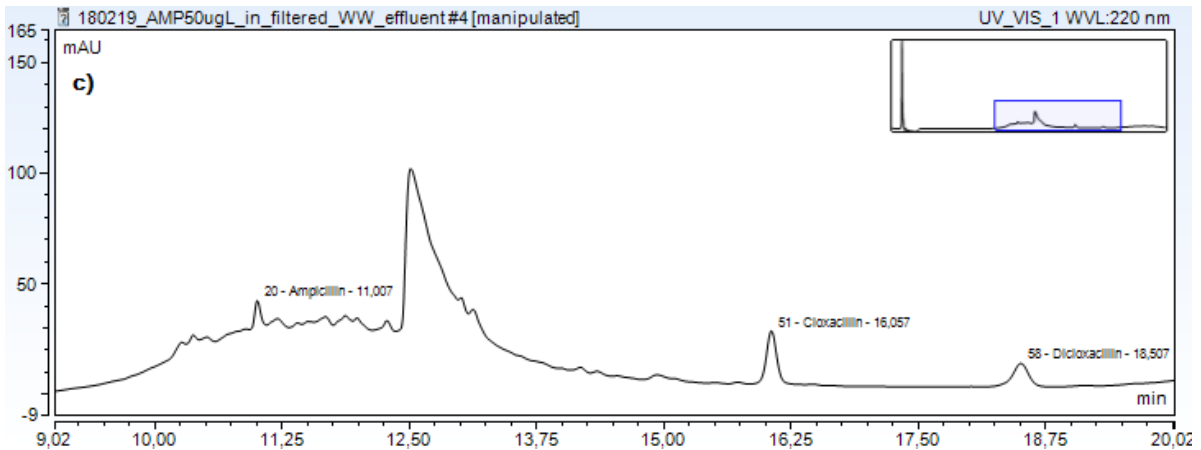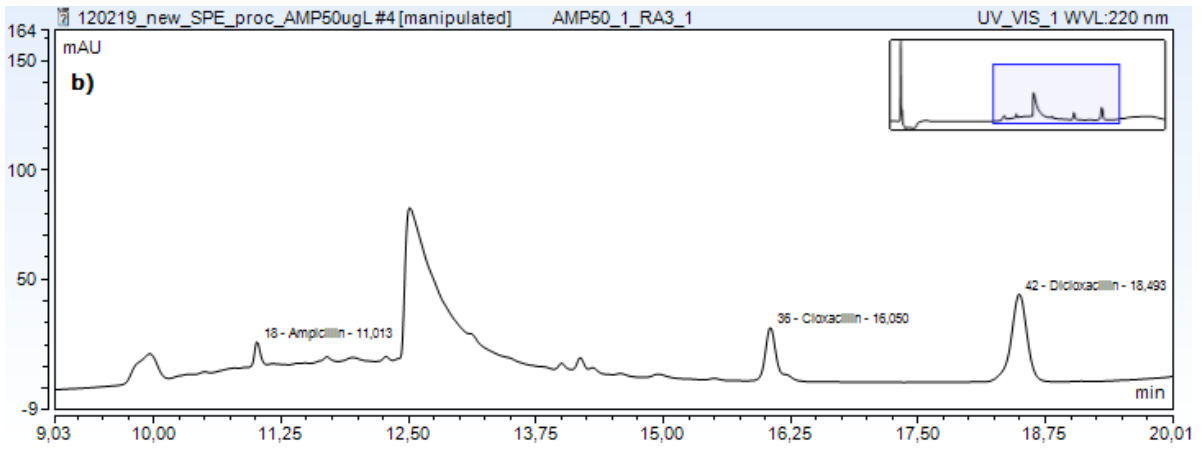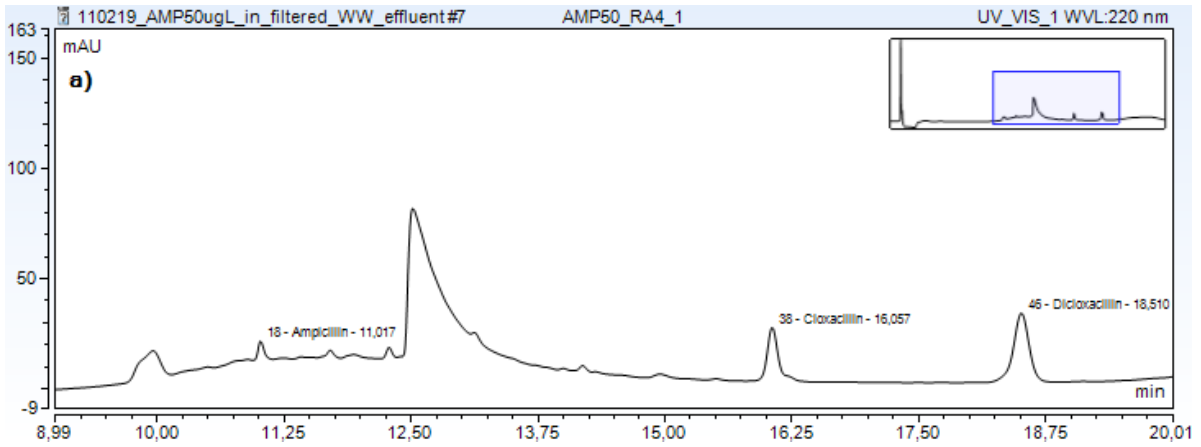
Figure 4.6: Chromatograms from filtered wastewater effluent extraction

a) qualitative test   b) first quantitative test   c) second quantitative test

Interestingly, Figure 4.6c has less noise than can be seen in Figure 4.4 and Figure 4.5. This despite the SBR being fed peptone in both cases. However, those two earlier figures show chromatograms of the matrix extracted from the SBR effluent, just a day after it had been inoculated with sludge. Likely, there was a greater mix of soluble compounds present in the

inoculum that were later removed through decanting and refilling with tap water. In addition, some of the extra noise in those earlier chromatograms could be explained by the biomass being less adapted to the substrate it was fed, and thus producing more complex waste products.

The observed clearing up of the background noise posed the question of whether a shorter SPE procedure, loading maximum 12 mL sample, could work on effluent from a lab-scale SBR. As this would be a much more efficient extraction (2 h compared to 6.5 h), this shorter procedure was tested.

4.1.4.3 Extracting B-lactams Using Short SPE procedure

As exemplified in Figure 4.7 there is minimal background noise in the chromatograms from these two tests. Thus, quantifying the peaks from the dilute solution (50 µg/L of both ampicillin and dicloxacillin) posed no problem.

At this point in the method testing, the simple SBR was fed a combination of peptone and starch, but the baseline is clearer than in any of the chromatograms in Figure 4.6. This could be due to less wastewater constituents being extracted by the short SPE procedure.

The results from the tests are shown in Table 4.6. Test 1 was on the more concentrated solution, while test 2 was on the dilute. All peak areas listed are calculated from relative peak areas, i.e. peak area of ampicillin or dicloxacillin divided by peak area of cloxacillin in the same chromatogram.
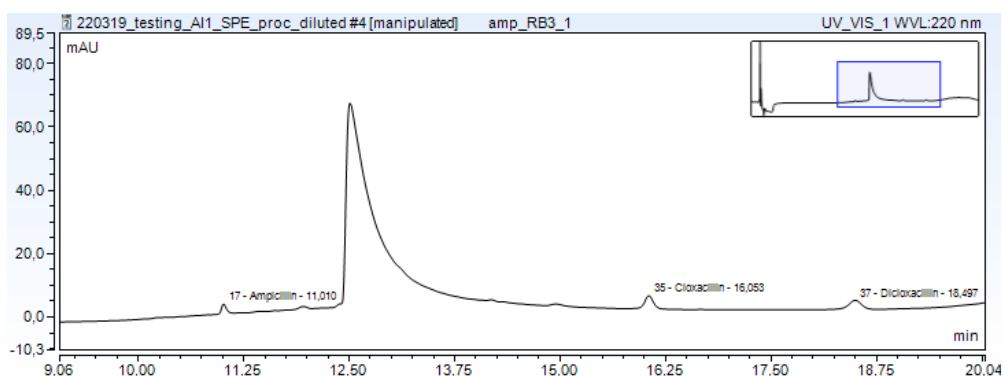

Figure 4.7: Chromatogram from dilute solution extracted by short SPE procedure

While the uncertainties for the recoveries overlap in the two tests, it does appear that the recovery is slightly higher for the more dilute solution. A possible explanation for this is that there was some overloading of the SPE sorbent for the more concentrated solution, causing more of the compounds to be washed out. Such overloading could also explain some of the lower recoveries for the previous tests using the long SPE procedure.

