Removal and Degradation Pathways of Sulfamethoxazole Present in Synthetic Municipal Wastewater via an Anaerobic Membrane Bioreactor

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ABSTRACT

Removal and Degradation Pathways of Sulfamethoxazole Present in Synthetic Municipal Wastewater via an Anaerobic Membrane Bioreactor

Claudia Lorena Sanchez Huerta

The current global water crisis in addition to continues contamination of natural water bodies with harmful organic micropollutants (OMPs) have driven the development of new water treatment technologies that allow the efficient removal of such compounds.

Among a long list of OMPs, antibiotics are considered as top priority pollutants to be treated due to their great resistance to biological treatments and their potential to develop bacterial resistance.

Different approaches, such as membrane-based and advance oxidation processes have been proposed to alleviate or minimize antibiotics discharge into aquatic environments. However most of these processes are costly and generate either matrices with high concentration of OMPs or intermediate products with potentially greater toxicity or persistence.

Therefore, this thesis proposes the study of an anaerobic membrane bioreactor (AnMBR) for the treatment of synthetic municipal wastewater containing sulfamethoxazole (SMX), a world widely used antibiotic. Besides the general evaluation of AnMBR performance in the COD removal and biogas production, this research mainly focuses on the SMX removal and its degradation pathway. Thus
SMX quantification was performed through solid phase extraction-liquid chromatography/mass spectrometry and the identification of its transformation products (TPs) was assessed by gas chromatography/mass spectrometry technique.

The results achieved showed that, working under optimal conditions (35°C, pH 7 and ORP around -380 to -420 mV) and after a biomass adaptation period (maintaining 0.85 VSS/TSS ratio), the AnMBR process provided over 95% COD removal and 95-98% SMX removal, while allowing stable biogas composition and methane production (≈130 mL CH₄/g COD_removed).

Kinetic analysis through a batch test showed that after 24 h of biological reaction, AnMBR process achieved around 94% SMX removal, indicating a first order kinetic reaction with K= 0.119, which highlights the high degradation capacity of the anaerobic bacteria.

Along the AnMBR process, 7 TPs were identified and possible degradation pathways were proposed. At low influent SMX concentrations (<10ppb), the only TPs detected was (1) Benzene sulfonamide N-Butyl. However, as the influent SMX concentration increased, it was possible to identify (2) Sulfanilamide, (3) Sulfisomidine and (4) 4-Aminothiophenol. Further degradation of compounds 2, 3 and 4 were detected after 9 hours of biological reaction in a batch test, producing three new intermediate products: (5) Aniline, (6) 4-Pyrimidinamine, 2,6-dimethyl and (7) Acetamide, N-(4-mercaptophenyl). Most of the detected TPs present a less complex structure than SMX, which can be associates with a lower toxicity.
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# TABLE OF CONTENTS

EXAMINATION COMMITTEE APPROVALS FORM ................................................................. 2  
COPYRIGHT .......................................................................................................................... 3  
ABSTRACT .......................................................................................................................... 4  
ACKNOWLEDGEMENTS .................................................................................................... 6  
TABLE OF CONTENTS ....................................................................................................... 7  
TABLE OF FIGURES .......................................................................................................... 10  
LIST OF TABLES ................................................................................................................. 12  
LIST OF ABBREVIATIONS ................................................................................................. 13  

Chapter 1 ........................................................................................................................... 15  
1. Introduction .................................................................................................................. 15  
   1.1. Background ........................................................................................................... 16  
      1.1.1. Pharmaceutically Active Compounds (PhACs) ............................................ 16  
      1.1.2. Sulfamethoxazole (SMX) ........................................................................... 17  
   1.2. Literature review ................................................................................................. 19  
      1.2.1. Membrane-based processes ...................................................................... 19  
      1.2.2. Advance Oxidation Processes (AOPs) ....................................................... 20  
      1.2.3. Biological Treatments ................................................................................. 22  
         1.2.3.1. Biological Aerobic Treatments ......................................................... 23  
         1.2.3.2. Biological Anaerobic Treatments .................................................... 24  

Chapter 2 ........................................................................................................................... 27  
2. Objective ...................................................................................................................... 27  
   2.1. Scope ..................................................................................................................... 27  
   2.2. Hypothesis ............................................................................................................. 28  

Chapter 3 ........................................................................................................................... 29  
3. Materials and Methodology ....................................................................................... 29  
   3.1. Wastewater Characterization ............................................................................ 29  
      3.1.1. Organic Micro-Pollutants .......................................................................... 30
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.</td>
<td>Anaerobic Membrane Bioreactor (AnMBR)</td>
<td>31</td>
</tr>
<tr>
<td>3.2.1.</td>
<td>Biological Treatment</td>
<td>31</td>
</tr>
<tr>
<td>3.2.2.</td>
<td>Anaerobic Activated Sludge Acclimatization</td>
<td>34</td>
</tr>
<tr>
<td>3.3.</td>
<td>OMPs</td>
<td>35</td>
</tr>
<tr>
<td>3.4.</td>
<td>Membrane Module</td>
<td>36</td>
</tr>
<tr>
<td>3.4.1.</td>
<td>Membrane Module Operation</td>
<td>36</td>
</tr>
<tr>
<td>3.4.2.</td>
<td>Ultrafiltration Membrane</td>
<td>37</td>
</tr>
<tr>
<td>3.4.3.</td>
<td>Membrane Cleaning</td>
<td>39</td>
</tr>
<tr>
<td>3.5.</td>
<td>Analytical Tests</td>
<td>40</td>
</tr>
<tr>
<td>3.5.1.</td>
<td>Hydrodynamic Analysis</td>
<td>40</td>
</tr>
<tr>
<td>3.5.2.</td>
<td>Physicochemical Analysis</td>
<td>41</td>
</tr>
<tr>
<td>3.5.3.</td>
<td>Chemical Oxygen Demand Analysis</td>
<td>41</td>
</tr>
<tr>
<td>3.5.4.</td>
<td>Liquid chromatography with Organic Carbon Detection LC-OCD</td>
<td>41</td>
</tr>
<tr>
<td>3.5.5.</td>
<td>Mixed Liquor Suspended Solids</td>
<td>44</td>
</tr>
<tr>
<td>3.5.6.</td>
<td>Biogas Analysis</td>
<td>46</td>
</tr>
<tr>
<td>3.6.</td>
<td>Organic Micro-Pollutant Quantification</td>
<td>47</td>
</tr>
<tr>
<td>3.7.</td>
<td>Transformation Products Identification</td>
<td>50</td>
</tr>
<tr>
<td>3.8.</td>
<td>Degradation Kinetics</td>
<td>52</td>
</tr>
</tbody>
</table>

Chapter 4

4. Results and Discussion

4.1. Temperature, pH and Oxido-Reduction Potential | 54
4.2. Hydrodynamic Analysis | 58
4.3. Chemical Oxygen Demand | 61
4.4. LC-OCD Analysis | 65
4.5. Suspended Solids | 68
4.6. Biogas Analysis | 70
4.7. Organic Micro-Pollutant Quantification | 77
4.7.1. Kinetic Analysis | 86
4.8. Transformation Products Identification | 91
4.8.1. Occurrence of TPs at different feeding SMX concentrations | 91
4.8.1.1. 0.01 ppm feeding SMX concentration | 93
4.8.1.2. 0.1 ppm feeding SMX concentration .............................................................. 96
4.8.1.3. 1 ppm feeding SMX concentration .............................................................. 100
4.8.1.4. 10 ppm initial SMX concentration ............................................................. 106
4.8.1.5. 100 ppm feeding SMX concentration ....................................................... 110
4.8.2. Kinetic Analysis ......................................................................................... 115
4.9. Degradation Pathway .................................................................................. 118

Chapter 5 ........................................................................................................... 121
  5. Conclusions .................................................................................................... 121
  5.1. Future work ............................................................................................... 124

REFERENCES ...................................................................................................... 125
APPENDICES ...................................................................................................... 131
TABLE OF FIGURES

Figure 1 Scheme of AnMBR system ........................................................................................................................................................................... 32
Figure 2 AnMBR setup .......................................................................................................................................................................................... 34
Figure 3 Hollow Fiber Membrane Module .................................................................................................................................................... 37
Figure 4 COD analysis .......................................................................................................................................................................................... 41
Figure 5 LC-OCD Scheme .................................................................................................................................................................................. 42
Figure 6 LC-OCD ............................................................................................................................................................................................... 43
Figure 7 MLSS analysis by the Standard Method 2540B ......................................................................................................................................... 46
Figure 8 Gas Chromatographer for Biogas Analysis ................................................................................................................................. 47
Figure 9 Sample preparation and quantification of SMX in LC/MS ............................................................................................................. 49
Figure 10 Sample preparation and identification of parent compound and TPs .......................................................................................... 52
Figure 11 Physicochemical parameters monitored to ensure the optimal conditions of the AnMBR .......................................................................................................................................................................................................................... 56
Figure 12 Temperature, pH and ORP variation along the 1st experimentation stage ................................................................................... 57
Figure 13 Variation of Flux and TMP along the experimental time ............................................................................................................. 59
Figure 14 Sludge leakage in the permeate cause due to glue detachment of membrane fibers ........................................................................... 60
Figure 15 COD concentration in influent and permeate; removal percentage ............................................................................................... 65
Figure 16 LC-OCD chromatography of MF and UF membrane in kinetics study ...................................................................................... 67
Figure 17 Fluctuation of TSS, FSS and VSS and VSS/TSS ratio .......................................................................................................................... 70
Figure 18 Calibration Curves for GC .................................................................................................................................................................... 71
Figure 19 Biogas composition and volume fluctuation ............................................................................................................................. 73
Figure 20 Biogas Composition ......................................................................................................................................................................... 76
Figure 21 Methane production in terms of COD removed ............................................................................................................................ 77
Figure 22 Calibration curves of pure SMX ....................................................................................................................................................... 78
Figure 23 Selected ion chromatograph of SMX and its isotope in a permeate sample ................................................................................... 79
Figure 24 Quantitative SMX analysis on permeate sample ............................................................................................................................ 81
Figure 25 Quantitative SMX analysis on influent samples .............................................................................................................................. 82
Figure 26 SMX fluctuation in influent and permeate along the AnMBR process ........................................................................................... 85
Figure 27 Average SMX concentration and removal percentage of SMX in stage 2nd and 3rd ........................................................................................................................................................................................................................................... 85
Figure 28 Kinetic analysis of SMX degradation and removal percentage ....................................................................................................... 87
Figure 29 Kinetic Order Correlation ............................................................................................................................................................... 88
Figure 30 Time course of the SMX concentration for the AnMBR treatment ............................................................................................... 90
Figure 31 Solvents comparison at 10ppb feeding SMX concentration ........................................................................................................... 94
Figure 32 Comparison of different sample extraction concentrations using MeOH plus 10% MeOH in MTBE at 10ppb feeding SMX concentration ........................................................................................................................................................................................................................................................................................................................................................................................................................................... 95
Figure 33 Peak 1, Benzenesulfonamide, N-Butyl; identified at 10ppb feeding SMX concentration ........................................................................................................................................................................................................................................................................................................................................................................................................................................................................................................... 96
Figure 34 Solvents comparison at 100ppb feeding SMX concentration ............................................................................................................ 97
Figure 35 Comparison of different sample extraction concentrations using MeOH plus 10% MeOH in MTBE at 100ppb feeding SMX concentration ........................................................................................................................................................................................................................................................................................................................................................................................................................................................................................................... 98
Figure 36 Peak 2, Sulfanilamide; identified at 100ppb feeding SMX concentration .............................................................................................. 99
Figure 37 Further degradation of TPs identified at 100ppb feeding SMX concentration .................................................................................... 100
Figure 38 Solvents comparison at Stage 2, 1ppm feeding SMX concentration ......................... 101
Figure 39 Solvents comparison at Stage 3, 1ppm feeding SMX concentration ......................... 102
Figure 40 Comparison of different sample extraction concentrations using DCM at 1ppm initial SMX concentration ................................................................................................................................ 104
Figure 41 Peak 3, Sulfsimidine; identified at 1ppm feeding SMX concentration .......................... 105
Figure 42 Peak 4, 4-Aminothiophenol; identified at 1ppm feeding SMX concentration .......... 105
Figure 43 Peak 5, Benzenamide or Aniline; identified at 1ppm feeding SMX concentration .................................................................................................................................................................................... 106
Figure 44 Solvents comparison at Stage 3, 10ppm feeding SMX concentration ...................... 107
Figure 45 Comparison of samples at 20k extraction concentrations using Acetone at 10ppm feeding SMX concentration ............................................................................................................................................................. 108
Figure 46 Peak 6, 4-Pyrimidinamine 2,6-dimethyl; identified at 10ppm feeding SMX concentration ....................................................................................................................................................... 109
Figure 47 Peak 7, Acetamide, N-(4-mercaptophenyl); identified at 10ppm feeding SMX concentration ................................................................................................................................................................................................. 109
Figure 48 Solvents comparison at Stage 3, 100ppm feeding SMX concentration ................. 110
Figure 49 Comparison of samples at different extraction concentrations using 20% MeOH in Acetone at 100ppm feeding SMX concentration ............................................................................................................................. 111
Figure 50 Comparison of occurrence of SMX and TPs on samples filtrated trough MF and UF membranes ................................................................................................................................................................................................. 112
Figure 51 Analytes Occurrence in samples extracted with Acetone and concentrated 20k times ................................................................................................................................................................................................. 114
Figure 52 Analytes Occurrence in samples extracted with 20%MeOH in Acetone and concentrated 20k times ................................................................................................................................................................................................. 115
Figure 53 Kinetic analysis of SMX transformation products generated along the AnMBR treatment ................................................................................................................................................................................................. 115
Figure 54 Fluctuation of the TPs occurrence along the AnMBR process .................................. 117
Figure 55 Main reaction pathway proposed for Sulfamethoxazole degradation through the AnMBR treatment ................................................................................................................................................................................................. 120
LIST OF TABLES