All recoveries in these two tests are higher than those shown for the previous tests on wastewater samples. This can likely be attributed to less loss of compound in the process compared to the long SPE procedure (only one rinse step, no refilling of test tubes). The recovery for ampicillin is comparable to the previous tests using the short SPE procedure on ultrapure water solutions. The precision is however much better for the dilute solution recovery in Table 4.6 than is seen for the dilute solutions in Table 4.2. Thus, it appears that precision is not necessarily lowered when less analyte is loaded onto the SPE cartridge, as was speculated above.

The success of the short SPE procedure was promising for further use. It would afford more flexibility, both by being less time-consuming and by allowing for smaller sample sizes. Thus, it made possible the study of adsorption of ampicillin to activated sludge, described in 4.2.

Table 4.6: Recovery for ampicillin and dicloxacillin, short SPE procedure

Test and compound	Average (n = 3) relative peak areas[1]					Recovery (%)[2]
	Parallel 1	Parallel 2	Parallel 3	Average	Recovery solution	
1						
Ampicillin	1.016 ± 0.007	1.021 ± 0.004	1.028 ± 0.003	1.02 ± 0.02	1.17 ± 0.01	87 ± 2
Dicloxacillin	3.34 ± 0.03	3.30 ± 0.05	3.26 ± 0.03	3.30 ± 0.09	3.45 ± 0.03	96 ± 3
2						
Ampicillin	0.4110 ± 0.0002	0.403 ± 0.004	0.408 ± 0.004	0.41 ± 0.01	0.454 ± 0.005	90 ± 2
Dicloxacillin	1.010 ± 0.002	0.98 ± 0.01	0.95 ± 0.02	0.98 ± 0.07	0.98 ± 0.02	100 ± 7

[1] Uncertainties calculated using equation 3.3

[2] Uncertainties calculated using input values (equation 3.2)

4.1.4.4 Recovery Ratio

The purpose of dicloxacillin in the method is to correct for recovery in unknown samples. This demands that there is a consistent ratio between the recovery for dicloxacillin and for ampicillin. The average of the ratios from the four tests reported above is 1.11 ± 0.02, where the uncertainty is calculated from equation 3.3. The precision for this value was considered acceptable. This ratio is used in the following, together with the measured recovery for dicloxacillin, to correct for recovery in unknown ampicillin samples.

4.2 Adsorption Studies

In this section, the results from the adsorption experiments are presented and discussed. The results are also put in a bigger context at the end of the section.

4.2.1 Exploring Isotherms from Literature

Table 4.7 shows the values predicted by the Freundlich and Langmuir isotherms in literature [11, 37], given an equilibrium ampicillin concentration of 1 mg/L. For calculating start concentrations and percent ampicillin remaining in solution, MLSS has been set to 1 g/L.

As can be seen, the isotherms by Jia, et al. [11] predict higher adsorption than the ones by Shen, et al. [37]. For the additional isotherms presented in the latter article, the difference is larger: the amount of ampicillin remaining in solution is predicted to be from 75% (BET isotherm) to nearly 100% (Tóth isotherm). For the Redlich-Peterson isotherm, which had the best fit to the data ($R^2 = 0.983$) [37], the predicted amount remaining is 96%. This is interesting, considering that adsorption has been shown to be a main removal path for ampicillin in activated sludge systems (see 2.3.1)

Table 4.7: Comparing literature isotherms (C_e = 1 mg/L, MLSS = 1 g/L)

Isotherm	Jia, et al. [11]			Shen, et al. [37]		
	C_s (mg/g)	C_0 (mg/L)	% remaining	C_s (mg/g)	C_0 (mg/L)	% remaining
Langmuir	11.6	12.6	7.9	0.330	1.33	75
Freundlich	6.57	7.57	13	1.21	2.21	45

A possible explanation for the difference in predicted removal is the dissimilar experiment designs employed. Jia, et al. [11] added 0.500 to 200 mg/L ampicillin to an activated sludge concentration of 1.000 g/L. Shen, et al. [37] centrifuged the activated sludge to "remove excess water" (p. 36) before adding a varying mass (0.3-0.03 g) of this to a constant volume of solution containing ampicillin. The ampicillin concentrations started higher in [37] than in [11], from 10 mg/L to 100 mg/L. Finally, in [11] the activated sludge was taken directly from a WWTP, whereas in [37] the sludge had been acclimated for a month prior to further use. Possibly, this acclimation, and the following centrifugation, altered the adsorbing capacity of the sludge.

4.2.2 Preliminary Adsorption Experiment

The MLSS of the simple SBR was measured to 0.53 ± 0.02 g/L (95% confidence, n=3).

The internal standard calibration done for ampicillin and dicloxacillin both had R^2 values of 1.000 with a standard error of 0.003 and 0.005, respectively. This points to a clear linearity between the response ratio and concentration ratio for both compounds in this range.

Table 4.8 shows the results of the experiment. The MLSS value listed is after dilution with tap water. C_e is the measured concentration of ampicillin in solution, calculated from equation 3.7. C_s is found from equation 3.8.

Based on the time intervals reported in [11, 37], seven hours are assumed to have been enough to achieve equilibrium in this experiment. Thus, the values listed in Table 4.8 are treated as equilibrium concentrations. A further assumption is that there have been no other removal mechanisms at play over the course of the experiment, such as biodegradation, chemical hydrolysis or photolysis. This assumption is based on the measures taken to avoid biodegradation and photolysis: inactivation of biomass by sodium azide and wrapping the tubes in aluminium foil to block light; as well as the stability of ampicillin to hydrolysis at neutral pH [27]. By these assumptions the calculation of C_s from equation 3.8 is justified.

The results show that only around 20% of the ampicillin was removed after seven hours. A possible explanation for this is that the amount of adsorbent was insufficient to remove more of the compound. A typical domestic wastewater plant has a MLSS of around 3 g/L [11], much higher than the MLSS used in this experiment. It is also possible that there was not complete mixing of the contents of the centrifuge tubes over the course of the experiment, leading to inadequate contact between the antibiotic and the sludge.

Table 4.8: Results from preliminary adsorption experiment[1]

Parallel	MLSS (g/L)	C_0 (mg/L)	C_e (mg/L)	C_s (mg/g)
1	0.201 ± 0.007	1.023 ± 0.005	0.82 ± 0.04	1.0 ± 0.2
2	0.201 ± 0.007	1.034 ± 0.005	0.80 ± 0.04	1.1 ± 0.2
3	0.201 ± 0.007	0.995 ± 0.005	0.83 ± 0.04	0.8 ± 0.2
Average	--	--	0.82 ± 0.07	0.97 ± 0.03

[1] Uncertainties calculated from uncertainty in input values (equations 3.1 and 3.2)

There was no pH-adjustment prior to SPE in the analysis of samples. However, the pH of the diluted sludge mixture after filtration was measured to be 7.7, only 0.2 above the target 7.5, so this likely did not have a big effect on the results.

Comparing the results from the experiment with the isotherms in [11, 37], the best fit of the data is to the Freundlich isotherm in [37]. This isotherm predicts a C_s value of 1.1 mg/g for a C_e value of 0.82 mg/L, as well as 79% remaining in solution at MLSS = 0.2 g/L.

In this first, preliminary experiment, the time to reach equilibrium was assumed. The next step of the thesis work was to actually determine this time interval, through observing adsorption over time. Furthermore, as a MLSS of 0.2 g/L is much lower than common real life WWTP concentrations, further experiments employed higher sludge amounts. The results of these experiments are described and discussed in the following.

4.2.3 First Study of Adsorption Kinetics

Figure 4.8 shows the ampicillin peak from the analysis of one of the 5 min samples. Chromatogram a is the result of measuring UV-absorption at 220 nm, while chromatogram b is from measuring at 205 nm.

As can be seen in both chromatograms, there is a broad peak, or combination of peaks, right at the tail of the ampicillin peak. This peak is not present in the calibration chromatograms, but can be seen in the analysis of the blanks. Possibly it is due to products from one or more degradation reactions in the sludge mixture over the course of the experiment. This would explain why it is not present in the calibration chromatograms, as these were prepared first. Its presence in the blank rules out the possibility of it being caused by ampicillin reactions.
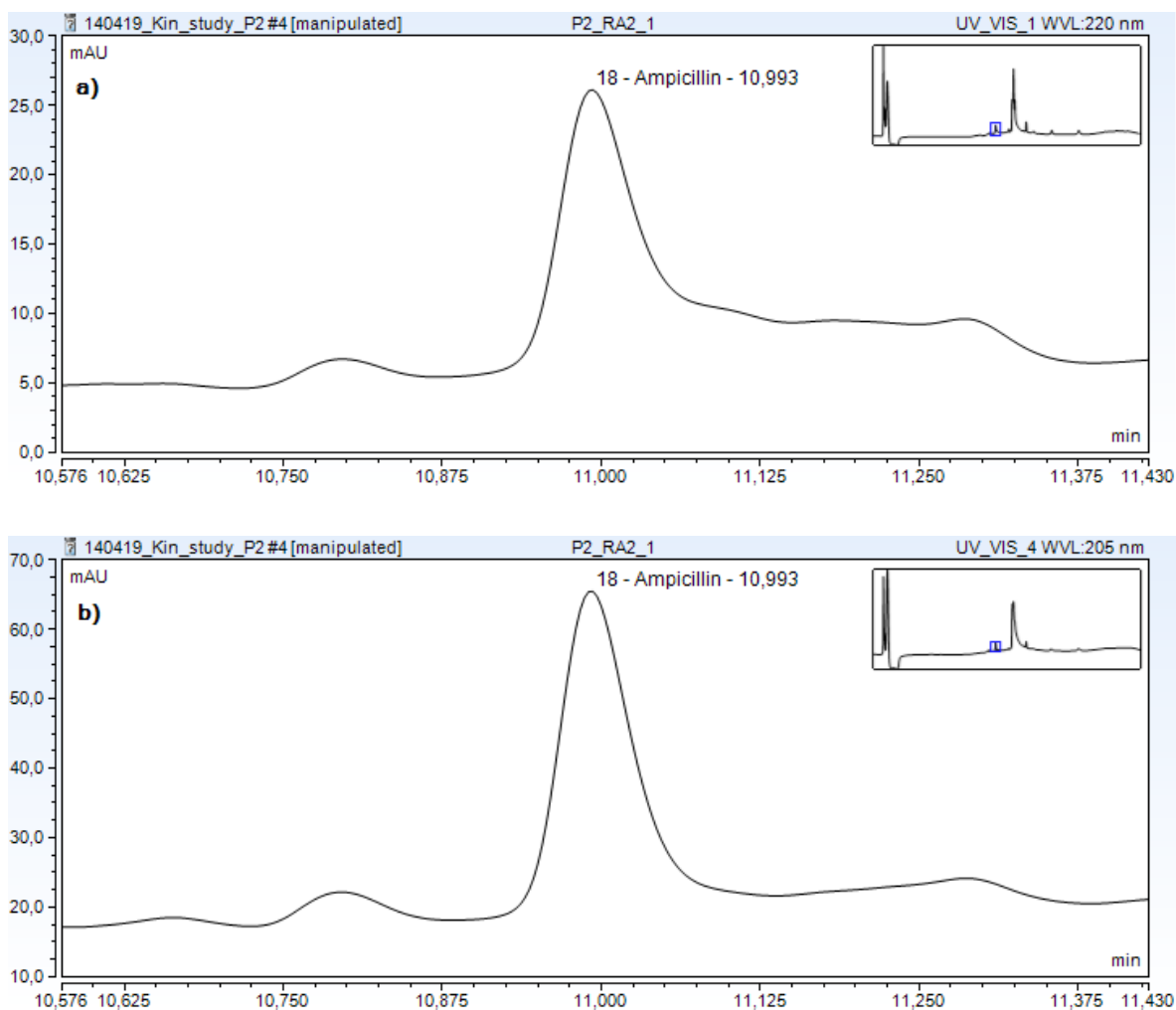
Figure 4.8: Chromatogram comparison, 220 nm (a) and 205 nm (b)

As Figure 4.8b shows, the broad peak is smaller relative to the ampicillin peak in the chromatogram from the 205 nm measurements. As this makes more precise quantification possible, all peak areas used to prepare the following results are from the 205 nm measurements.

The internal standard calibrations for ampicillin and dicloxacillin yielded R^2 values of 1.000 with a standard error of 0.008 and 0.006, respectively. This again shows linearity.

Figure 4.9 gives the plot of remaining ampicillin vs. time. The first datapoint, at t = 0, is a theoretical value. The error bars reflect 95% confidence, calculated from equation 3.3 with n = 4. Possible outliers have been tested using Chauvenet's criterion [51], and none were rejected.

The shrinking of the error bars with time might be explained by more homogenous mixing of sludge and ampicillin in the different parallels over time. This would cause more similar adsorption in the flasks later in the experiment.
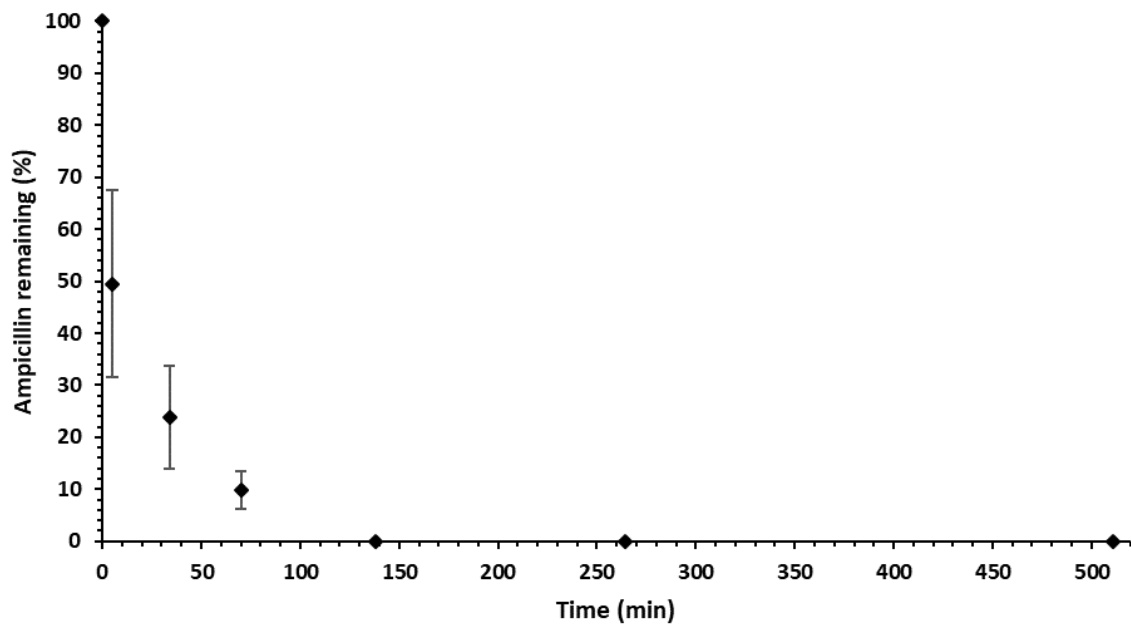
Figure 4.9: Ampicillin remaining plotted against time, first kinetics study

As can be seen, no remaining ampicillin was detected after the third sampling. Part of this could be due to issues with quantification caused by the broad peak described above. Figure 4.10 shows a comparison of the chromatograms from one of the 5 min samples and one of the 8.5 h samples. The possible presence of ampicillin in the latter sample, as well as in the 2 h and 4 h samples, is considered impossible to quantify. Attempting to subtract the blank sample from the chromatograms does not improve the quantification.

Being unable to quantify ampicillin after 1 h makes it unclear if and where equilibrium was achieved in the experiment. Two things are apparent, however. The initial rate of removal is high, and much more ampicillin has been removed than in the preliminary adsorption experiment. This can be attributed to the higher MLSS (3 g/L vs. 0.2 g/L).