Table 1 Wastewater concentrated stock recipe [47] .............................................................................. 29
Table 2 Sulfamethoxazole properties ............................................................................................................ 31
Table 3 Properties and operational parameters of DOW-UF hollow-fiber membrane (51) ..38
Table 4 Kinetic SMX Degradation Analysis ................................................................................................. 87
Table 5 Identification of the main SMX Transformation Products formed in the AnMBR process and their respective chemical structures ................................................................................... 92
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOPs</td>
<td>Advance Oxidation Processes</td>
</tr>
<tr>
<td>AnMBR</td>
<td>Anaerobic Membrane Bioreactor</td>
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<tr>
<td>BP</td>
<td>Biopolymer</td>
</tr>
<tr>
<td>BPA</td>
<td>Bisphenol-A</td>
</tr>
<tr>
<td>BB</td>
<td>Building Blocks</td>
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<tr>
<td>CBZ</td>
<td>Carbamazepine</td>
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<tr>
<td>COD</td>
<td>Chemical Oxygen Demand</td>
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<tr>
<td>CDOC</td>
<td>Chromatographable Organic Carbon</td>
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<tr>
<td>CDEO</td>
<td>Conductive-Diamond Electrochemical Oxidation</td>
</tr>
<tr>
<td>CPS</td>
<td>Counts per Second</td>
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<tr>
<td>DCF</td>
<td>Diclofenac</td>
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<tr>
<td>DCM</td>
<td>Dichloromethane</td>
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<tr>
<td>DW</td>
<td>Distilled Water</td>
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<tr>
<td>EA</td>
<td>Ethyl Acetate</td>
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<td>EDCs</td>
<td>Endocrine-Disrupting Compounds</td>
</tr>
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<td>FSS</td>
<td>Fixed Suspend Solids</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<tr>
<td>HS</td>
<td>Humic Substances</td>
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<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
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<td>LMW</td>
<td>Low Molecular Weight</td>
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<tr>
<td>MBR</td>
<td>Membrane Bioreactor</td>
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<td>MeOH</td>
<td>Methanol</td>
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<td>MF</td>
<td>Microfiltration</td>
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<td>ML</td>
<td>Mixed Liquor</td>
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<td>MLSS</td>
<td>Mixed Liquor Suspended Solids</td>
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<td>MS</td>
<td>Mass Spectrometry</td>
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<tr>
<td>MTBE</td>
<td>Methyl Tert-Butyl Ether</td>
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<tr>
<td>MW</td>
<td>Molecular Weight</td>
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<td>MWCO</td>
<td>Molecular Weight Cut-Off</td>
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<td>NF</td>
<td>Nanofiltration</td>
</tr>
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<td>NOM</td>
<td>Natural Organic Matter</td>
</tr>
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<td>OCD</td>
<td>Organic Carbon Detection</td>
</tr>
<tr>
<td>OMP</td>
<td>Organic Micropollutants</td>
</tr>
<tr>
<td>ORP</td>
<td>Oxidation-Reduction Potential</td>
</tr>
<tr>
<td>PhACs</td>
<td>Pharmaceutically Active Compounds</td>
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<tr>
<td>PFCS</td>
<td>Perfluorinated Compounds</td>
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<tr>
<td>PPCPS</td>
<td>Pharmaceuticals and Personal Care Products</td>
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<tr>
<td>RO</td>
<td>Reverse Osmosis</td>
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<tr>
<td>SMX</td>
<td>Sulfamethoxazole</td>
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<tr>
<td>SMX-d4</td>
<td>Isotope of Sulfamethoxazole</td>
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<tr>
<td>SW</td>
<td>Sea Water</td>
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<td>SPE</td>
<td>Solid Phase Extraction</td>
</tr>
</tbody>
</table>
TIC  Total Ion Chromatograms
TPs  Transformation Products
TSS  Total Suspended Solids
UF   Ultrafiltration
UVD  Ultra Violet Detection
VSS  Volatile Suspended Solids
WWTP Wastewater Treatment Plants
Chapter 1

1. Introduction

Nowadays the world is undergoing rapid social and environmental changes. The unceasingly population growth, the contamination of natural water bodies and the intensive use of water for irrigation and industrial purposes, in addition to the uneven distribution of water resources as well as the recurrent droughts periods, caused by global warming [1, 2, 3], have generated that currently around one-fifth of the world’s population (1.2 billion people) lives under water scarcity [4, 5].

In order to alleviate the current global water crisis, different approaches have been analyzed. The reuse and reclamation of wastewater has been proposed as a potential option to reduce water stress by serving as an unconventional water source [6]; however, its acceptance has been diminished due to the presence of organic micropollutants (OMPs) which are not efficiently removed in the conventional wastewater treatment plants (WWTPs) [7,8], thus entering to the environment.

In addition, in the last decades the presence of organic micropollutants, in WWTPs’ permeates, as well as in natural aquatic environments, has emerged as an increasing concern due to a large list of organic micropollutants (OMPs), which includes: hormones, endocrine-disrupting compounds (EDCs), surfactants, perfluorinated compounds (PFCs), pesticides, pharmaceuticals and personal care products (PPCPs) among others, are continuously discharged into the environment by different routes, including industrial and domestic permeates.
Despite the fact that the discharge concentration of these OMPs might be low, its continuous input may generate long-term high concentration, which represents a risk to human health and to the environment [9, 10].

1.1. Background

1.1.1. Pharmaceutically Active Compounds (PhACs)

Different studies have confirmed the presence of pharmaceutically active compounds (PhACs) in different natural water bodies as result of its incomplete metabolism in the body when used for human therapy or veterinary practice, as well as the improper disposal of hospital and industrial permeates.

PhACs have been recognized as potentially hazardous compounds due their ubiquity, persistence and biologically active nature; with endocrine disruption functions previously reported [17].

Despite most pharmaceuticals compounds present limited biodegradability, due to their nature; it has been reported that some pharmaceuticals are partially degraded by microorganisms in conventional WWTPs [11]. However, some others, like antibiotics present a great resistance to biological treatments, as they have been specifically designed for the elimination of bacteria. Therefore, most antibiotics are insufficiently removed by WWTPs, being released in their parent and metabolite forms (with different properties such as greater toxicity or persistence) into the aquatic environments at concentrations of ng/L to μg/L [11, 12, 13, 14, 15 and 16].
The most commonly detected antibiotics in wastewaters are the tetracyclines, penicillins and sulfonamides [16].

1.1.2. Sulfamethoxazole (SMX)

Sulfonamides are synthetic antibiotics derived from sulfanilic acid which act as bacteriostatic, inhibiting the synthesis of dihydrofolic acid [18].

Sulfamethoxazole (4-amino-N-(5-methylisoxazol-3-yl)-benzenesulfonamide) is a representative compound of the sulfonamide family or sulfa drugs. It is a world widely used synthetic antibiotic drug derived from sulfanilic acid; which acts as bacteriostatic by inhibiting the synthesis of dihydrofolic acid. It is usually prescribed for the treatment of human and animal infections, and as food additives in livestock productions.

When used as antibiotic, the unmetabolized SMX (around 15-25%) is excreted from the human or animal body being release into the wastewater and eventually discharged into conventional WWTPs [19, 22].

SMX, an amphoteric polar compound, has a relative large molecule (C_{10}H_{11}N_{3}O_{3}S, molecular weight 253.28 g/mol) that consists of two aromatic rings and a low $K_d$ value, which explains its occurrence and transport in natural waters. The SMX has also two protonated states, which varies with the pH, and different ionic states that have a remarkable effect on its reactivity [11]. The $pK_a$ values of the sulfamethoxazole are: $pK_{a1} = 1.6$ and $pK_{a2} = 5.7$ [23], which means that the sulfamethoxazole has a cationic form at pH < 1.6, and an anionic form at pH > 5.7.
These chemical properties, in addition to its biologically active nature and its design (which targets the elimination of bacteria) make of SMX a refractory and persistent compound. Thus, SMX is barely removed through conventional biological treatments in WWTPs, being able to persist in the environment for more than a year [22]. SMX is considered as an indicator of antibiotic contamination due to its ubiquitously occurrence in aquatic environments (with over 73% of frequency detection), being reported in WWTP’s permeates at concentrations higher than 1.9 µg/L [22, 23], and even in drinking water at trace levels (0.03-0.48 µg/L) [24].

Although SMX has not been ascribed, until now, to any specific human health adverse effects. Different studies have demonstrated its capacity to be accumulated in various organisms [22, 25] and the potential development of bacterial resistance [26] due to the long-term exposure to low concentrations of this antibiotic [27, 28, 29]. Other authors had suggested that SMX can also induce genetic mutations and chronic effects [30].

Therefore, it is of great importance to investigate and understand the antibiotic microbial metabolism and ultimate fate, in order to develop new efficient technologies that limit the release of such pharmaceutical pollutants and their toxic metabolites, into the environment.
1.2. Literature review

An important stage to prevent OMP’s water contamination is the efficient removal of such components from wastewater.

Since antibiotics, such as sulfamethoxazole, offer great resistance to natural biodegradation and conventional wastewater treatments (on average, activated sludge provides only 24% removal of sulfa-drugs antibiotics [31]), different approaches have been proposed to alleviate and/or to minimize their entrance into the environment.

The processes that have captured more interest in the antibiotic treatment due to their high removal efficiency are: membrane-based processes and advance oxidation processes (AOPs).

1.2.1. Membrane-based processes

Membrane-based processes have great application in drinking water and wastewater treatments for the removal of organic and inorganic compounds as well as microorganisms. Recently, different studies have demonstrated the efficiency of pressure-driven membrane processes (nanofiltration (NF) and reverse osmosis (RO)) in the removal of PhACs [32, 33].

The degree of efficiency of the membrane-based process mainly depends on the membrane features as well as on the properties of the pollutant itself. Low-pressure membranes, microfiltration (MF) and ultrafiltration (UF) membranes, present limited input in the treatment of pharmaceutical compounds with molecular weight
(MW) around 100-500 Da due to their large pore size higher than 0.01 µm (equivalent MW cut-off around 10 kDa).

In contrast NF and RO membranes have demonstrated high efficiency in the treatment of OMPs polluted water, since these membranes involve two separation mechanisms. The first, size exclusion mechanism, allows the retention of molecules with a MW greater than the molecular weight cutoff (MWCO) of the membranes (NF ≈ 200-1000 Da; RO < 100) providing over 85% removal of PhACs [32]. The second mechanism, diffusion, is capable to retain charged molecules (even with MW smaller than the membrane’s MWCO) due to the presence of electrical interactions between the molecule and the charged membrane surface; Radjenović J., et at. (2008) had proven that most negative charged pharmaceuticals can reach up to 95% rejection, while 90% removal is achieved for positive charge molecules [32, 33].

Although, membrane-based processes provide a strong physical barrier against pharmaceuticals, and OMPs in general, this processes do not resolve the presence of OMPs in the environment, as this processes produces brines containing high concentrations of OMPs which would raise the concern of safe disposal practices and post treatments.

1.2.2. Advance Oxidation Processes (AOPs)

Advance oxidation processes are attractive alternatives to conventional water treatments in the abatement of bio-recalcitrant OMPs.
AOPs’ potential to degrade OMPs relies on the promotion of highly oxidative hydroxyl radicals (·OH), by the combination of different oxidants such as ozone or hydrogen peroxide with ultraviolet (UV) or visible irradiation and a catalyst such as metal ions (TiO$_2$) or semiconductors.

Among AOPs, Photo-Fenton, Ozonation, chemical oxidation and TiO$_2$ photocatalysis, have reported high performance in the elimination of various PhACs from environmental aqueous matrices due to their capability of reducing OMPs (PhACs) molecules and discomposing them until CO$_2$ and H$_2$O.

Sometimes, the complete mineralization of PhACs may be reached depending on the properties of the pollutant itself, the aqueous matrix composition and the selected oxidation process (potential of hydroxyl radical formation and/or dosage of oxidants or light radiation applied). However, the incomplete depletion of PhACs might result in the formation of different transformation products (TPs) with significantly different physical, chemical and toxicological properties, which might make them more resistant to the treatments and more dangerous to the environment [16, 24].

For example, Trovó AG, et al. (2009) investigated the degradation of antibiotics in different aqueous matrix, distilled water (DW) and sea water (SW), through photo-Fenton catalysis. The results showed that, under the same treatment conditions, the antibiotics’ and TPs’ toxicity in DW was reduced from 20% to 85%; while for SW matrix, despite 45% antibiotics mineralization was achieved, the toxicity of the TPs increased from 16% to 86%, which reflects that intermediates generated in SW are
different to those in DW [15]. In the other hand, Martín de Vidales MJ, et al. (2012) reported that conductive-diamond electrochemical oxidation (CDEO) is an effective technology to reduce pharmaceuticals from water sample to values under 0.1µg/dm³, describing the formation of nine TPs whose concentration depends on the initial parent compound concentration. Similarly, photo-chemical oxidation can lead to the total elimination of parent compound. Nevertheless, it is not able to completely mineralize the TPs.

Ozone, also has been proven to be highly effective in the abatement of PhACs, however high dose of ozone is required to attain the complete mineralization of the intermediates generated along the process [24].

Therefore, the use of AOPs should be carefully considered and analyzed before applying it on real scale basis. In the same direction, new approaches that prevent TPs formation or allows the complete elimination of the parent compound and its TPs must be studied.

1.2.3. Biological Treatments

As previously reported, physical and chemical processes do not resolve completely the entrance of PhACs (and OMPs, in general) in the environments, at the same time that this processes sometimes may generate the formation of different TPs, that can present higher toxicity and impact in the environment, in comparison with the compounds in their parent form [35, 36, 37].
In the other hand several studies have demonstrated the limitations of conventional aerobic and anaerobic sludge-based water treatment in the biodegradation of antibiotics, which allow very limited removal of this compounds.