The Freundlich isotherm in [37] gives a C_0 of 1.03 mg/L if C_e is set to 0.06 mg/L and MLSS to 3 g/L. A C_0 of 1 mg/L, as in this experiment, would thus give a C_e of 0.05-0.06 mg/L according to this isotherm.
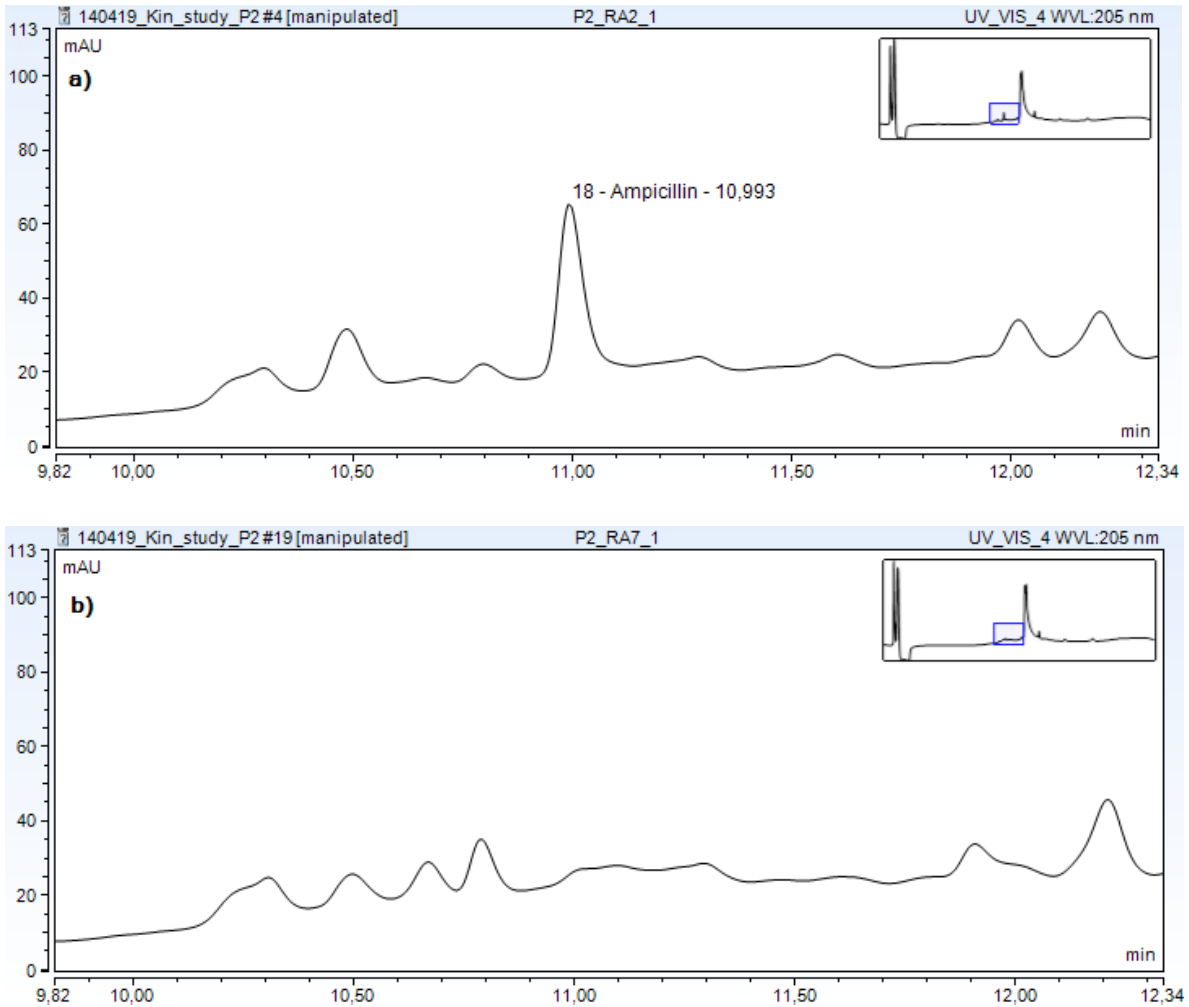
Figure 4.10: Chromatograms from 5 min (a) and 8.5 h (b) samples

As values of 0.08-0.10 mg/L are measured for the 1 h samples in this experiment, it is possible that values of 0.05-0.06 mg/L could have been measured for the later samplings, had there been less issues with quantification. So, there is a possibility that both the results from the preliminary adsorption experiment and this present experiment are in line with the Freundlich isotherm in [37], and thus that the ampicillin was adsorbed to the activated sludge according to similar mechanics. However, concluding on this is not possible. The results from this experiment could also easily fit the isotherms in [11], which predict a C_e of 0.013 mg/L (Freundlich) and 0.019 mg/L (Langmuir) for the same conditions.

If there were other removal mechanisms than adsorption at play in the experiment, the appearance of complete adsorption of the ampicillin after 1 h can also be explained by this.

The inconclusive results from this experiment prompted the decision to repeat the experiment with a lower MLSS. This was done in combination with an experiment to attempt to set up adsorption isotherms for ampicillin onto activated sludge.

4.2.4 Second Kinetics Study and Adsorption Isotherm Study

The broad peak on the tail of ampicillin observed in the previous experiment is, while smaller, present in the chromatograms from the experiments described here as well. The smaller size indicates some correlation between this peak size and the amount of sludge. As in the previous experiment, better quantification is achieved by use of the 205 nm chromatograms. Further improvements have been reached through subtracting the blank sample chromatogram from the rest of the chromatograms in each run.

The strong indications of linearity in the calibrations done previously for the relevant concentration range (4.1.1, 4.2.2 and 4.2.3) justifies using single point calibration in these experiments.

4.2.4.1 Second Kinetics Study

Figure 4.11 shows the variation in remaining ampicillin over time. The data for the figure has been prepared the same way as for Figure 4.9.
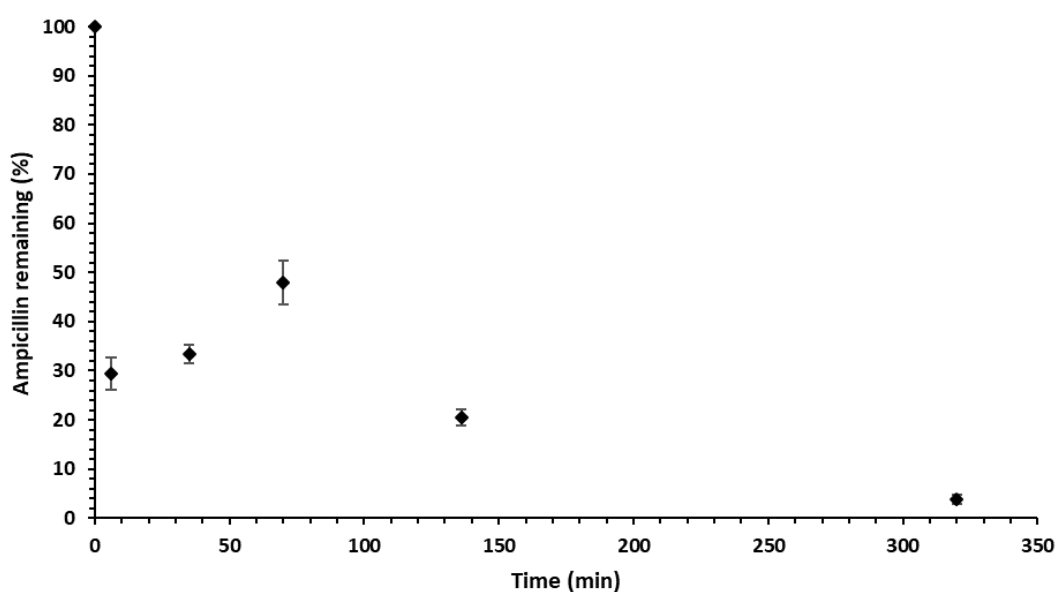

Figure 4.11: Ampicillin remaining plotted against time, second kinetics study

The precision of the measurements, reflected in the error bars, is better for this experiment than the previous. A possible explanation for this is the lower MLSS employed here. The inhomogeneous nature of sludge could cause more variable adsorption, correlating with the amount of sludge present.

Another difference from the previous experiment is the presence of ampicillin in solution at all points in the experiment, and at higher concentrations for the 30 min, 1 h and 2 h samples. This may again be explained by the smaller amount of adsorbent present. The better quantification achieved in this experiment can also have had an effect on both this and the precision.

The clear issue with the results is the apparent increase in dissolved ampicillin concentration going from the 5 min sample to the 1 h sample. This goes against the expectation that the concentration will decrease over time as the system moves towards equilibrium, which would cause a plot more similar to the one in Figure 4.9. Two possible explanations are identified that may have caused this issue, and both are related to pH.

The 1 h and 2 h samples were analysed first. When adjusting pH, the start-pH of both were judged from experience to be in a normal range (approximately 8.2-8.3). However, when analysing the 5 min and 30 min samples, it was noted that the pH was quite high (over 9). Thus, the start-pHs for the rest of the samples were registered. The variation of pH over time is shown in Figure 4.12, where the data points for the 1 h and 2 h samples are approximations based on experience, and the $t = 0$ data point is an assumption based on the pH measured for the negative control samples (9.6, more on this below).
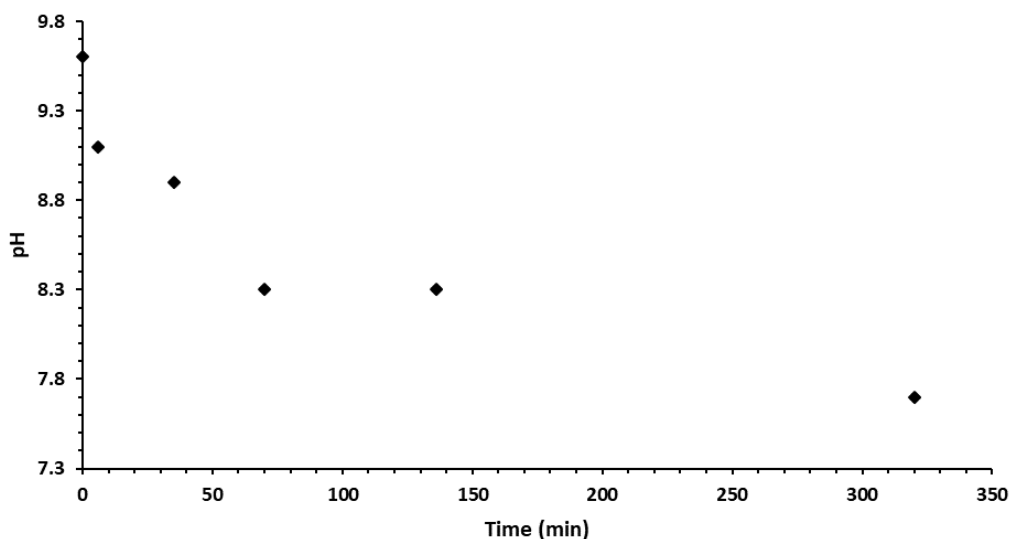

Figure 4.12: Variation in sample pH over time

As can be seen, the pH of the samples is reduced over time. The pH measured for the negative control is higher than any of the others despite it standing on the bench for the duration of the experiment. From this it can be surmised that the reduction in pH is due to some process associated with the sludge. It is likely not related to the ampicillin, as the same pH was measured in the blank samples, and the pH was high in the negative control samples containing ampicillin.

After sampling, the samples were quickly separated from the sludge by centrifugation, and through further treatment, freezing, thawing and preparation for analysis, they were left at the measured pH for approximately four hours. Knowing the higher susceptibility of ampicillin to hydrolysis at pH 9 [27], it is probable that the lower concentrations measured for the 5 min and 30 min samples were due to chemical hydrolysis occurring in the time between centrifugation and pH adjustment.

Another possible explanation is that the higher pH early in the experiment has had an effect on the adsorption itself, with more adsorption occurring at higher pH and then more ampicillin being released from the sludge as pH lowered.

The reason for the high pH at the start of experiment is unclear, but a possible cause is the addition of sodium azide. The pKb of the azide is 9.3 [56], making it a weak base. It is however uncertain if 0.03 M of the azide is capable of increasing pH to the level observed, especially as there is expected to be a degree of buffering capacity in sludge mixtures such as the one prepared. pH above 8.5 was not measured for the previous experiment (4.2.3), despite the presence of the same amount of sodium azide. As the sludge mixture was less diluted there, it should have had a greater buffering capacity. Furthermore, in the previous experiment the sludge mixture stood on a shake table overnight, likely allowing the sodium azide and the sludge to equilibrate prior to addition of ampicillin.

Disregarding the first two measurements in Figure 4.11, it is still difficult to conclude if and where equilibrium was achieved. The initial rate of removal of ampicillin appears lower than in the first kinetics study, an indication that the adsorption rate for ampicillin in the two experiment conditions is dependent on the adsorbent concentration.

As equilibrium was achieved after 4-5 hours in the literature [11, 37], it is likely that equilibrium was achieved in the present experiment as well. Under this assumption, the data from the 5 h measurements have been used to prepare adsorption isotherms.

4.2.4.2 Adsorption Isotherm Study

The MLSS from the SBR, prior to dilution and addition of sodium azide, was measured to 2.8 ± 0.1 g/L (95% confidence, n=3).

Table 4.9 shows the measured and calculated data from the adsorption isotherm experiment. The analysis results for sludge mixture A3 were not quantifiable and are thus not included. The data listed for sludge mixture K are the results from the 5 h sampling in the second kinetics experiment.

As in the preliminary adsorption experiment, the C_s values have been calculated using equation 3.8, again under the assumption that no other removal mechanisms occurred over the course of the experiment. The single point calibration for the 5 h samples was used to determine C_e.

Linear isotherm plots of the eight data points in Table 4.9 are shown in Figure 4.13. The error bars for each point reflect the uncertainties listed in the table, after transformation according to error propagation rules (equation 3.2 and the log transformation rule [46, p. 114]). A trendline has been added. The uncertainties listed for the slope and y-intercept (95 % confidence), and the uncertainty for the R^2 values (standard error) are calculated by the regression analysis tool in Microsoft Excel.

Table 4.9: Data from adsorption isotherm experiment (MLSS = 0.99 ± 0.04 g/L)[1]

Sludge mixture	Parallel	C_0 (mg/L)	C_e (mg/L)	C_s (mg/g)
A1	1	2.691 ± 0.005	0.060 ± 0.003	2.7 ± 0.1
	2	2.613 ± 0.005	0.060 ± 0.004	2.6 ± 0.1
K	1	0.97 ± 0.03	0.044 ± 0.002	0.93 ± 0.04
	2	1.02 ± 0.03	0.041 ± 0.002	0.98 ± 0.05
	3	0.99 ± 0.03	0.037 ± 0.003	0.96 ± 0.05
	4	1.01 ± 0.03	0.031 ± 0.002	0.99 ± 0.05
A2	1	0.570 ± 0.002	0.008 ± 0.003	0.57 ± 0.02
	2	0.542 ± 0.002	0.012 ± 0.003	0.53 ± 0.02

[1] Uncertainties calculated from uncertainty in input values (equations 3.1 and 3.2)
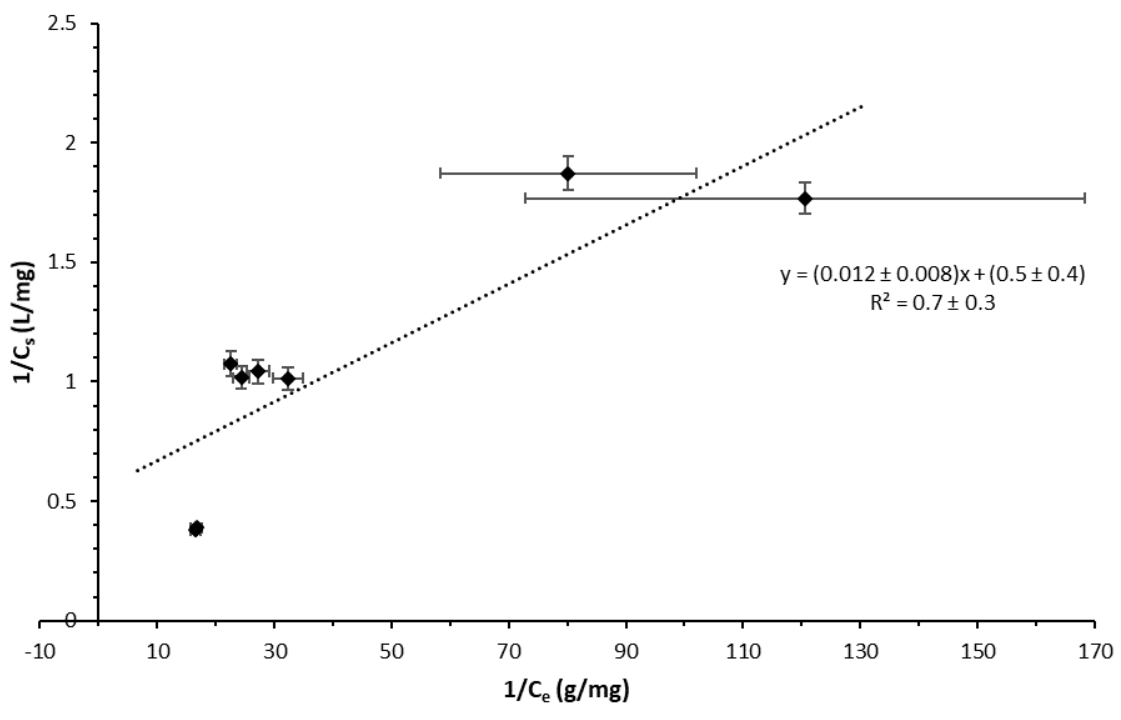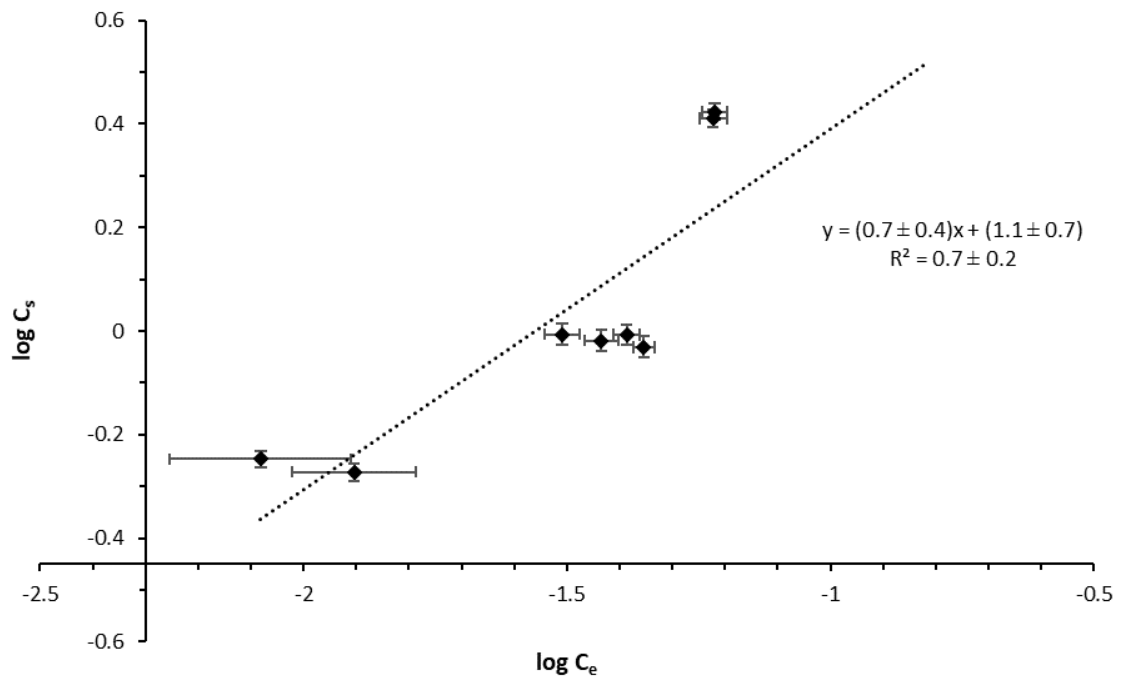
Figure 4.13: Linear isotherm plots