1.2.3.1. Biological Aerobic Treatments

Recently, different studies have been carried on in order to improve and/or enhance the efficiency of biological processes in the abatement of PhACs. Marco-Urrea E, et al. (2009) [38] reported high efficiency in the removal of different OMPs, including persistent PhACs, though white-rot fungi via one or more extracellular enzymes (laccases, lignin peroxidases, manganese dependent peroxidases), indicating that the potential of the performance depend on fungal species and their enzymatic system.

Meanwhile, Yang S, et al. (2013) compared the elimination efficiency of four pharmaceutical compounds through nitrifying activated sludge, whole-cell pure fungus culture and fungal extracellular enzyme extract, obtaining that activated sludge provide from 5% to 40% elimination of the target PhACs after 22h of reaction, from which 25% of the removal of hydrophobic compounds (such as BPA) is due to biosorption; however for compound such as SMX approximately a 15% of total SMX removal was attained, and negligible biosorption was observed. Whole-cell fungus allowed partial removal of BPA and DCF after 10h of incubation, to further increase the removal rate at 72h; in contrast, insignificant degradation of CBZ and SMX was reported. Finally, fungal extracellular enzyme extract showed a
great performance in the removal of BPA (>96% removal); however, other OMPs displayed negligible degradation through the extracellular enzyme extract [39].

In general, Baran W., et al. (2011) have reported an efficiency of approximately 24% removal of sulfonamide drugs by means of diverse conventional biological-mechanical treatments [40].

1.2.3.2. Biological Anaerobic Treatments

Likewise, anaerobic processes present some limitations in the treatment of organic micropollutants; showing low efficiency in the removal of hormones, plasticizers and some pharmaceutical compounds. However different studies have demonstrated that anaerobic processes possess high capacity in the removal of certain antibiotics such as sulfamides (sulfamethoxazole).

Carballa M., et al. (2007) had reported efficiencies above 90% for the removal of Sulfamethoxazole and Trimethoprim during anaerobic digestion under thermophilic and mesophilic conditions [41]; Likewise, Xue W., et al. (2010) reported the integration of aerobic-anaerobic treatments for the successful removal of Trimethoprim, 4-nonilphenol and caffeine (70% removal)[42]; and Musson S.E., et al. (2010) showed a fast and important removal (>65%) of progesterone, ethynil-estradiol, metoprolol tartrate and other antibiotics under anaerobic treatments [43], concluding that the removal efficiency of OMPs is governed in a large extend by
the pollutant physicochemical properties such as molecular weight, hydrophobicity size an charge, but also by the primary metabolism and the type of biomass.

In addition the literature has reported that the integration of anaerobic treatments with low pressure membranes processes (such as microfiltration or ultrafiltration), will results in a technologically and economically viable treatment process; as it combines the advantages and high removal rates of OMPs obtained by the anaerobic treatments at the same time that uses membranes as biomass retention method.

Despite, no clear effects in the enhancement of the OMPs has been proved for the MBR system; Radjenovic J., et al. (2008)[32], and Clara M., et al. (2011) [44] have demonstrated that under similar operational conditions membrane bioreactor processes, in comparison with conventional activated sludge (CAS), achieves similar and sometimes better removal of antibiotics due to the high sludge retention time (obtained by the membrane separation process) allows the development of slower growing bacterial species, which could lead to an improvement in the antibiotics removal.

Several publications demonstrate that the main elimination pathway of most pharmaceutical compounds is the biological transformation, while adsorption into activated sludge (either aerobic or anaerobic) in negligible for most antibiotics such as sulfamethoxazole [45].

Additionally, UF membrane processes are widely recognized for their efficiency in the removal of conventional pollutants and high molecular weight organics (which can be posteriorly degrade) as well as pathogens. Thus UF membrane bioreactors
are highly demanded to increase the treatment efficiency and to offer high quality permeates [46].

Therefore, the purpose of this research is to study the treatment of synthetic wastewater polluted with sulfamethoxazole through an anaerobic MBR, in order to evaluate the efficiency of this system in the elimination of antibiotics with similar nature and characteristics to those for SMX.
Chapter 2

2. Objective

The main goals of this research are:

- To assess the efficiency of an anaerobic membrane bioreactor (AnMBR) in the removal of OMP, specifically the antibiotic sulfamethoxazole, present in synthetic wastewater.
- To identify the transformation products (TPs) formed along the process and propose the biodegradation pathways of SMX.

2.1. Scope

The analysis is made at laboratory scale using synthetic wastewater.

The OMP assessed is the antibiotic Sulfamethoxazole (SMX).

The process selected for the removal of SMX is an AnMBR using an ultrafiltration membrane.

Toxicology analysis of parent compound and TPs are not considered in this research.
2.2. Hypothesis

- Anaerobic activated sludge with a proper acclimation is expected to be an effective method for SMX removal.
- It is proposed that the system will allow over 70% removal of SMX; either by biodegradation or biotransformation.
- It is no expected to obtain the complete mineralization of Sulfamethoxazole due to its complex structure.
- It is suggested that the bond that connects both benzene rings of SMX would be broken, creating different transformation products.
Chapter 3

3. Materials and Methodology

3.1. Wastewater Characterization

The wastewater used for this experimentation is synthetic municipal wastewater produced in the laboratory. The recipe used to prepare the concentrated stock of synthetic wastewater is the one proposed by Wei C-H, et al. (2014) [47], which contains organic and inorganic substances as well as trace metals (Table 1).

Table 1 Wastewater concentrated stock recipe [47]

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inorganics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Ammonium Chloride</td>
<td>NH4Cl</td>
<td>1.02</td>
</tr>
<tr>
<td>2 Ferrous Sulfate</td>
<td>FeSO4·7H2O</td>
<td>0.464</td>
</tr>
<tr>
<td>3 Potassium Phosphate</td>
<td>KH2PO4</td>
<td>1.872</td>
</tr>
<tr>
<td>4 Urea</td>
<td>CH4N2O</td>
<td>7.34</td>
</tr>
<tr>
<td>5 Diamagnesium phosphate</td>
<td>MgHP04·3H2O</td>
<td>2.32</td>
</tr>
<tr>
<td><strong>Organics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Milk powder</td>
<td></td>
<td>9.295</td>
</tr>
<tr>
<td>2 Starch</td>
<td></td>
<td>9.76</td>
</tr>
<tr>
<td>3 Yeast</td>
<td></td>
<td>4.179</td>
</tr>
<tr>
<td>4 Peptone</td>
<td></td>
<td>1.392</td>
</tr>
<tr>
<td>5 Sodium acetate</td>
<td></td>
<td>6.345</td>
</tr>
<tr>
<td><strong>Trace Metals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Chromium chloride</td>
<td>CrCl3-6H2O</td>
<td>0.04096</td>
</tr>
<tr>
<td>2 Cooper chloride</td>
<td>CuCl2-2H2O</td>
<td>0.04288</td>
</tr>
<tr>
<td>3 Manganese sulfate</td>
<td>MnSO4-H2O</td>
<td>0.00864</td>
</tr>
<tr>
<td>4 Nickel sulfate</td>
<td>NiSO4-6H2O</td>
<td>0.02688</td>
</tr>
<tr>
<td>5 Lead chloride</td>
<td>PbCl2</td>
<td>0.008</td>
</tr>
<tr>
<td>6 Zinc chloride</td>
<td>ZnCl2</td>
<td>0.01664</td>
</tr>
</tbody>
</table>

*Recipe to prepare 4L of wastewater concentrated stock containing around 8000mg of COD/L

All the chemicals used to prepare the synthetic municipal wastewater were purchased from Sigma-Aldrich ® and Fluka® Analytical with at least 98% purity.
The organic load of the wastewater (used as influent for the assessed process) in terms of chemical oxygen demand (COD) was maintained around 800 mg of COD/L, typical value of municipal wastewater [41]. To reach this organic load, the synthetic wastewater concentrated stock (with approximately 8,000 mg of COD/L) was diluted ten times with tap water. Such synthetic wastewater was used to directly feed the anaerobic biological reactor during the first two months of operation for assimilation, thereafter the wastewater was spiked with different concentrations of SMX along the experiment.

3.1.1. Organic Micro-Pollutants

The OMP used in this research (SMX) was spiked into the synthetic wastewater at different times and concentrations.

A 1000 ppm sulfamethoxazole concentration stock was prepared through mixing 50 mg of SMX into 50 mL of Methanol. After its complete mixing, the stock solution was stored at 4°C in an amber glass in order to avoid any type of degradation.

This stock solution was used for spiking the wastewater. The first concentration assessed was 10 ppb; posteriorly this concentration was increased to 100 ppb and finally 1000 ppb (1ppm).

Subsequently, 2 g of SMX were diluted into 40ml of methanol in order to achieve a stock solution with 50,000ppm SMX concentration. This second stock solution was used to further increase the SMX concentration in the wastewater to 10ppm and 100ppm, avoiding high doses of methanol into the system.
The analytical grade Sulfamethoxazole (99.85%, CAS: 723-46-6, 4-amino-N-(5-methylisoxazol-3-yl)-benzenesulfonamide) (Table 2), used to spike the wastewater, was provided by Fulka® Analytical. Meanwhile, the methanol with LC-MS grade was purchased from Fisher Scientific®.

Table 2 Sulfamethoxazole properties

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>Structure</th>
<th>CAS No.</th>
<th>M.W.</th>
<th>pKa 1</th>
<th>pKa 2</th>
<th>Log Kow</th>
<th>Kd*</th>
<th>Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMX</td>
<td>C_{10}H_{11}N_{3}O_{3}S</td>
<td><img src="image" alt="" /></td>
<td>723-46-6</td>
<td>253.3</td>
<td>1.6</td>
<td>5.7</td>
<td>0.89</td>
<td>-0.22</td>
<td>-</td>
</tr>
</tbody>
</table>

Log Kow (42), Kd (43)*Under pH = 7 and 35 °C

3.2. Anaerobic Membrane Bioreactor (AnMBR)

3.2.1. Biological Treatment

The treatment train proposed in this research is Anaerobic Membrane Bioreactor (AnMBR).

The first step, a biological treatment, was based on anaerobic activated sludge. Therefore, a glass reactor with 2L capacity was inoculated with anaerobic activated sludge, previously used by Wei C-H., et al., (2015) (47) which consisted of a mixture of sludge from a real anaerobic digester in Riyadh, and camel manure from Jeddah, Saudi Arabia. The optimum anaerobic environment and completely homogeneous composition inside the bioreactor was achieved through a controller system that was programmed to maintain 35°C temperature; pH value of 7; and 200 rpm of stirring (Figure 1 and 2).
Figure 1 Scheme of AnMBR system
The bioreactor was operated in a continuous mode. The feeding was intermittent, with one cycle of 1.5 minutes: 4 seconds of feeding at flow rate of 35 mL/min, and 146 seconds of pause. The hydraulic retention time (HRT) was controlled at 24 hr, after which, the mix liquor (ML) was directly leaded to a sidestream crossflow membrane module which allowed the permeate filtration and the recirculation of the anaerobic activated sludge.

The biogas formed through the degradation process, was collected in a bas-bag (Figure 2).
3.2.2. Anaerobic Activated Sludge Acclimatization

The first stage of this project was proposed to acclimatize the anaerobic activated sludge to the operational conditions (temperature, pH, organic load, etc.).

At first, it was proposed to maintain the bioreactor at 22°C temperature, in order to assess the performance of the system at low temperature (thus low energy consumption). However, due to some operational complications and a low microbial community growth rate, it was decided to operate the biological reactor at optimum temperature (35°C).
With the increase of temperature and the extra seeding of the bioreactor with pre-acclimatized sludge, the bioreactor was operated in a continuous mode during a period of 2 months. Along the first month, the bioreactor was fed with synthetic wastewater with an organic load concentration of 1600 mg COD/L in order to increase the microbial community growth rate; during the second month the organic load of the synthetic wastewater was reduced to 800 mg COD/L to simulate real municipal wastewater.

3.3. OMPs

After 15 days of acclimatization, the bioreactor was run for 25 days more to verify its stability under the operation parameters (Temperature 35°C; pH 7; Flux 3.011 LMH). Thereafter, the bioreactor was fed with wastewater spiked with low concentration of SMX, later this concentration was increased.

The experimentation was divided in four stages: the acclimatization of the activated sludge to the operational conditions; the pre-run of wastewater spiked with low concentrations of SMX, to adapt the activated sludge to the type of feeding; the run of different SMX concentrations passing from trace to high concentrations (which exceed the concentrations found in real municipal wastewater); and the four stage, where the degradation kinetics were studied.

During the acclimatization period no SMX was spiked in the wastewater, posteriorly in the second experimentation stage, the first SMX concentration assessed was 10ppb. After 10 days of operation, the concentration was increased to 100ppb and
finally to 1000 ppb. During the third stage the concentrations studied were in the sequence of 10ppb, 100ppb, 1000ppb, 10000ppb and 100 000ppb.

Finally, for the kinetics analysis one single dose of SMX at target initial 100 ppm was directly injected into the bioreactor and sampling was performed at 0h, 1h, 2h, 3h, 6h, 12h, 18h, 24h, 36h, 48h, 72h, 96h, and 124h after the SMX spike.

3.4. Membrane Module

After the biological treatment, the mixed liquor was pumped from the bioreactor directly into a side-stream crossflow membrane module for filtration.

3.4.1. Membrane Module Operation

The system used in this research is a side-stream membrane bioreactor. Therefore, after the biological anaerobic treatment, which main target was the removal of the major quantity of organic load, the produced mix liquor was pumped into an ultrafiltration membrane system, which allowed the removal of solid materials product of the biological process (Figure 3).

The mixed liquor was pumped from the bioreactor to the membrane module with a peristaltic pump Cole-Parmer® at 32.60 L/h (tubing size 18). To maintain a constant flux operation, another peristaltic pump was placed after the membrane module in suction mode. This second pump worked in cycles of 10 minutes: 9 minutes of suction at 1.1-1.2mL/min flow rate (tubing size 16), and 1 minute of pause. To monitor the membrane performance, two barometers were placed at the entrance of the membrane module and exit of the permeate line, respectively.
To reduce the membrane fouling, a pump for biogas recirculation was placed at the entrance of the membrane module. It allowed the continuous pumping of biogas into the membrane at 16 L/h flow rate, to form a gas scouring along membrane surface.