top: Freundlich   bottom: Langmuir

It is clear from the R^2 values in the plots that neither of the linear isotherm equations are good fits for the data. In addition, there is large uncertainty connected to some of the data points, throwing the results further into question.

The values found for the Freundlich and Langmuir isotherm parameters are listed in Table 4.10. Their uncertainties are calculated using error propagation rules (equation 3.2 and the antilog rule [46, p. 114]), with the uncertainties from the regression analysis as the input values.

Comparing with the isotherms from literature (Table 2.1), the parameters found for the Freundlich isotherm can fit those found in both articles, due to the large uncertainties. For the Langmuir isotherm, the parameters fit neither. Both Langmuir isotherms in the articles have a larger $C_{s,max}$ and a smaller K_L than listed in Table 4.10. As $C_{s,max}$ is the theoretical maximum adsorption capacity [34], this could mean that the sludge used in this thesis work had different adsorption capabilities than the sludge used in either of those studies.

However, it is difficult if not impossible to conclude anything from this adsorption isotherm study. Possibly a larger amount of data points, spread over more C_0 values, would give better results. A non-linear regression approach using the sum-of-least-squares method with the problem solver in Microsoft Excel was attempted, but from a qualitative evaluation of the resulting plots (seen in Appendix C), it is unclear whether these isotherms gave better fits.

Other isotherms than Freundlich and Langmuir could fit better with the data points, however the preparation and evaluation of these are considered to be beyond the scope of this thesis.

Table 4.10: Calculated isotherm parameters

Isotherm	Parameter	Value
Freundlich	K_F (mg/g)(L/mg)^{nF}	12 ± 19
	n_F	0.7 ± 0.4
Langmuir	$C_{s,max}$ (mg/g)	2 ± 1
	K_L (L/mg)	44 ± 44

4.2.4.3 Possibility of Chemical Degradation

A representative chromatogram from the analysis of the negative control samples is shown in Figure 4.14. The measured average remaining ampicillin in the negative control is 23% $\pm$ 35% (n = 3, 95% confidence). The large uncertainty is due to one of the samples having a concentration over twice that of the two others. A test by Chauvenet's criterion [51] shows that this result cannot be disregarded as an outlier.

Regardless of the large uncertainty, the value clearly shows that there has been substantial removal of ampicillin in the negative control despite the absence of sludge. As previously noted, the pH of the negative control was measured to 9.6 prior to adjustment. The ampicillin was left at this high pH for more than seven hours. It is reasonable to assume that a substantial part of the ampicillin removal has been due to chemical hydrolysis in the basic solution. Extracellular enzymes excreted by the activated sludge, and not removed by 0.45 μm filtration, may also have played a part.

Thus, while the pH was reduced over time in the sludge mixture used for kinetics and adsorption isotherm studies (Figure 4.12), some removal of ampicillin through chemical hydrolysis must be assumed for those samples as well. And so, the use of equation 3.8 to calculate C_s in 4.2.4.2 is not entirely justified, making the results from the adsorption isotherm experiment even more inconclusive.
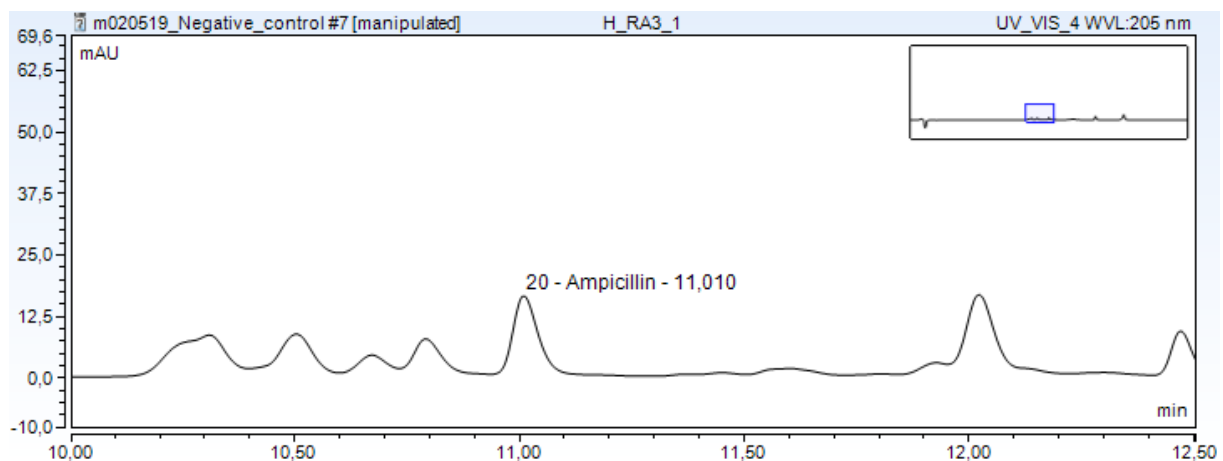

Figure 4.14: Chromatogram from negative control

Figure 4.15 shows representative chromatograms from the sludge mixtures A1 (a), K (b) and A2 (c). The four peaks to the left of ampicillin appear to decrease with decreasing start concentration of ampicillin. As the blank has been subtracted from all three chromatograms in the figure, these peaks were not present in solutions without ampicillin. This indicates that these peaks are caused by products from ampicillin degradation. The peaks are also present in the negative controls, as exemplified in Figure 4.14.

Thus, it appears that there has been chemical degradation of ampicillin in all samples, and that this has contributed to the removal of the β-lactam over the course of the experiments.

Exactly which products cause the peaks to the left ampicillin is unclear. However, as hydrolysis causes addition of an -OH group to a molecule, it is expected that the product will be more polar than the parent compound. A hydrolysis product of ampicillin would thus have a shorter retention time in the reverse phase HPLC column, consistent with the peaks appearing to the left of ampicillin.

4.2.5 Summary and the Bigger Picture

The adsorption studies described above are inconclusive when it comes to time of equilibrium and adsorption isotherms, as well as on how much removal is actually due to adsorption. There is however one clear take-away from the results: at activated sludge concentrations of 1-3 g/L, even when the sludge is inactivated by sodium azide, more than 90% of ampicillin is removed from solution. In the case of 3 g/L sludge, this removal occurs within the first hour of exposure. For 1 g/L sludge, the results show a likely 50% removal within the first hour, with the remaining ampicillin down to 4% after five hours.

In the context of the larger research project this thesis is part of, where activated sludge will be exposed to both ampicillin and ampicillin resistance genes, it is important to know the removal of the β-lactam. The results above give some indications on this.

In a larger context, the results show that in biological WWTPs, even a HRT of five hours should be enough for removal of most of the ampicillin. And so, the antibiotic will not be released to natural water systems. However, it should be noted that even with the removal found in this thesis and other research, the presence of ampicillin at sub-inhibitory concentrations in biological wastewater treatment plants could still promote antibiotic resistance among the bacteria present there.
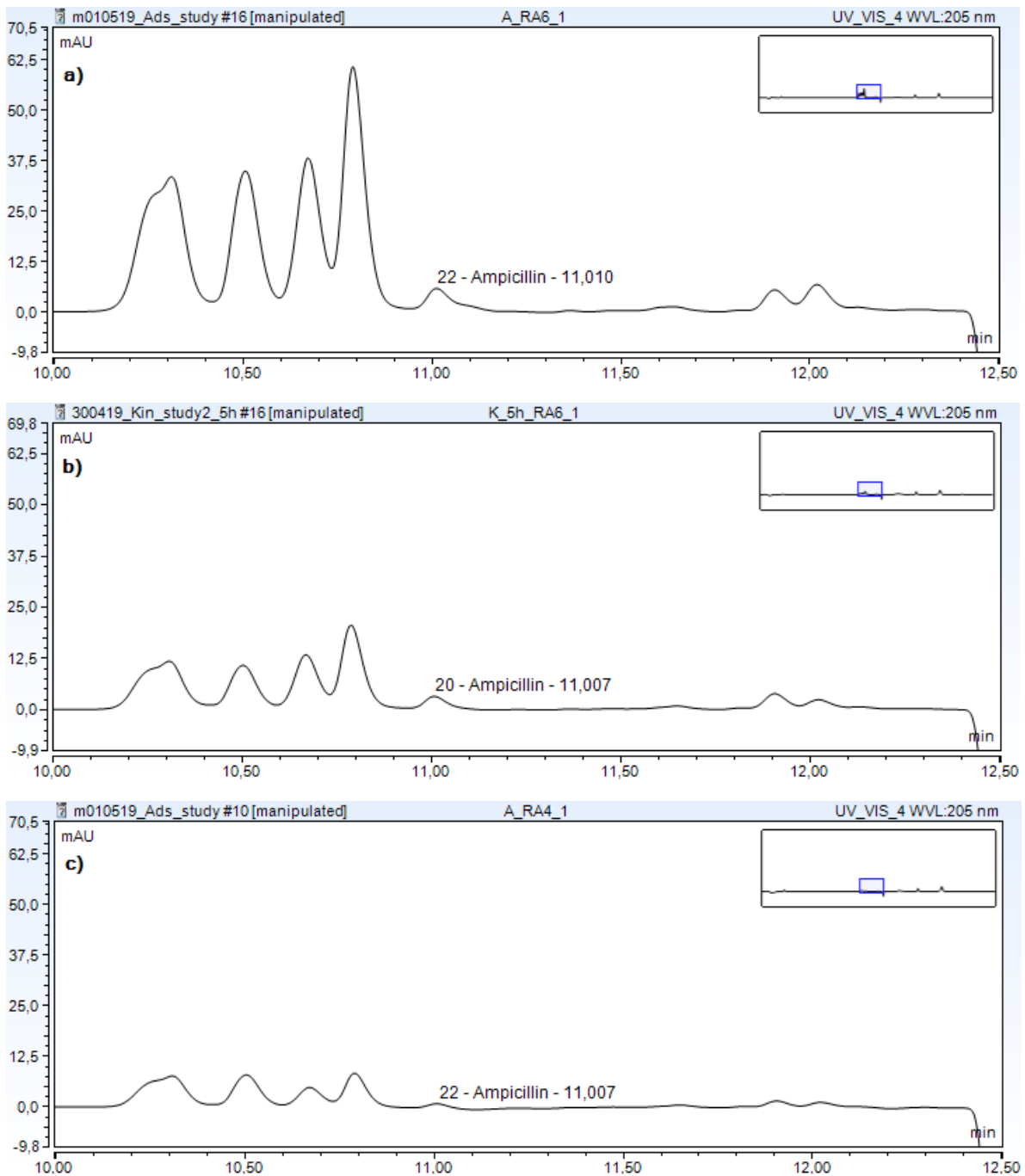
Figure 4.15: Chromatograms from adsorption isotherm experiment

a) A1, $C_0 \approx 2.6$ mg/L   b) K, $C_0 \approx 1.0$ mg/L   c) A2, $C_0 \approx 0.5$ mg/L

Furthermore, this measured removal is for ampicillin dissolved in the effluent. If the removal by adsorption is high, the ampicillin will follow the sludge. Common uses for wasted sludge in Norway is as fertiliser and to produce biogas [57, pp. 518-530]. This use makes it less than ideal to have antibiotics remaining in the sludge. Yet, several of the processes used to hygienise and stabilise sludge prior to use as fertiliser involve exposing it to higher temperatures [57]. According to Mitchell, et al. [27], the hydrolysis half-life of ampicillin decreases with increase

in temperature. In addition, as the time the sludge spends in the hygienisation and stabilisation processes is usually more than five days [57], some hydrolysis of ampicillin even at neutral pH and ambient temperature will occur. This of course requires that the adsorption does not stabilise the ampicillin towards chemical degradation. If not, it does not seem highly probable that much intact ampicillin will be spread onto fields along with the fertiliser.

4.3 Suggestions for Further Work

Ideas for possible further work on the topics in this thesis are presented here.

4.3.1 Analysis Method

The long SPE procedure developed for larger sample sizes has been proven to work, but yields lower recovery for β-lactams than the short procedure. As reflected in the name, it is also an inefficient procedure that takes 6.5 h to apply 56 mL sample each onto six cartridges. This is due to the sequential run of the RapidTrace®. If further use of the SPE method on larger samples is desired, a different setup than the RapidTrace® should be investigated, which loads samples in parallel instead of in order. This could possibly improve recovery as well. It will also allow for use of larger SPE cartridges with more sorbent, reducing the risk of overloading.

A possible simplification of the method could be the use of only one internal standard, added at the start of sample preparation, instead of two.

The 1:1 dilution of the SPE eluate is practical for adding internal standard and calibration solutions, but decreases analyte concentration. The addition is explained by Benito-Peña, et al. [8] as a step to avoid peak distortion during HPLC. Thus, it should not be dispensed with entirely, but the feasibility of adding less water could be assessed.

Finally, other studies analysing ampicillin (e.g. [11, 39]) utilise mass spectrometry as detector for HPLC. Shifting from UV to MS could offer better detection, as well as the option of identifying unknown compounds in the chromatograms, e.g. the possible chemical degradation products discussed in 4.2.4.3. If such a change in detector is desired, it is important to be aware that the TFA used in the mobile phases is known to cause signal suppression in MS [58].

4.3.2 Adsorption Studies

As most of the results from the adsorption studies are inconclusive, these experiments should be repeated. The pH in the sludge should be controlled through use of a buffer.

While pH control could reduce the hydrolysis issue, checking for this is still important for further adsorption studies. In addition, checking for biodegradation can be done through use of caffeine, see e.g. [9].

An option for the kinetics studies is fitting the results to kinetic models. This is done by Chitongo, et al. [35] for adsorption of antibiotics to activated carbon.

When setting up new adsorption isotherms, a higher number of samples should be used, spread out over a range of start concentrations. If non-linear regression is applied, other isotherms than Langmuir and Freundlich may be fitted to the data.

Finally, instead of measuring adsorption to activated sludge by proxy through the concentration in the liquid, it could be interesting to develop a method for analysing this directly. A possible basis for this can be found in [40], where a method for extracting antibiotics from manure is described.

5 CONCLUSIONS

The analysis method adapted and developed for ampicillin in the first part of this thesis work has been shown to work satisfactorily in both test and research context. Testing has been done using ampicillin concentrations in the 0.050 to 1.0 mg/L range. When applied for adsorption studies, the method has measured ampicillin concentrations down to 10 µg/L.

Extraction and upconcentration by SPE can be done for small or large samples, but in the latter case the process is time-consuming and yields lower recovery. Still, recovery from the SPE method has been measured to 75% or greater for all compounds extracted from wastewater effluent.

The HPLC method gives reliable detection of all β-lactams tested. Retention time for ampicillin is 11 minutes. As variability in the analysis results have been discovered, the use of internal standards is necessary to increase accuracy and precision. For the final wastewater effluent solutions tested, the uncertainty in recovery for ampicillin is ± 2%, with 95% confidence.

When the method is used on complex samples, some issues with quantification have been encountered due to overlapping peaks in the chromatograms. Subtracting blank chromatograms mitigates this. Suggestions for improving the method includes employing MS instead of UV detection.

*

The results from the adsorption studies performed in the second part of the thesis work are largely inconclusive.

Time of equilibrium is not clearly identified; however, the study of adsorption over time indicate a sludge-dependent removal rate for ampicillin in 1-3 g/L MLSS. The results from the adsorption isotherm experiment give a poor fit to the Freundlich and Langmuir isotherms. Issues of chemical degradation have been identified, calling into question some of the conclusions on adsorption.