3.4.2. Ultrafiltration Membrane

The membrane, used in this research, is a lab-scale side-stream crossflow Ultrafiltration Membrane, provided by DOW Filmtech®, which has a hollow fiber structure manufactured from Polyvinylidene fluoride (PVDF). PVDF gives high strength and chemical resistance to the membrane allowing a long useful membrane life time.
This membrane is a low pressure membrane with nominal pore diameter of 0.03 µm which is useful for the removal of bacteria, viruses and particulates including colloids. The effective filtration area of the first membrane module was 310cm². Because of the occurrence of membrane fiber leakage due to glue detachment, a new membrane module, with same characteristic, was required; the effective filtration area of this second module was 352.86cm².

The membrane fibers used, have a filtration flux rate of 40-120 L/m²/h for fresh water at 25°C. The range of operating pH is between 2 to 11 units, at 40°C of maximum temperature (51). Properties and operational characteristics of this membrane are summarized in Table 3.

<table>
<thead>
<tr>
<th>Membrane Characteristics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structure</strong></td>
<td>Hollow Fiber</td>
</tr>
<tr>
<td><strong>Material</strong></td>
<td>Hydrophilic PVDF</td>
</tr>
<tr>
<td><strong>Pore diameter</strong></td>
<td>30 nm</td>
</tr>
<tr>
<td><strong>Fiber inner diameter</strong></td>
<td>0.7 mm</td>
</tr>
<tr>
<td><strong>Fiber outer diameter</strong></td>
<td>1.3 mm</td>
</tr>
<tr>
<td><strong>Fiber length</strong></td>
<td>48.5 cm</td>
</tr>
<tr>
<td><strong>Fiber number</strong></td>
<td>16</td>
</tr>
<tr>
<td><strong>Effective filtration area</strong></td>
<td>310 cm²</td>
</tr>
<tr>
<td><strong>Filtrate Flux @ 25°C</strong></td>
<td>40-120 L/m²/hr</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td>1-40°C</td>
</tr>
<tr>
<td><strong>Max. inlet module pressure ( @ 20°C)</strong></td>
<td>6.25 bar</td>
</tr>
<tr>
<td><strong>Max. operating TMP</strong></td>
<td>2.1 bar</td>
</tr>
<tr>
<td><strong>Max operating air scour flow</strong></td>
<td>12 nm³ /hr</td>
</tr>
<tr>
<td><strong>Max backwash pressure</strong></td>
<td>2.5 bar</td>
</tr>
<tr>
<td><strong>Operating pH</strong></td>
<td>2-11</td>
</tr>
<tr>
<td><strong>TSS (max.)</strong></td>
<td>100 mg/L</td>
</tr>
<tr>
<td><strong>Expected filtrate turbidity</strong></td>
<td>≤0.1 NTU</td>
</tr>
<tr>
<td><strong>Expected filtrate SDI</strong></td>
<td>≤2.5</td>
</tr>
</tbody>
</table>
3.4.3. Membrane Cleaning

Generally anaerobic activated sludge, used in AnMBR processes, presents high fouling potential to the membrane.

Membrane fouling can deplete the membrane performance causing a drop in the permeate volume production and reducing the membrane selectivity capacity. Therefore, a peristaltic pump (Cole-Parmer® at 16 L/h, tubing size 18) was placed before the membrane module, in order to allow the injection of biogas into the module which helped minimizing the membrane fouling.

To extend the useful life-time of the membrane and maintain the permeate flow rate over 1.56 mL/min, at least once every two weeks a mechanical cleaning was conducted: the biogas flow rate was increased and the crossflow was enhanced (by doubling the sludge flow rate for 5 min), meanwhile mechanical shaking of the membrane module was carried out to decrease the blocking.

Chemical cleaning, that included: soaking the membrane module for 72h in a solution of 1000 mg/L Sodium Hypochlorite and then rinsing it with tap water, was implemented every four months of continuous operation.
3.5. Analytical Tests

To assess the performance of the system proposed, different parameters were analyzed.

3.5.1. Hydrodynamic Analysis

Despite the fact that the hydrodynamic performance of the AnMBR system is not one of the main targets in this research, flux and transmembrane pressure (TMP) were monitored in daily bases to assess the performance of the membrane module.

Flux is the transfer rate of mass or volume through a specific membrane surface, in other words, it is the amount of material that pass through each unit area of the membrane per unit of time (L/m²/h) (Equation 1); Flux is directly correlated with the driving force or transmembrane pressure (defined as the pressure difference between the influent/recirculation flow and the permeate flow; Equation 2) and with the hydraulic resistance offered by the membrane.

\[ J_p = \frac{Q_p}{A} \]  
\[ \text{Jp: Flux} \quad \text{Qp: Permeate flow rate} \quad \text{A: Membrane area} \]

\[ TMP = \frac{P_i + P_r}{2} - P_p \]  
\[ \text{TMP: Transmembrane pressure} \quad \text{Pi: Influent pressure} \]  
\[ \text{Pr: Recirculation pressure} \quad \text{Pp: Permeate pressure} \]
3.5.2. Physicochemical Analysis

Diverse physicochemical parameters such as temperature, pH and oxidation/reduction potential were monitored in order to ensure the optimal condition inside the biological reactor.

3.5.3. Chemical Oxygen Demand Analysis

The efficiency of the AnMBR in the removal of organic matter was analyzed through the measurement of the Chemical Oxygen Demand (COD); which was assessed using HACH® kits and a HACH® Spectrophotometer DR5000 (method 8000) (Figure 4).

![COD analysis](image)

**Figure 4 COD analysis**

3.5.4. Liquid chromatography with Organic Carbon Detection LC-OCD

The LC-OCD analysis is a rapid and sensible method that allows the separation of chromatographable organic carbon (CDOC) into fractions of different molecular
weight such as biopolymer (BP), humic substances (HS), building blocks (BB), low molecular weight (LMW) acids and low molecular weight neutrals [56].

Liquid Chromatography (LC) with Organic Carbon Detection (OCD), Organic Nitrogen Detection (OND) and Ultra Violet Detection (UVD) was used to analyze the rejection efficiency of the UF membrane and to characterize the organic matters remaining in the AnMBR permeate (Figure 5).

The LC-OCD analysis involved the assess of: a) AnMBR permeate samples filtered by the UF membrane with a pore size of approximately 30nm and b) samples from the bioreactor supernatant filtered through 0.45 µm (MF filters) GMF Filter media from Whatman™. After the samples were filtrated, by MF or UF membranes, they were diluted 60 times with Mili-Q® water to achieve less than 20 mg COD/L (LC-OCD requirement.

1mL sample was then injected in the LC-OCD at a flow rate of 1.1 mL/min. The system used two chromatography columns in series: a Toyopearl HW65 followed by a Toyopearl HW 50 resin provided by Tosoh Bioscience LLC.
Once the sample eluted from the column, the first detector was a non-destructive UV detector, with fixed wavelength at 254 nm. Posterior to the UVD, the sample stream was divided into two streams, the first stream carbonates were converted into carbonic acid through the sample acidification with phosphoric acid at flow rate of 0.2 mL/min, then the organic carbon detector was ran. The second stream was directly sent to the organic nitrogen detector (OND) (which was driven in parallel to the OCD), where all the organic nitrogen was converted into nitrates and measured at 220nm wavelength (Figure 6).
3.5.5. Mixed Liquor Suspended Solids

Likewise, the mixed liquor suspended solids (MLSS) and the composition of the produced biogas were analyzed.

The MLSS were measured following the Standard Method 2540B proposed by APHA, AWWA, WEF (2005) (45). A mixed liquor sample with 5-10mL volume was filtrated through a pre-rinsed and pre-dried 0.75 µm diameter Glass Fiber Filters, provided by Whatman™ (CAT NO. AP4004700).

After the ML filtration, the filter was placed in the oven at 105°C for 2h, in order to remove all the moisture and to be able to measure the mass of the total suspended solids (TSS). The sample was then placed in the oven at 550°C for another 2h so as to allow the burning of the organic portion of the sample; in this way the Fixed Suspend Solids (FSS) were obtained. The volatile suspended solids (VSS) were then calculated as the subtraction of the TSS minus the FSS (Equations 3, 4 and 5; Figure 7).

\[
\frac{X_1 - X_F}{V} = \text{Total Suspended Solids} = \text{TSS} \quad \ldots (3)
\]

\[
\frac{X_2 - X_F}{V} = \text{Fixed Suspended Solids} = \text{FSS} \quad \ldots (4)
\]

\[
\frac{X_1 - X_2}{V} = \text{Volatile Suspended Solids} = \text{VSS} \quad \text{or} \quad \text{TSS} - \text{FSS} = \text{VSS} \quad \ldots (5)
\]

Where:
- \(X_F\): weight of the filter
- \(X_1\): sample weight after 105°C for 2h
$X_2$: sample weight after 550°C for 2h
3.5.6. Biogas Analysis

The biogas, in the other hand, was collected in a gas bags (purchased from Calibrated Instruments, INC.) and analyzed with a gas chromatograph SRI 310C® (Figure 8) with a Silica GE column at 100°C for the analysis of H₂, O₂, N₂ and CH₄, using Argon as gas carrier; and at 80°C for the CO₂ analysis, with Helium as gas carrier. The sample injection volume was 250μL.

The total gas volume was calculated by the gas-bag method proposed by Ambler and Logan (2011) which is based on adding into the gas bag, a spike of the gas with lower concentration in the sample. The spike's volume should be around 20-30% of
the initial total gas volume, in order to produce an appreciable change in the area of

the gas at lower concentration in the GC chromatogram [53].

3.6. Organic Micro-Pollutant Quantification

The SMX concentration in the influent and permeate of the AnMBR system was assessed through a Dionex® Autotrace 280 solid-phase extraction (SPE) followed by liquid chromatography/mass spectrometry (LC/MS) technique (Figure 9).

For SPE technique, 100 and 1000mL of permeate sample at 1, 10, 100 and 1000 times concentration were taken, diluted with Milli-Q® water and spiked with 100 µL of deuterated SMX (d4-SMX) with concentration of 1 mg/L, which served as reference to measure the recovery rate of the SPE.
The prepared samples were then injected at flow rate of 1 mL/min and filtrated through Cartridge Filters Oasis® HLB 6cc (500mg) from Waters. Cartridge Filters Oasis® HLB are specialized in the extraction of organic compounds, as they are made from two monomers N-vinylpyrrolidone (hydrophilic) and lipophilic divinylbenzene (hydrophobic) which provide them with a high capacity for the retention of polar compounds, such as SMX (24).

Once the compounds were retained inside the cartridge filter, they were eluted through organic solvents (MeOH and 10% MeOH in MTBE) at 1 mL/min flow rate.

All the reagents and solvents used for processing and extracting the samples are analytical grade (>99% purity) and HPLC purity.

After the sample was extracted and concentrated by SPE, the 10 mL eluted sample was vaporized and further concentrated into 1mL sample by a Turbo Vap® concentration workstation at 39°C and under Nitrogen gas atmosphere.

The SMX quantification was carried out through a Liquid Chromatograph Agilent Technologies® 1260 Infinity coupled with a Mass Spectrometer AB SCIEX QTRAP 550 equipped with a Luna 5u C18 (2) 100 A Phenomenex® column (No. 00F-4252-E0) with dimensions of 150 x 4.60 mm and 5 µm pore size. A sample volume of 20µL was injected in each run at 0.8 mL/min flow rate. The mobile phase used was a positive phase composed by A) 0.1% Formic Acid in Extra Pure Water and B) 0.1% Formic Acid in Methanol (as the Formic Acid donates only one H⁺ enhancing the ionization of molecules); the mobile phase gradient involved 65% A held for 0.5 min, then stepped down to 50% at 0.51 min and decreased linearly to 5% at minute 8. It
is held at 5% for 6 mins, then stepped up to 65% A for an equilibration step of 4 min, giving a total running time of 18 mins. The quantification analysis and data process was carried on through the software Analyst® Instrumental Control and Data processing Software Version 1.6 by AB Sciex.

The LC/MS instrument detection limit (IDL) is 1 ppb (1µg/L); however due to the use of SPE it is possible to concentrate the real sample and attain a method detection limit of SMX under this conditions of approximately 0.01 ppb (10 ng/L).

Figure 9 Sample preparation and quantification of SMX in LC/MS
3.7. Transformation Products Identification

A first effort of transformation products' identification was done with the LC/MS, used for the SMX quantification. However due to the presence of high noise and the lack of a digital library, this first effort failed, thus forcing the use of a GC/MS for the identification of the SMX's TPs.

The accurate identification of SMX and its transformation products, formed along the degradation treatment process, was performed through a gas chromatograph (GC) Agilent Technologies® 7890A and a mass spectrometer (MS) Agilent Technologies® 5975C inert XL EI/CI with Triple-Axis Detector (Figure 10).

For the GC/MS analysis, the sample preparation involved the injection of 2 L water sample in the SPE at flow rate (1 mL/min) for maximum recovery. After the Cartridge Filters Oasis® HLB 6cc (500mg) were conditioned, the sample was loaded on a cartridge and its elution was performed by different solvents.

In the second stage of experimentation (the pre-run of wastewater spiked with low concentrations of SMX), methanol (MeOH), acetone, methyl tert-butyl ether (MTBE), Di-chloromethane (DCM), 10% MeOH in MTBE- and Hexane were used as solvents. In the third stage of experimentation (the run of trace and high concentrations of SMX), to achieve further accuracy and sharp peaks in the analysis, Acetone, DCM, 20% MeOH in Acetone and 20% MeOH in DCM solutions were used as solvents, since MeOH acts as proton donor solvent helping in the disruption of H-bonding on the Oasis HLB sorbent. The cartridge conditioning and sample elution was done with the same solvent.
Once the samples were extracted and concentrated by the SPE AutoTrace 280 DIONEX®, the 10 mL sample was vaporized and further concentrated into 0.5/0.1mL sample (for the 2\textsuperscript{nd} and 3\textsuperscript{rd} experimentation stage respectively) by a Turbo Vap\textsuperscript{®} concentration workstation at 39°C, under Nitrogen gas atmosphere.