A high degree of ampicillin removal is found for MLSS in the 1-3 g/L range. After five hours, 4% of the original ampicillin concentration remained in 1 g/L. In 3 g/L, no remaining ampicillin was measured after 1 h. This is favourable as it means less release of the antibiotic to natural water systems. Removal through adsorption does however bring up issues for sludge reuse.

This thesis can be used in further work where analysis of ampicillin from water is required, and as a basis for further study of the fate of ampicillin in activated sludge.

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APPENDIX

A: Note on the Method Development

It was discovered after completion of method testing that there was a small error in how the adjustment for dilution due to pH adjustment was done. This adjustment was done by weight, assuming negligible change in density of the concentrations. Instead of multiplying the original concentration with the ratio of the mass of the original solution to the mass of the diluted solution, the original concentration was multiplied with the ratio of the mass of the whole (container and solution) before and after dilution.

Correcting for this in the case of the wastewater samples was straightforward, as there was available data on the mass of the solutions and/or mass of containers. However, in the case of the pure water samples (4.1.2), some estimation was required.

The numbers listed for all testing of the method (4.1) are the corrected values, except for the testing of internal standards (4.1.3) where no concentrations or assumptions of concentrations were used in calculating recovery, and the validation solutions (Table 4.1) that did not go through pH adjustment (V1-V3, V5 and V7).

The corrected values did not however vary much from what was initially calculated, as the dilution in all cases was small. Thus, the conclusions drawn from the results are the same.

B: Analysis Procedure

Here, the procedure made for the analysis method is presented.

Making solutions for SPE and HPLC

Following is the recipes for the solutions used in SPE and HPLC.

SPE solutions

1. Ultrapure water
2. Phosphate buffer: dissolve 0.0170 mol $H_2PO_4^-$ and 0.0330 mol HPO_4^{2-} in 1000 mL ultrapure water. Check pH of buffer, should be 7.5. Adjust with 2 M NaOH.
3. Phosphate buffer (pH 7.5) with 5 % methanol (10 mL methanol in 200 mL buffer)
4. Methanol
5. Methanol with 0.05 M tetrabutylammonium hydrogen sulphate (TBA): dissolve 3.3954 g TBA in 200 mL methanol

HPLC solutions (mobile phases)

1. Acetonitrile (ACN) with 0.01 % TFA: dissolve 0.05 mL = 50 μL trifluoroacetic acid (TFA) in 500 mL acetonitrile
2. Water with 0.01 % TFA: 0.100 mL = 100 μL TFA in 1000 mL ultrapure water

Analysis

Following is the analysis procedure for β-lactams in wastewater.

SPE procedure

NOTE: RapidTrace® reagent lines must be primed prior to running samples, and cleaned afterwards using clean-up procedure.

1. Add a known concentration of recovery standard dicloxacillin to the sample
2. Adjust pH of sample to 7.5, making sure to measure either mass or volume of solution before and after adjustment, to account for dilution. Samples will normally have a pH higher than 7.5, so adjust with dilute HCl solution (pH ≈ 2). Remember to calibrate the pH meter before using
3. Prepare samples for SPE. Add around 13 mL sample to each test tube. Prepare a dummy sample to place in the first position on the RapidTrace® rack. Also prepare an equal number

of test tubes to hold the SPE eluates. Place all tubes in the rack and place the rack in RapidTrace®

4. Place one Oasis® MAX cartridge per sample in the RapidTrace®. Put a used cartridge in the first position (for the dummy sample)

5. Run the RapidTrace® using the chosen procedure (e.g., Setup Racks → Assign AMP_AI1.SPE to samples → Run/Monitor → Run the procedure)

Preparing for HPLC

1. Add 2.00 mL pure water solution to each test tube containing SPE eluate. This can be pure water, internal standard (cloxacillin) solution or calibration solution depending on the nature of the sample. Mix well using vortex mixer

2. Filter each sample into marked HPLC vials, using glass syringe and GHP syringe filters. Cap vials. Wash syringe and cannula with 1:1 water:methanol solution between each sample, also rinse once with sample before aspiration and filtration

HPLC

1. Open purge valve

2. Gradually increase flow up to 2 ml/min, keeping note of pressure

3. Purge for 5 min with pure water

4. Purge for 1 min (2 mL/min) with ACN with 0.01% TFA

5. Purge for 1 min (2 mL/min) with pure water with 0.01% TFA

6. Gradually reduce flow to 0.31 mL/min

7. Close purge valve

8. Place samples in autoinjector. Include one "sample" containing ultrapure water

9. Make and run start sequence in Chromeleon™, injecting the water sample and using "startup" instrument method

10. Make and queue dummies sequence, injecting water at least thrice using "amp_method_v2" instrument method

11. Make and queue sequence for analysing samples, injecting each at least thrice, using "amp_method_v2" instrument method

12. Make and queue end sequence, injecting water and using "shutdown" instrument method

13. If running overnight, make sure that instrument is set up to run Smart Shutdown at end of queue

C: Regression, Adsorption Isotherms

Figure I shows a comparison of the linear and non-linear regression results for the Freundlich isotherm.
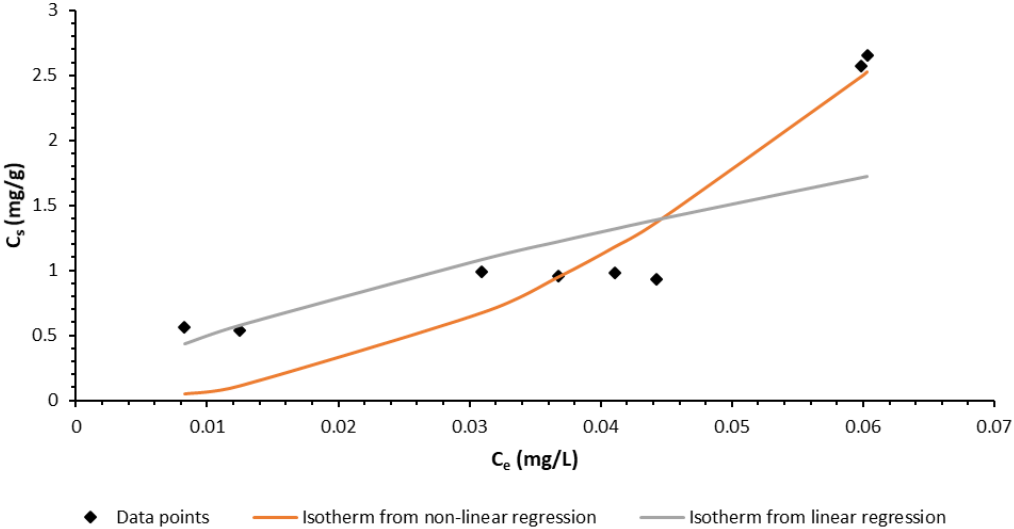

Figure I: Regression, Freundlich

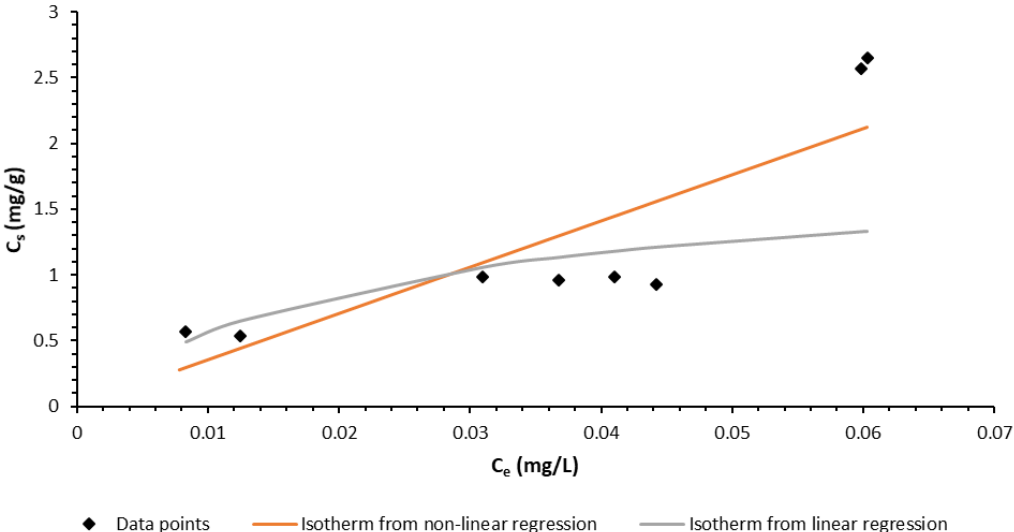Figure II shows a comparison of the linear and non-linear regression results for the Langmuir isotherm.

Figure II: Regression, Langmuir