5µL sample was injected, at 1.5 mL/min Helium carrier gas flow rate, into the Gas Chromatograph. The GC columns employed were Agilent J&W GC Column 122-0732 with maximum 260°C and Agilent J&W HP-5ms with maximum 320°C in the 2\textsuperscript{nd} and 3\textsuperscript{rd} stage, respectively. The oven program started at an initial temperature of 60°C for 3 min, and ramped until 260°C/320°C with a heating rate of 20°C/min and remained at 260°C/320°C for 5 min.

The TPs identification were determined by comparing the mass spectra of samples to standards from the NIST library 2.0 version. Among matched compounds with potential SMX fragments structure, only those with more than 70% matching probability were selected as transformation products.
3.8. Degradation Kinetics

The SMX degradation kinetics study, involved the injection of a single dose of SMX at 100ppm concentration into the bioreactor followed by sampling at different reaction times. The spike was done after the bioreactor was washout from the previous SMX concentration assessed (10ppm in Stage 3).
After the bioreactor was spiked with SMX, 50 mL sample of mixed liquor were directly taken from the bioreactor at different reaction times: 0h, 1h, 2h, 3h, 6h, 12h, 18h, 24h, 36h, 48h, 72h, 96h, and 124h.

The samples were then centrifuged at 15,000g at 22°C for 15 minutes. Afterwards, the supernatant was filtrated through 0.45 µm GMF Syringe Filter from Whatman™ and storage at 4°C until posterior evaluations.

For quantification analysis, 0.1mL of the samples were taken and diluted 100 and 1000 times using Milli-Q® water. The 100ml diluted samples were then spiked with 100µL of deuterated SMX (d4-SMX, 1ppm), that served as reference to measure the recovery rate of SMX during the SPE extraction process.

The prepared samples were then processed through the SMX quantification process previously described, using MeOH followed by 10% MeOH in MTBE as solvents.

In the other hand, the presence of TPs were elucidate through the TPs identification process, previously defined. In this case, 30mL sample was diluted into 70mL Milli-Q® water and posterior injected into the solid phase extraction system, where 20%MeOH in acetone was used as solvents to condition the cartridges and to elude the samples. The samples were concentrated and further vaporized until 0.1mL volume, which were then injected into the GC/MS system to proceed with the TPs identification.
Chapter 4

4. Results and Discussion

The treatment train proposed in this research consisted in an anaerobic MBR with external UF hollow fiber membrane.

The experimentation was divided in four stages: a) the acclimatization of the activated sludge to the operational conditions; b) the first run of wastewater spiked with low SMX concentrations to adapt the activated sludge to the type of feeding; c) the run of different SMX concentrations passing from trace to high SMX concentrations; d) and the evaluation of degradation kinetics.

4.1. Temperature, pH and Oxidation-Reduction Potential

Along the four stages of experimentation, physicochemical parameters such as temperature, pH and oxidation/reduction potential (ORP) were monitored, in daily bases, in order to ensure the optimal anaerobic environment and the completely homogeneous composition inside the bioreactor (Figure 11).

As previously described, the bioreactor was maintained at 200 rpm of stirring, to ensure the homogeneity inside the bioreactor. In the other hand, it was proposed that the temperature of the bioreactor could be maintained at room temperature (22-26 °C) without compromising the efficiency of the anaerobic bacteria (that commonly present its optimum efficiency at high temperature around 35°C) therefore, during the first eleven days of operation the temperature was maintained around 22-24°C; and no pH control was provided. Along this period, the wastewater
fed contained an organic load of 800 mg COD/L without spiking SMX. In this period the ORP fluctuated from 228 to -230 mV (as no pH control was provided), and the chemical oxygen demand (COD) removal rate obtained was quite low (under 50%); consequently, on day 9th, 100ml of pre-acclimatized activated sludge were seeded into the bioreactor.

Due to the presence of air leaks into the anaerobic bioreactor, the bacterial community presented a slow growing rate causing that the COD removal continued being low. Therefore, after fixing the air leakages, the temperature was raised and maintained (from day 11th until the end of the experimentation) at 35°C; the automatized pH control was started using an alkali solution of NaOH at 1M concentration; and the organic load of the feed wastewater was increased to 1600mg COD/L, in order to speed up the growth rate of the bacterial community and to allow the stabilization of COD removal (Figure 12).

By day 24th, the pH and temperature presented a constant behavior maintaining values of around 7 and 35°C, respectively. Likewise, the ORP value decreased to -430mV. The ORP value is related with the bacterial capacity to uptake the organic matter, as more negative the ORP value more degradation potential.

Despite the bioreactor efficiency increased when maintaining the bioreactor under this operational conditions, the COD removal rate remained low; therefore, the bioreactor was inoculated with extra 250ml of activated sludge and the organic load at the influent wastewater was decreased to 800mg/L.
Figure 11 Physicochemical parameters monitored to ensure the optimal conditions of the AnMBR
After the second addition of activated sludge, the bioreactor was maintained under stable conditions (pH 7; temperature 35±0.5°C; stirring 200 rpm; ORP from -411 to -424mV). At day 106th, a pH disturbance was observed: the pH value started rising up until 8 causing a further decrease of the oxidation-reduction potential (-467mV). Hence, the alkali pH control was stopped, and the acid pH control started with an HCl solution at 1M concentration.

From day 131st to day 141st, the AnMBR operation was paused because the membrane presented some leakages problems which forced the need of a new membrane module. On day 142nd the new membrane module (with same characteristics as the previous one) was connected and the AnMBR operation was restarted. From this point until the end of the experimentation, no big changes on
the pH and temperature were registered, however the ORP value presented some fluctuations (from -339 to -397 mV) when the wastewater was spiked with SMX at 100ppm, due to the high antibiotics concentration could cause inhibition of the bacterial activity.

4.2. Hydrodynamic Analysis

Despite the hydrodynamic performance of the AnMBR system is not one of the main targets of this research, the flow rate per unit membrane area (flux) and transmembrane pressure (TMP) were monitored in daily bases to assess the performance of the membrane module (Figure 13).

In the first operational days the transmembrane pressure and flux presented high variations, due to unstable operation conditions, until day 8th when it was established to maintain a nominal permeate flow rate of 1.1-1.2 mL/min, which meant a flux of around 3.01 L/m²/h.

During the acclimatization period (first experimentation stage), the TMP presented high values ranging from over 2.5 to 7.5 kPa, when maintaining flux at 3.01L/m²/h. After day 33rd, when the membrane was chemically cleaned with 1000mg/L Sodium Hypochlorite solution, the TMP was reduced to 0.21 kPa and maintained (until day 80th) under 2.5 kPa, thanks to the concentration of suspended solids in the bioreactor remained constant (around 3 500 mg TSS/L) and continuous mechanical cleaning of the membrane was performed (mechanical cleaning consisted on enhancing the crossflow by doubling the sludge and biogas flow rate during 5 min at the time that mechanical shaking of the membrane module was performed). Around
day 63rd, the flux suffered a drop from 3.01 to 2.79L/m²/h due to intense fouling; despite the application of mechanical cleaning, the flux did not recover.

By day 72nd the rate of the suction pump, in the permeate side, was increased from 1.1 to 1.2 mL/min, thereby the flux was mended to 3.01L/m²/h. Around day 95th a similar behavior was presented; once again, as the mechanical cleaning did not offered a significant improvement in the flux, the suction pump rate was increased this time from 1.2 to 1.3 mL/min.

![Figure 13 Variation of Flux and TMP along the experimental time](image)

At the beginning of the third experimental phase (day 103rd), despite the flux reached 3.23mL/min, the TMP suffer a great increase from 1.41 to 4.9, 10.7, 14.5, until 17.5 kPa on day 117th, when chemical cleaning was performed and the TMP was recovered. However new drop of the flux in conjunction with strong TMP fluctuation forced a second chemical cleaning on day 127th; the strong chemical
cleaning caused the detachment of glue from the membrane fiber, generating strong leakage of sludge into the permeate (Figure 14).

In spite of the efforts to re-glue the fibers, a new membrane module was designed and connected to the system by day 142\textsuperscript{nd}. The suction pump rate was then re-adjusted at 1.1 mL/min and after day 192\textsuperscript{nd} at 1.2 mL/min. From this point until the end of the experimentation the TMP was maintained, by periodic mechanical cleaning, on a range between 0.1 and 4±0.5 kPa and flux at 2.67 L/m\textsuperscript{2}/h, despite the fact that during this experimental stage the increase of the influent COD allowed that total suspended solids in the bioreactor doubled its concentration, which means an increasing in the fouling potential.
4.3. Chemical Oxygen Demand

The chemical oxygen demand was measured in order to monitor the efficiency of the bioreactor in the removal of the organic content.

Figure 15 shows the COD concentration in the influent and permeate, as well as the removal percentage obtained. During the first 15 days of acclimatization, the COD content was not measured in the permeate because it was expected to obtain very high variations as the anaerobic activated sludge was not adapted yet neither to the operational conditions nor to the specific synthetic wastewater composition.

During the first 15 days of operation, the influent wastewater was prepared with a COD content of 800 mg COD/L. However in order to increase the bacterial community growth rate, from day 15th to day 35th, the organic content of the influent wastewater was augmented around to 1600 mg COD/L. Along this period, the percentage of organic matter removal was low, about 50-80%, producing a permeate with 200-600 mg COD/L.

Once the acclimatization period ended, the influent wastewater was maintained with a COD content of 800 mg COD/L. As the activated sludge concentration was increased and already adapted to the type of wastewater fed, the AnMBR process showed high efficiency (over 95%) in the removal of the organic content, allowing permeates with 25-35 mg COD/L.
From the AnMBR process, the anaerobic biological process is the one that permitted the removal/degradation of the major quantity of organic matter, while the UF membrane acts as a sludge water separation role by producing high quality permeate free of suspended solids.

The second experimentation stage involved the spike of the influent wastewater with SMX at 10, 100 and 1000 µg/L concentration (from a 1000 mg/L SMX in methanol stock solution; previously mentioned in chapter 3) at day 40th, 61st and 73rd, respectively.

The first two SMX concentrations assessed did not present a great impact on COD concentration neither in the influent nor in permeate. However after day 73rd, when the influent wastewater was spiked with 1 mg SMX/L, the organic content of the influent raised over 2000 mg COD/L due to the contribution of methanol (1 ml methanol in 1 L equals 1200 mg/L COD), which was used to prepare the concentrate stock of SMX (1000mg/L).

Despite the organic content of the influent wastewater increased almost three times (from 800 to 2110 mg COD/L), the permeate presented 90mg COD/L initially and after maintaining the feeding of 1ppm SMX/L for somedays, the COD concentration in the permeate decreased to 25-30 mg COD/L. This showed the high efficiency of the biological bioreactor which offered a COD removal over 97%. Such high efficiency is also reflected in the ORP value which did not present great fluctuations throughout the stage being on the range of -418 ±20 mV.
At day 111\textsuperscript{th} the COD removal suffered a slight drop to 94\% due to the sudden increase of the pH (to 7.81) and the decrease of the ORP of the anaerobic activate sludge. As soon as the pH was re-established at 7, by acid pH control solution, the ORP was recovered (≈ -411mV) and the COD removal returned to over 97\%.

In the third experimental stage, the SMX stock solution used for spiking the wastewater with high SMX concentrations (10 and 100ppm) had an initial concentration of 50,000 mg SMX/ L in methanol therefore, the methanol introduction into the AnMBR system (when spiking 10 mg SMX/L) did not represented a high organic load thus the influent was maintained around 800mg COD/L.

On day 176\textsuperscript{th} the SMX spiking was suspended for a week in order to wash out the SMX and its TPs form the anaerobic bioreactor and proceed with the degradation kinetics study. In this week, the organic load of the wastewater was augmented to 2600mg COD/L approximately, to allow the adaptation of anaerobic activated sludge to high influent COD expected in initial degradation kinetics study.

By day 183\textsuperscript{rd}, the kinetics analysis started by the spike of a single 4 ml dose of stock solution (with 50,000mg SMX/L in MeOH) directly into the bioreactor, which raised the initial COD to over 3100 mg COD/L. In this fourth stage, the influent and permeate flows were stopped and manual sampling of mixed liquor directly from the anaerobic bioreactor was carried on at different reaction times.

The reduction of the COD along the kinetic analysis is observed in Figure 15 from day 183\textsuperscript{rd} to day 188\textsuperscript{th}. Along this period it is also possible to observe the
contribution of the membrane module in the removal of the organic load. Filtration though UF membrane provided approximately 40% more COD removal than MF membrane, which could indicate that the majority of the COD in permeate is due to the presence of biopolymers (i.e., proteins and polysaccharides).

After the kinetics analysis, the study of the AnMBR in continues operation mode was restored (day 189th) and its performance on the removal of 100ppm SMX concentration was assessed. The constant addition of high SMX/MeOH concentrations generated that the influent COD ranged from 3200 to 2800 mg COD/L.

The continuous input of SMX at 100 ppm, produced a shock in the bacterial metabolism causing that its ability to reduce the organic load was diminish to 65-75% of COD removal (505-1138mg COD/L in the permeate). After one week of sludge adaptation to this SMX concentration, the AnMBR was able to increase its removal capacity and produce permeates with an organic content under 250mg COD/L (>92% COD elimination).
UF membrane, in comparison of the MF membrane, is expected to provide higher rejection of colloids; however none of this filtration processes is expected to offer any type of resistance against the pass of Sulfamethoxazole molecules, due to the large pore size of MF and UF membranes.

Liquid chromatography with online organic carbon detection (LC-OCD) was carried on in order to analyze the performance of UF with respect to MF membranes, in the removal of organic matter.

Figure 16 shows the LC-OCD chromatograms acquired from permeate samples obtained after 96h and 124h of anaerobic degradation reaction, and filtration through MF and UF membranes.
From the chromatograms, the main differences between MF and UF permeate were the biopolymers (mainly polysaccharides and proteins). For all signals including organic carbon detection (OCD), organic nitrogen detection (OND) and Ultra-Violet light detection (UVD), UF permeates showed much lower peak intensity of biopolymers than MF permeate, indicating UF nearly rejected all biopolymers present in anaerobic sludge and thus provided stable permeate quality.
Figure 16 LC-OCD chromatography of MF and UF membrane in kinetics study
4.5. Suspended Solids

In order to monitor the biomass concentration inside the bioreactor, the analysis of mixed liquor suspended solids (MLSS) was carried on following the Standard Method 2540B proposed by APHA, AWWA, WEF (2005) [52].

As previously mentioned, during the acclimatization period, the biomass concentration inside the bioreactor was very low therefore no sampling for MLSS analysis was taken.

During the initial 30 days of the second experimentation stage, the biomass concentration and composition was very stable with approximately 3,500mg/L of TSS from which, about 83.2% was VSS (organic fraction) and just 16.8% was FSS (inorganic portion).

With the increase in the SMX concentration to 1ppm, the MeOH entrance in the influent wastewater also increased, enhancing the biomass growth rate. Thus, the TSS reached 5300 mg/L including 4420mg VSS/L and 880mg FSS/L, at the end of the second experimentation stage. The VSS/TSS ratio was maintained around 85%±0.03 in the final 20 days of the second experimentation stage (Figure 17).

In the 3rd stage, the sludge concentration maintained roughly stable level, around 5000 mg/L with the VSS/TSS ratio of around 83%, until spiking SMX concentration at 10 ppm. Thereafter, with the increase in the organic load of the influent wastewater over 3200mg COD/L, the AnMBR sludge doubled its concentration to 10800 mgTSS/L with 70.4% and 29.6% of VSS and FSS, respectively. Later this
concentration started slightly decreasing to 10300 mg TSS/L (68.7% VSS; 31.1% FSS) and finally until 97800 mg TSS/L (76.9% VSS; 23.1% FSS), on day 203rd. Further decrease in the biomass concentration would be expected due to inhibition of the bacterial activity if the experimentation carry on with the continuous spike of high SMX concentrations.

Despite the fluctuation of the total suspended solids, the active biomass composition (VSS/TSS ratio) was preserved around 85% with a slight decrease until 70% at the 3rd stage, when the SMX spiked concentration was raised until 100 mg/L. The decrease of the biomass concentration could be explained due to the continue input of high concentrations of SMX (100 ppm) could have caused changes in the bacterial metabolism and inhibition of its degradation capacity and growth. Therefore, in the last experimentation stage of this research the biomass reduced its ability of COD removal (65% of COD removal), in the same way that the biogas production decreased considerably.
4.6. Biogas Analysis

In any anaerobic microbiological process, the bacterial community growth and energy requirements will depend in the microbial metabolism of organic material (COD) in a free-oxygen atmosphere which will result in the production of biogas and the development of biomass (CH$_4$+CO$_2$+biomass).

In order to determine the composition and volume of the biogas produced through the AnMBR process, two gas chromatograph SRI 310C$^\circledR$ were used. Before running the biogas samples, calibration curves for every analyte presented in the biogas mixture (CH$_4$, CO$_2$ and N$_2$) were generated by the injection of a series of known volume of the pure gas into the GC and the measurement of its peak area.

Figure 18 shows the calibration curves for CH$_4$, CO$_2$ and N$_2$. Within the range of assessed volumes, the three calibration curves presented a linear response ($R^2$ of
0.9985, 0.9747 and 0.901, respectively) therefore, it was possible to use the regression line equation to calculate the volume of each one of the analytes present in biogas mixture. The total volume of the biogas sample was obtained by following the gas-bag method proposed by Ambler and Logan (2011) [53].

Figure 19 shows the variation of the total volume of biogas along the operation time. During the first 40 days of operation, the biogas production was very low due to instability in the bioreactor, which caused the lack of enough biogas sample to run the gas-bag method in the GC; therefore, no data about the volume of the biogas produced along this period is presented.
After day 40th, when the bioreactor was fed with synthetic wastewater spiked with 10ppb SMX, the biogas production improved and it was possible to start the measurement through the GC. From figure 19, it is possible to see that as the concentration of SMX spiked into the bioreactor raised from 10 ppb at day 40th, 100 ppb at day 61st and 1000ppb at day 73rd, the biogas volume also increased from 309mL/d to over 4750 mL/d. This could be explained due to the entrance of methanol, presented in the stock SMX solution, strongly contributed to the influent COD (12, 120 and 1200 mg COD /L from methanol contribution for spiked SMX concentration of 10, 100 and 1000 ppb). As the organic load in the influent increased, the biogas production (and biomass growth) also augmented directly.

The same behavior was observed in the third experimentation stage, at 100ppb SMX concentration the biogas production was maintained around 2042 mL/d. As the SMX concentration augmented to 1ppm and 10ppm, the biogas production went up to 6088mL and 5954mL respectively, to later start decreasing until 390mL, when the bioreactor was spiked with 100ppm SMX. From this it can be concluded that the continuous input of high concentrations of SMX (> 10ppm) can develop inhibition in the bacteria capacity to digest the organic matter and to produce biomass and biogas.
The analysis of the biogas composition (calculation of the percentage of each component CH₄, CO₂ and N₂ inside the gas mixture) was carried on through the regression line equations obtained with the calibration curves (Equation 6, 7 and 8).

\[ y_{CH} = 0.1383 \times x \]  \hspace{1cm} \text{(6)}

\[ y_{CO} = 0.386 \times x \]  \hspace{1cm} \text{(7)}

\[ y_{N2} = 0.4471 \times x \]  \hspace{1cm} \text{(8)}

*Regression line equations for Methane, Carbon Dioxide and Nitrogen*
The biogas generated in the AnMBR process was mainly composed by methane (CH₄), nitrogen (N₂) and carbon dioxide (CO₂). In the first 40 operational days the biogas presented different conformation, being primarily composed by Nitrogen (> 50%) and Methane (< 45%), presenting low concentrations of Carbon Dioxide (< 5%), as well as trace concentrations of Oxygen, which was an indicator of instable performance inside the anaerobic MBR and explained the fluctuation of the ORP along this period (Figure 20).

As the bioreactor was settled at specific operational parameters and acclimatized to the type of influent wastewater, the biogas production increased and its composition was maintained. From day 41ˢᵗ, the biogas bulk was principally formed by Methane reaching more than 90% of the total volume. In the other hand the Nitrogen concentration was reduced to less than 15% and the Carbon Dioxide remained as the tracer gas in the bag with less than 5% of the total volume. This gas composition was maintained despite the influent wastewater was spiked with SMX.

In the day 75ʰ, after the SMX concentration was raised to 1ppm, an increase in the concentration of CH₄ took place, going from 88% to over 95%; this could be due to the presence of methanol in the SMX concentrated stock.

Slight variation in the gas composition were also present around day 103ʳᵈ, 144ᵗʰ and 168ᵗʰ when 10ppb, 1ppm and 10ppm SMX concentrations were spiked. The antibiotic at this concentration could had caused a shock in the bacterial metabolism creating a fluctuation in composition of the biogas produced.
The energy recovery from the AnMBR treating synthetic wastewater, expressed as methane production in terms of milliliters of methane per gram of COD removed, was calculated through Equation 9, once the total gas volume and the gas composition were obtained (Figure 21).

\[
\frac{(Total\ Volume_{Biogas} \times \%CH_4)}{(COD_i - COD_e)} = mL \ CH_4/g \ COD_{removed}
\]

Along the first 70 days of operation, when the SMX concentration was between 10 and 100 ppb, the methane production in terms of COD removal ranged from 45 to 130 mL CH₄/g COD_removed. As the SMX concentration increased to 1000 ppb (raising the influent organic load from 800 mgCOD/L to over 2000 mgCOD/L) the methane content in the biogas also increased, reaching a maximum of 281 mL CH₄/g COD_removed at day 79th. At this stage, the bioreactor had a stable operation, producing approximately 2.12 L of biogas per day. This biogas rich in methane (94.6 % CH₄) can indicate the good balance between the methanogen and the acidogen bacteria, as suggested by Saddoud A., et al. (2007) [54].
In the 3rd experimentation stage, a similar behavior was observed: around 62-180 mL CH$_4$/g COD$_{removed}$ were obtained when the AnMBR was spiked with 10 and 100 ppb SMX. Posteriorly, as the SMX concentration and organic loading rate increased, the biomass and methane production increased as well until 4420 mg VSS/L and 310 mL CH$_4$/g COD$_{removed}$ (at day 156th). The high organic loading rate would enhance the biomass growing rate and promote the conversion of more substrate (influent COD) into methane. The energy produced by the assessed
AnMBR system reached levels comparable with the theoretical value of 382 mL CH4/g COD\textsubscript{removed}, which is consistent with previous studies [55].

Greater SMX concentrations (10ppm and 100ppm) allowed around 100 mL CH4/g COD\textsubscript{removed} initially, which further decreased probably due to the inhibition of the bacterial activity caused by the high and continuous SMX concentrations.

![Figure 21 Methane production in terms of COD removed](image)

4.7. Organic Micro-Pollutant Quantification

Before starting the quantification analysis in the LC/MS, a calibration curve for pure SMX (Figure 22) was obtained in order to ensure the accuracy of the results.
The SMX calibration curve presented a linear response with $R^2$ of 0.989, thus it was possible to use the regression line equation to estimate the real SMX concentration in the influent and permeate.

As previously mention in chapter 3, the quantitative analysis and data process of SMX concentration was done via the Analyst® Instrumental Control and Data processing Software Version 1.6 by AB Sciex. Figure 23 illustrates the selected ion chromatograph of a permeate sample (at day 103rd, when the wastewater was fed at 10ppb SMX concentration), where the red color represents the SMX isotope spiked as a standard for recovery control of SPE process, and blue color represents the SMX concentration present in the sample.
Sulfamethoxazole, with m/z of 254 as precursor ion and m/z of 156 as product ion, was detected approximately at 5.16±0.02 min retention time, while the SMX isotope (m/z of 260 as precursor ion and m/z of 160 as product ion) was detected at 5.15±0.02 minutes, serving as a good reference control for SMX.

Figures 24 and 25 illustrate the quantification process of permeate and influent samples respectively. From these figures is noticeable the difference in the peak intensity, which goes from 9.1436e6 cps (counts per second) in the influent to 8e4 cps in permeate, approximately two orders of magnitude. Despite the peak intensity gives some information about the SMX concentration in the samples, for more accurate results in the quantification analysis, the integration of the area under the
peak was processed in Excel and the real SMX concentration was thereby obtained based on calibration curve and recovery ratio from the SMX isotope.

The performance of the AnMBR in the removal of SMX throughout the different experimental stages is presented in figure 26, showing the SMX concentration in the influent and permeate of the system as well as the percentage of removal.

In general, the AnMBR process provided a very stable removal efficiency of SMX, achieving permeates with low SMX concentrations. At stage 2, whenever the bioreactor was spiked with a new SMX concentration (10 ppb, 100 ppb, 1 ppm, 10 ppm and 100 ppm), the AnMBR produced permeates with SMX concentrations 20 times less than the initial SMX concentration in the influent wastewater. As the system got adapted to the concentrations assessed, the removal efficiency increased and permeates presented a SMX concentration 50 times less than the initial one.
Figure 24 Quantitative SMX analysis on permeate sample
Figure 25 Quantitative SMX analysis on influent samples
For example, in the second experimental stage (day 40\textsuperscript{th}) when the influent was spiked with 10µg SMX/L (real SMX concentration in the influent wastewater ≈ 9.881 µg SMX/L) the average permeate SMX concentration obtained, from day 40\textsuperscript{th} to day 46\textsuperscript{th}, was around 0.48112 µg SMX/L, 20-21 times less than the initial concentration. Posteriorly, from day 47\textsuperscript{th} to day 50\textsuperscript{th}, the permeate concentration was reduced to around 0.3381 µg SMX/L (30 times less than the initial concentration) and finally after day 50\textsuperscript{th} (ten days after the initial 10ppb SMX spike) to 0.23995 µg SMX/L, around 50 times less than the influent wastewater.

Similar behavior was observed when spiking 100ppb, 1ppm and 10ppm SMX concentrations in the influent. However, at 10ppm of initial concentration the system efficiency got a slight diminish providing permeates with concentrations ranging from 1.217 to 0.515 mg SMX/L (less than 8 to 19 times less than the influent concentration). This proves the wide capacity of the AnMBR bacterial community to easily adapt and to obtain high biodegradation/biotransformation rates from this antibiotic.

The reported experimental results highlight the AnMBR effectiveness in the removal of SMX as the elimination efficiency obtained was over 80% for all the concentrations assessed, achieving a removal percentage of around 95-98% at concentrations between 10 to 1000ppb. Considering that the actual SMX concentration in real wastewater is approximately 1.9ppb [22, 23], we can conclude that the treatment process proposed in this research is a suitable and viable option to treat wastewaters containing sulfamethoxazole (Figure 26).
As the influent concentration increases to 10ppm and 100ppm, the system effectiveness present a slight reduction reaching around 90% and 85% which could have been caused by changes generated in the bacterial metabolism due to the antibacterial characteristics of SMX. Such behavior was not only reflected in the lower SMX removal but also in other parameters such as the biogas production, which presented a slight change in its composition but a strong drop in its volume (passing from 6L to less than 1L of total biogas; Figure 19), and the decrease on the active biomass concentration (Figure 17). Despite the changes in the AnMBR performance are relatively small (SMX removal varying from 98% to 88%), it is expected that, by increasing the SMX concentration spiked in the influent wastewater, the performance might diminishes even more (Figure 27).

Regardless of the 95-98% of SMX removal obtained in this processed, this does not mean that the SMX was completely mineralized. The biotransformation of the parent compound (SMX) into different TPs is expected and therefore analyzed in the next section.
Figure 26 SMX fluctuation in influent and permeate along the AnMBR process

Figure 27 Average SMX concentration and removal percentage of SMX in stage 2nd and 3rd
4.7.1. Kinetic Analysis

The kinetic study, previously described in chapter 3, was accompanied by the quantification of the parent compound and the identification of the SMX degradation intermediates.

The sulfamethoxazole biodegradation capacity in the AnMBR was examined for an initial SMX concentration of approximately 100 ppm. Figure 28 illustrate the kinetics of the SMX degradation achieved by the anaerobic bioreactor, showing a relatively rapid and linear behavior, with an acceptable removal rate (94%) at 24h of reaction; after 24h no significant variation in the SMX biodegradation rate was achieved. At 36h, 96.8% SMX removal was attained, obtaining a maximum degradation rate of 97.8% at 72h of reaction.

From the quantitative analysis it was possible to obtain the experimental SMX concentration at the beginning of the 4th experimentation stage, which was over 105 ppm. After 3h of reaction, approximately 25% of the initial concentration was reduced to 74 ppm to further achieve 31.8 ppm, 22.6 ppm and 13.5 ppm (68.2%, 77.32% and 86.5% removal) at 6, 9 and 18 hours, respectively.

5.5 ppm SMX concentration is achieved after 24h of reaction; from this point until the end of the kinetic experiment the removal efficiency was maintained between 94 to 97%, reaching a minimum concentration of 2.18 ppm after 96 hours of biological reaction (Table 4). Thus, the biological treatment proposed in this research was not able to provide the complete elimination of SMX.
Table 4 Kinetic SMX Degradation Analysis

<table>
<thead>
<tr>
<th>Reaction Time (h)</th>
<th>SMX concentration (PPB)</th>
<th>% SMX Remaining</th>
<th>% SMX Removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100000</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>105221.70</td>
<td>105.22</td>
<td>-5.22</td>
</tr>
<tr>
<td>3</td>
<td>74000.17</td>
<td>74.00</td>
<td>25.99</td>
</tr>
<tr>
<td>6</td>
<td>31785.99</td>
<td>31.78</td>
<td>68.21</td>
</tr>
<tr>
<td>9</td>
<td>22670.20</td>
<td>22.67</td>
<td>77.33</td>
</tr>
<tr>
<td>12</td>
<td>22937.29</td>
<td>22.93</td>
<td>77.06</td>
</tr>
<tr>
<td>18</td>
<td>13488.45</td>
<td>13.48</td>
<td>86.51</td>
</tr>
<tr>
<td>24</td>
<td>5548.48</td>
<td>5.54</td>
<td>94.45</td>
</tr>
<tr>
<td>36</td>
<td>3206.845</td>
<td>3.21</td>
<td>96.79</td>
</tr>
<tr>
<td>48</td>
<td>3139.49</td>
<td>3.14</td>
<td>96.86</td>
</tr>
<tr>
<td>72</td>
<td>2281.96</td>
<td>2.28</td>
<td>97.72</td>
</tr>
<tr>
<td>96</td>
<td>2181.83</td>
<td>2.18</td>
<td>97.82</td>
</tr>
</tbody>
</table>

Figure 28 Kinetic analysis of SMX degradation and removal percentage
In figure 29 the data obtained was fitted with different order kinetics correlations, observing that the SMX degradation kinetics have the best agreement with the first order kinetic model, presenting a linear correlation with $R^2$ of 0.938.

\[ \text{Kinetics Correlation} \]

Considering a first order decay, the time course of the SMX biodegradation in the AnMBR can be expressed by equation 10[57]:

\[ \frac{dC}{dt} = -kt \]
In practice, the first-order rate constant $k$ is often substituted by a half-life, $H$ (equation 11)

$$H = \frac{\ln 2}{k} \quad \ldots (11)$$

In this way, the first-order equation becomes written as:

$$\frac{dC}{dt} = -\frac{0.693}{H} C \quad \ldots (12)$$

If the degradation rate remains constant during the biodegradation process, the residual concentration $C(t)$ is given by an exponential equation of the form [58]:

$$C(t) = C_0 \times e^{-kt} = C_0 \times e^{-0.693t/H} = C_0 \times 0.5^{t/H} \quad \ldots (13)$$

$C = \text{SMX concentration}$ $k = \text{first-order rate constant}$

$C_0 = \text{extrapolated initial SMX concentration}$ $t = \text{reaction time}$

From equation 13 is possible to appreciate that the logarithm of the concentration is a linear function of time.
Then, the first-order reaction constant $k$ can be calculated by fitting experimental data points from the degradation phase to a single exponential (Figure 30).

$$C(t) = 1.024 \times e^{-0.119t}; \quad k=0.119$$

It is important to consider that the AnMBR process may not lead to the total SMX mineralization, but to the formation of other compounds that compete during the depletion reaction for the bacterial degradation. Generally, the amount of intermediates increase with the initial concentration [24], therefore the first-order reaction constant might differ when varying the initial SMX concentration.
4.8. Transformation Products Identification

In the second experimentation stage it was proposed to perform the transformation products (TPs) identification throughout the LC-MS used for the quantification analysis. However, due to the lack of an accurate digital library for the LC-MS, the identification by this method was difficult.

Therefore, in order to identify the SMX transformation products formed along the AnMBR treatment and to propose a possible degradation pathway, GC-MS total ion chromatograms (TIC) were obtained and the mass spectra of main TIC peaks was compared with standard spectrum in NIST library 2.0 version.

This section explains the occurrence of different SMX transformation products according to the feeding SMX concentration by comparing samples prepared with different extraction solvents (to improve the peak intensity) and samples at different extraction concentrations. Finally, a kinetic analysis is presented to indicate the occurrence of each analyte along the degradation reaction; broad calculations about the intensity of each analyte are offered.

4.8.1. Occurrence of TPs at different feeding SMX concentrations

During the AnMBR treatment different feeding SMX concentrations (0.01, 0.1, 1, 10 and 100ppm) were analyzed, obtaining various TPs in each concentration. A total of seven peaks were detected and identified by the GC-MS. Table 5 summarizes the main characteristic of the TPs identified along the 2nd, 3rd and 4th experimentation stages.
Table 5 Identification of the main SMX Transformation Products formed in the AnMBR process and their respective chemical structures

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Chemical Formula</th>
<th>MW</th>
<th>CAS number</th>
<th>GC Elution Time (min)</th>
<th>Influent SMX concentration where it was detected</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Benzene sulfonamide, N-Butyl-</td>
<td>C\textsubscript{10}H\textsubscript{15}NO\textsubscript{2}S</td>
<td>213</td>
<td>3622-84-2</td>
<td>11.1 – 11.2</td>
<td>0.01, 0.1, 1, 10, 100 ppm</td>
<td><img src="image1" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>2</td>
<td>Sulfanilamide</td>
<td>C\textsubscript{6}H\textsubscript{8}N\textsubscript{2}O\textsubscript{2}S</td>
<td>172</td>
<td>63-74-1</td>
<td>12.2 -12.5</td>
<td>0.1, 1, 10, 100 ppm</td>
<td><img src="image2" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>3</td>
<td>Sulfisomidine</td>
<td>C\textsubscript{12}H\textsubscript{14}N\textsubscript{4}O\textsubscript{2}S</td>
<td>278</td>
<td>515-64</td>
<td>14.5 -15.5</td>
<td>1, 10, 100 ppm</td>
<td><img src="image3" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>4</td>
<td>4- Aminothiophenol</td>
<td>C\textsubscript{6}H\textsubscript{7}NS</td>
<td>125</td>
<td>1193-02-8</td>
<td>8.1 – 8.2</td>
<td>1, 10, 100 ppm</td>
<td><img src="image4" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>5</td>
<td>Aniline</td>
<td>C\textsubscript{6}H\textsubscript{7}N</td>
<td>93</td>
<td>62-53-3</td>
<td>5.05-5.5</td>
<td>1, 10, 100 ppm</td>
<td><img src="image5" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>6</td>
<td>4-Pyrimidinamine, 2,6-dimethyl-</td>
<td>C\textsubscript{6}H\textsubscript{9}N\textsubscript{3}</td>
<td>123</td>
<td>461-98-3</td>
<td>7.5</td>
<td>10, 100 ppm</td>
<td><img src="image6" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>7</td>
<td>Acetamide, N-(4-mercaptophenyl)-</td>
<td>C\textsubscript{9}H\textsubscript{9}N\textsubscript{OS}</td>
<td>167</td>
<td>1126-81-4</td>
<td>10.76</td>
<td>10, 100 ppm</td>
<td><img src="image7" alt="Chemical Structure" /></td>
</tr>
</tbody>
</table>
4.8.1.1. 0.01 ppm feeding SMX concentration

The first feeding SMX concentration assessed, in the 2nd and 3rd experimentation stages, was 10ppb, which was representative of SMX concentration in real wastewater [22, 23].

For the analysis, at 10ppb feeding SMX concentration, the samples were prepared with different solvents: Methanol (MeOH), Dichloromethane (DCM), Ethyl Acetate (EA), Methyl tert-butyl ether (MTBE), Acetone and MeOH plus 10% MeOH in MTBE (Figure 31), obtaining that compound 1 (Benzene sulfonamide, N-Butyl-) was detected under all tested solvents at the same retention time.
Further analysis, at different sample extraction concentrations (1k, 20k and 120k time’s concentration) was carried on with MeOH plus 10% MeOH in MTBE (Figure 32) in order to improve the signal and to obtain a more accurate detection of TPs products.

The analysis of 10ppb feeding SMX concentration showed the presence of only one transformation product detected at 11.16 minutes retention time, even under 120k concentrating times, identified with over 76% matching probability as Benzenesulfonamide, N-Butyl with a M+ of 77 (Figure 33).
Figure 32 Comparison of different sample extraction concentrations using MeOH plus 10% MeOH in MTBE at 10ppb feeding SMX concentration
Figure 33 Peak 1, Benzenesulfonamide, N-Butyl; identified at 10ppb feeding SMX concentration

4.8.1.2. 0.1 ppm feeding SMX concentration

The feeding SMX concentration spiked in the influent wastewater was increased to 100ppb in order to analyze the potential occurrence of other biotransformation products.

From the comparison of the chromatogram intensity (Figure 34) of samples prepared with different solvents (DCM, EA, Acetone and MeOH plus 10% MeOH in MTBE), it was obtained that all tested solvents showed similar TIC profiles including
two peaks: peak 1 at retention time 11.1-11.2 min, and peak 2 at retention time 12.2-12.4 min.

The comparison of different sample extraction concentrations (1k and 20k time's concentration), under the same solvent MeOH plus 10% MeOH in MTBE (Figure 35), allowed the identification of peak number 2.

When concentrating the sample 1000 times, just peak 1 (Benzenesulfonamide, N-Butyl) was detected. However, by increasing the sample extraction concentration at
20,000 times, the GC-MS was able to detect the second peak presented at trace amounts.

Figure 35 Comparison of different sample extraction concentrations using MeOH plus 10% MeOH in MTBE at 100ppb feeding SMX concentration

Peak 2 was identified as Sulfanilamide, with around 68% matching probability, having a retention time of 12.3 minute and a M+ of 172 (Figure 36).
By comparing samples extracted under the same solvent and concentration times but different reaction times, when the biological bioreactor was operated under continuous mode, it was observed that the TPs suffer a posterior decrease in their peak intensity, which can suggest further degradation.

Figure 37 illustrates the variation of Benzenesulfonamide, N-Butyl (peak 1) incidence. When continuously spiking the bioreactor at 100ppb SMX concentration, at day 63\textsuperscript{rd} (when the bioreactor was just spiked with this initial concentration) peak 1 presented an intensity of over 360,000,000; posteriorly by day 68\textsuperscript{th} the
intensity got around 900 times reduced (≈400,000) to finally reach an intensity of around 100,000 at day 71\textsuperscript{st}.

![Figure 37 Further degradation of TPs identified at 100ppb feeding SMX concentration](image)

**4.8.1.3. 1 ppm feeding SMX concentration**

In stage 2, the occurrence of TPs at 0.01, 0.1 and 1ppm feeding SMX concentration was evaluated with MeOH, MTBE, DCM, EA, Acetone and MeOH plus 10% MeOH in MTBE. The comparison of 1ppm feeding SMX concentration chromatograms,
extracted with these solvents, showed that DCM and EA offered higher resolution spectra (Figure 38).

In the other hand, stage 3 involved the analysis of TPs at 0.01, 0.1, 1, 10 and 100 ppm feeding SMX concentration with MeOH, DCM, EA, Acetone, 10% MeOH in MTBE, 20% MeOH in Acetone and 20% MeOH in DCM as extraction solvents. Figure 39 demonstrates that 20% MeOH in Acetone and DCM are the solvents that offered higher recovery and sharper peaks in the TICs; therefore the analysis of samples extracted with these solvents were preferred as the accuracy in the results is higher.
Figure 39 Solvents comparison at Stage 3, 1ppm feeding SMX concentration

The GC-MS chromatograms obtained with DCM at extraction concentrations of 900, 4000 and 20,000 times, made possible the identification of peaks 1 and 2 in addition of three new peaks (Figure 40).

These new peaks identified as: 3) Sulfisomidine, 4) 4-Aminothiophenol and 5) Aniline were found at retention time of 14.5min, 8.16min and 5.5 min, with...
matching probability of 45.5%, 66.5% and 41.4%, presenting a M* of 214, 125 and 93, respectively (Figure 41, 42 and 43).

Besides the TPs identification at 1ppm feeding SMX concentration, Figure 40 shows the variation of peak 1, 2, 3, 4 and 5 at different biodegradation times: at stage 2 on day 79th (first day of spiking 1ppm concentration) and day 88th; at stage 3 on day 149th and 158th; for it is possible to observe in general that at day 158th the intensity of most peaks increased compared with day 149th, which could be explained due to the increase in the bacterial capacity to degrade SMX which causes the direct intensification in the production of TPs.
Figure 40 Comparison of different sample extraction concentrations using DCM at 1ppm initial SMX concentration
Figure 41 Peak 3, Sulfisomidine; identified at 1ppm feeding SMX concentration

Figure 42 Peak 4, 4-Aminothiophenol; identified at 1ppm feeding SMX concentration
Figure 43 Peak 5, Benzenamide or Aniline; identified at 1ppm feeding SMX concentration

4.8.1.4. 10 ppm initial SMX concentration

The analysis of TPs at 10ppm SMX feeding concentration was carried on at stage 3 using DCM, Acetone, EA, MeOH, 20% MeOH in Acetone and 20% MeOH in DCM as solvents. Figure 44 illustrates the TIC chromatograms obtained with each one of the extraction solvents showing that acetone provides higher intensities for all the detected peaks.
Figure 44 Solvents comparison at Stage 3, 10ppm feeding SMX concentration

Figure 45 displays the chromatograms of samples from day 189th and 191st (extracted with acetone at 20k concentration times) presenting very similar behavior, except for slight shifts on peaks 2, 3 and 5.

The high feeding SMX concentration (10ppm) in addition to the high sample extraction concentration (20k times) allowed the detection of other two possible degradation products.
Peak 6 found at 7.5min retention time was identified, with 74% matching probability, as 4-Pyrimidinamine 2,6-dimethyl-, having a M+ equal to 123 (Figure 46). In the other hand, peak 7 was detected at 10.56min retention time being recognized as Acetamide, N-(4-mercaptophenyl) with a matching probability of 67.1% and an M+125 (Figure 47).

Figure 45 Comparison of samples at 20k extraction concentrations using Acetone at 10ppm feeding SMX concentration
Figure 46 Peak 6, 4-Pyrimidinamine 2,6-dimethyl; identified at 10ppm feeding SMX concentration

Figure 47 Peak 7, Acetamide, N-(4-mercaptophenyl); identified at 10ppm feeding SMX concentration
4.8.1.5. 100 ppm feeding SMX concentration

The last feeding SMX concentration assessed in stage 3 was 100 ppm. For this study 20% MeOH in Acetone and 20% MeOH in DCM were used as extraction solvents due to the addition of MeOH to the solvent would enhance the disruption of H-bond in the SPE cartridge filters allowing to increase the recovery of the analytes present in the sample.

From the analysis of 100 ppm initial SMX concentration the presence of the 7 peaks previously detected was confirmed (Figure 48).

Figure 48 Solvents comparison at Stage 3, 100 ppm feeding SMX concentration
Figure 49 reflects the importance of obtaining a high extraction concentration in order to allow the accurate identification of the transformation products. In this figure is possible to notice that only extraction over 1k times concentrations offer chromatograms with some perceptible peaks; however just concentrations over 10k times will allow the recognition of the 7 TPs identified in this study.

Figure 49 Comparison of samples at different extraction concentrations using 20% MeOH in Acetone at 100ppm feeding SMX concentration
In order to evaluate the contribution of the UF membrane in the removal of SMX, a brief comparison of the occurrence of SMX on sample filtrated through the MF and UF membrane is presented in Figure 50.

This figure shows that the presence of TPs and their peaks’ intensity is very similar in permeates obtained by both filtration processes. However, the use of UF membrane will be reflected in the GC/MS chromatograms which presented lower rate of noise, due to the removal of organic matter that causes it.

Figure 50 Comparison of occurrence of SMX and TPs on samples filtrated through MF and UF membranes
Summarizing the occurrence and detection of transformation products generated along the AnMBR treatment, Figure 51 and 52 present a broad quantitative analysis of the 7 TPs identified; showing at feeding 10ppb SMX concentration the presence of only Benzene sulfonamide N-Butyl, considered at this point as the main transformation product.

As the feeding SMX concentration increases to 100ppb, the second peak corresponding to Sulfanilamide is detected with a ratio of 30% respect to the first peak area intensity. Further increase of the feeding SMX concentration to 1, 10 and 100ppm will lineally diminish the production of Benzene sulfonamide N-Butyl, but exponentially enhance the production of Sulfanilamide (in a ratio of 18.9, 99 and 520 times the peak area intensity of Sulfanilamide/Benzene sulfonamide N-Butyl, respectively). Therefore, it can be stated that at high SMX concentration in the influent wastewater, the main degradation pathway through the AnMBR treatment will be the sulfamethoxazole transformation into Sulfanilamide.

Sulfanilamide is expected to further degraded onto peak 4 (4-Aminothiophenol) and posteriorly to peak 5 (Aniline).

Finally, feeding SMX concentration from 1 to 100ppm would allow the identification of peak 3 corresponding to Sulfisomidine. The presence of Sulfisomidine with area intensity ratio respect to peak 1 Benzene sulfonamide N-Butyl ranges from 0.2 at 1 ppm, 7.7 at 10ppm and 99 at 100ppm. It is proposed that this compound was additionally degraded and transformed into peak 6 (4-Pyrimidinamine, 2,6-
dimethyl) and 7 (Acetamide, N-(4-mercaptophenyl)) which were detected at initial SMX concentration of 10ppm and 100 ppm in a fluctuating ratio around 0.02 for 4-Pyrimidinamine, 2,6-dimethyl- and 0.02-0.05 for Acetamide, N-(4-mercaptophenyl) with respect to Sulfsisomidine.

Figure 51 Analytes Occurrence in samples extracted with Acetone and concentrated 20k times
4.8.2. Kinetic Analysis

As previously mentioned, the kinetic analysis included the monitoring of SMX TPs, in order to study their appearance along the reaction and their further degradation.

The transformation products’ occurrence at different reaction times (from zero to 124h) of the AnMBR treatment is presented in Figure 53 and 54. Figure 54 clearly displays the occurrence of peaks 1, 2 and 4 at reaction time zero, which indicates the strong capacity of the anaerobic bacteria to rapidly breakdown the SMX molecule and transform it mainly into Sulfanilamide, 4-Aminothiophenol and Benzene sulfonamide N-Butyl. As the SMX concentration got reduced, the concentration of
these TPs increased inversely proportional in the first 9h of reaction. Posteriorly at
hour 12, the peaks 2, 4 and 1 decreased to around 2, 47 and 24% of the maximum
peak intensity reached at 9hr, respectively. This results indicate that Sulfanilamide
is the main transformation product formed in the SMX degradation process through
an AnMBR.

The 3rd peak, Sulfisomidine, presented the second higher peak intensity, after
Sulfanilamide, being detected after 6hr of reaction. Its peak intensity further
augmented until hour 48, when it exhibited a constant behavior that lasted until the
end of the kinetic analysis.

Finally peak 5 Aniline, was detected from time 9 until time 124 hours and
maintained, along this reaction time, at concentration similar to that from Benzene
sulfonamide N-Butyl.

Around 9.2 minute’s retention time, a high intensity peak was detected in the GC/MS
chromatogram (Figure 53). Despite its high intensity, the comparison of its mass
spectra with the NIST library standards identified it as 1-Dodecanol, with only 28%
matching probability. Due to the absence of this compound along the TPs’
occurrence assessment at continuous AnMBR operation, this peak was considered
as noise.
Figure 53 Kinetic analysis of SMX transformation products generated along the AnMBR treatment

Figure 54 Fluctuation of the TPs occurrence along the AnMBR process
4.9. Degradation Pathway

The results previously described helped to identify a possible degradation pathway for Sulfamethoxazole throughout an AnMBR treatment.

From Figure 51 is possible to observe that at low influent SMX concentration (<10ppb, typical SMX concentration in real wastewater), the main SMX degradation pathway was the transformation of sulfamethoxazole into Benzene sulfonamide N-Butyl (peak 1), which could have been generated either by the cleavage of the β- and ε-position of the SMX molecule and the posterior attachment of a 4 carbon chain to the NH-bonding; or by the cleavage of the β- position and the isoxazole ring opening.

As the feeding SMX concentration increased to 100ppb, 1ppm and 10ppm, the second peak corresponding to Sulfanilamide (peak 2) was broadly detected by the GC/MS system at around 1.3x10^6 CPS intensity. Sulfanilamide could have been originated by hydrolysis, which allowed the breakdown of the ε-position cleavage site of the SMX molecule.

When the feeding SMX concentration raised up to 100 ppm, the kinetic analysis showed that, at reaction time ≈zero, the AnMBR oxidation process resulted in a complex mixture of intermediates, in which three direct oxidation pathways were operating simultaneously: (a) formation of Benzene sulfonamide N-Butyl due to the breakdown of the isoxazole ring; (b) hydrolysis of the SMX’ ε-position cleavage producing Sulfanilamide, and (c) either the rupture of the δ-position cleavage
directly from the SMX molecule or the breakdown of the Sulfanilamide’ amine group, which yield to the formation of 4-Aminothiophenol (peak 4).

Further degradation of the 4-Aminothiophenol molecule was detected in the kinetic analysis after 9 hours of reaction. 4-Aminothiophenol could have been posteriorly desulfurized into Aniline (peak 5), which has been reported to be possible to degrade until its complete mineralization by means of biological reactions [19].

Another tentative degradation pathway could be outlined from the kinetic analysis, which after 6 hours of reaction showed the presence of Sulfisomidine (peak 3). This TPs could be a product of the SMX isoxazole ring breakdown and the further addition of an extra carbon and nitrogen atom into the ring. It is proposed that Sulfisomidine undergoes additional degradation by the hydrolysis of the δ-position cleavage of Sulfisomidine molecule yielding the formation of 4-Pyrimidinamine, 2,6-dimethyl- and Acetamide, N-(4-mercaptophenyl) (peak 6 and 7, respectively).

The metabolic pathway proposed for sulfamethoxazole degradation by the AnMBR treatment, based on the TPs identified, is presented in Figure 55.
Figure 55 Main reaction pathway proposed for Sulfamethoxazole degradation through the AnMBR treatment
Chapter 5

5. Conclusions

Based on the experiments performed and results achieved in this research, it is possible to conclude as follows:

Periodic mechanical cleaning of the membrane module, consisting in the enhancement of the crossflow in parallel with the mechanical shaking of the module, will (a) reduce the membrane fibers blockage, (b) allow constant permeate flux at low transmembranal pressure, and (c) extend the useful life time of the UF membrane module.

Optimal performance of the AnMBR treatment was obtained at 35°C temperature, 7±0.2 pH and -380-420MV of oxidation/reduction potential; offering high organic matter degradation rates that reached efficiencies of over 95% total COD removal.

From the AnMBR system, the biological treatment (based on anaerobic activated sludge) was the process that allowed the degradation of the major fraction COD and SMX presented in the influent synthetic wastewater. In the other hand, UF process did not offer any type of improvement in the removal of SMX; however this process contributed to increase the permeate quality due to its capacity to reject biopolymers (polysaccharides and proteins) present in the anaerobic sludge.
Under stable operational conditions the bioreactor maintained a VSS/TSS ratio of 0.85 indicating a stable sludge composition; with slight variations presented wherever the bioreactor was spiked with different concentrations of SMX.

Likewise, low SMX spiking concentration did not have a significant effect on the anaerobic treatment performance in terms of biogas production. The AnMBR presented a stable biogas composition with approximately 75-97% of methane, >5% carbon dioxide and 0-21% nitrogen. The methane production in terms of COD removal ranged from 45 to 130 mL CH4/g CODremoved during the entire experimentation. The fluctuation of the biogas composition and methane production is explained due to the increase in the influent methanol concentration.

This research work has shown that the treatment of sulfamethoxazole in a complex aqueous matrix by an anaerobic MBR led successfully to the relatively rapid degradation and/or transformation of SMX.

The assessment of the AnMBR in the removal of SMX indicates an initial efficiency of 82%, however after a period of acclimatization and bacterial adaptation to different SMX concentrations, the AnMBR system was able to reach 95% removal efficiency of this OMP compound. The kinetic analysis showed that after 24h of biological reaction, the AnMBR process was able to achieved over 94% removal of the initial SMX concentration, which indicates the relatively high capacity of the anaerobic bacteria in the degradation/transformation of SMX and compounds with similar structure.
As expected, the AnMBR treatment did not accomplish the complete mineralization of Sulfamethoxazole (due to its complex structure) leading to the formation of different TPS. Along the AnMBR process, seven TPs were identified and possible degradation pathways were proposed.

At low influent SMX concentrations (<10ppb, which is the SMX concentration in real wastewater) the main/only transformation product detected was (1) Benzene sulfonamide N-Butyl. As the influent SMX concentration increases, new degradation pathways were registered such as the formation of (2) Sulfanilamide, (3) Sulfisomidine and (4) 4-Aminothiophenol. Through the kinetic analysis it was possible to detect further degradation of these TPs and the presence of three new intermediate products after 9 hours of biological reaction.

Most of the detected TPs present a less complex molecular structure than SMX, which can be associates with a lower toxicity. Therefore, it can be concluded that for the treatment of wastewaters, with SMX in its composition or other compounds with similar structure, the proposed AnMBR treatment is a viable and sustainable alternative as it provides a relatively high quality permeate.
5.1. Future work

In this field further analysis of the transformation products toxicity is suggested in order to analyze the pros and cons of the AnMBR process.

The study and monitoring of changes in the bacterial community metabolism could help the understanding of SMX removal and improve the efficiency of the biological treatment to its further use in the treatment of wastewaters polluted with compounds with similar SMX structure.

Mass balance studies, which includes the quantification of each of the identified TPs, would help to clarify the contribution of each degradation process (biodegradation, adsorption, transformation, complete mineralization) that allows the removal of SMX and its TPs.

Advance oxidation processes such as ozone, photocatalysis and Fenton processes are proposed as tertiary treatments to achieve the complete mineralization of OMPs and their TPs.
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Degradation pathway of peak 1, Benzene sulfonamide, N-Butyl.

Degradation pathway of peaks 2, 4 and 5, Sulfanilamide, 4-Aminothiophenol and Aniline, respectively.
Degradation pathway of peaks 3, 6, and 7, Suldisomidine, 4-Pyrimidinamine 2,6-dimethyl and Acetamide, respectively.