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Amoxicillin Removal by Pre-Denitrification Membrane Bioreactor (A/O-MBR): Performance Evaluation, Degradation By-Products, and Antibiotic Resistant Bacteria

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ABSTRACT

Membrane bioreactors (MBR) are one of the treatment technologies with the potential to remove emerging compounds from wastewater. The present work evaluated the efficiency of an MBR pilot system in removing amoxicillin and by-products from synthetic wastewater using a continuous flow pre-denitrification MBR (A/O-MBR) pilot unit. The system operated in three phases: (1) synthetic wastewater and hydraulic retention time (HRT) of 40 h; (2) adding amoxicillin 100 µg L⁻¹ to the influent, and (3) varying flowrate to HRT of 20 h. Liquid chromatography coupled to high resolution mass spectrometry analysis confirmed the presence of five amoxicillin degradation by-products in the influent sample, indicating that the amoxicillin molecule had already hydrolysed in the feed tank and no by-products were quantified in A/O-MBR-treated effluents. The addition of amoxicillin did not affect chemical oxygen demand (COD) or dissolved organic carbon (DOC) removal efficiencies. Respirometry showed that amoxicillin level did not inhibit heterotrophic bacteria metabolism. The change in HRT reduced the DOC removal (from 84% to 66%) but did not influence COD (>94%) or total nitrogen (>72%). The amoxicillin and by-products total removal decreased from 80% to 54% with HRT change. Adsorption and biodegradation represented the largest removed fraction of the antibiotic in the A/O-MBR system (68%). Ecotoxicity assays showed *P. fluorescens* was more resistant and *E. coli* less resistant to amoxicillin residues at effluent sample matrix.

Keywords: Bacteria resistance; Degradation by-products; Emerging contaminants; Micropollutants; Removal mechanisms.

Introduction

Different chemical substances have been detected in aquatic ecosystems at concentrations lower than µg L⁻¹ or ng L⁻¹. Most are not eliminated or bio-transformed as they are persistent, bioactive and bioaccumulative (Das *et al.*, 2017). These substances are called micropollutants. Some micropollutants are known as emerging

contaminants because they are not currently covered by water quality regulations. They are considered potential threats to environmental ecosystems, human health, and safety because their medium- and long-term effects are unknown (Lafarré *et al.*, 2008).

Beek *et al.* (2016) showed the occurrence of human and veterinary pharmaceuticals in environmental matrices of 71 countries, indicating that this is a worldwide concern. As a consequence of increased consumption and low body uptake, these compounds have been detected in various aquatic environments. Many are not fully removed in conventional biological wastewater treatment plant, so that their continuous discharge leads to chronic exposure to these compounds for aquatic organisms (Virkutyte; Varma; Jegatheesan, 2010). This has led to calls for the use of advanced post-treatment technologies, such as membrane bioreactors (MBRs), to achieve sustainable protection of the environment (Westerhoff *et al.*, 2005; Grandclément *et al.*, 2017);.

MBR systems are an attractive technology with several advantages, including a high pollutant removal compared to conventional activated sludge treatment systems. MBR technology has been recognized as a key process for enabling water reuse in urban area in many countries (Le-Clech, 2010; Subtil *et al.*, 2013; Taheran *et al.*, 2016). It produces a high-quality effluent as a result of the membranes' capacity for retaining biomass for a long time. In addition, pollutants with molecular weights greater than the membrane's threshold are retained due to the sieving effect of the latter, thereby increasing contact time with microorganisms inside the MBR for their degradation (Lalit Goswami *et al.*, 2018).

There are different processes for removing pharmaceuticals via an MBR system. These include biodegradation, sorption on sludge, and physical retention by membranes (Besha *et al.*, 2017; Tambosi *et al.*, 2010). The physical-chemical characteristics of the pharmaceutical compounds and the MBR operating conditions determine the main mechanisms and efficiencies, but sometimes the results are not satisfactory for the removal of many recalcitrant micropollutants. In addition, degradation by-products may also be target compounds. These may be formed by natural degradation of the parent compound or by (bio)chemical or photochemical processes by which the parent compound was submitted.

Epidemiologies and pharmaceutical preferences vary from country to country, so micropollutant management must respond to these variations. The Brazilian experience of pharmaceutical consumption shows that amoxicillin is the most commonly used

penicillin antibiotic (Bertoldi et al., 2016). Amoxicillin is a β -lactam group penicillin antibiotic, with a broad spectrum of action against both Gram-positive and Gram-negative bacteria (Kaur et al., 2011). This class of antibiotics works by disrupting bacteria cell walls during reproduction (Baghapour et al., 2014). The vast number of prescriptions given for amoxicillin is related to its effectiveness in treating most pathogenic bacteria, its low cost, its few side effects, and its use in both human and veterinary medicine (Elizalde-Velázquez et al., 2016). When ingested, only a small fraction of the active substance is metabolized and about 80 to 90% is excreted unchanged, reaching wastewater treatment plants and water bodies (Bound et al., 2004; Na et al., 2019).

Amoxicillin in the environment may cause drug allergies, toxicological problems, and selection of antibiotic-resistant bacteria (Gavrilescu et al., 2015). For example, *Pseudomonas* is a ubiquitous Gram-negative bacterium with outer membrane permeability extremely restrict comparing to *E. coli*, which gives low efficiency for antibiotic permeation (Naghmouch et al., 2012; Nikaido, 1998;). However, the resistance modulatory activity of amoxicillin and other beta-lactamic antibiotics is more pronounced with Gram positive organisms such as *Bacillus subtilis* and *Staphylococcus aureus* (Gbedema et al., 2011).

These risks, direct or indirect, impact human health through passive and active antibiotic consumption, and are the basis for regulations and the definition of maximum residue limits for antibiotic usage. In the United States, the tolerance level for amoxicillin in milk and uncooked bovine tissue is 0.1 ppm (10 ng g⁻¹) (Elizalde-Velázquez et al., 2016). Currently, in Brazil, there are no regulations for controlling pharmaceutical residues in the environment.

Examining the fate and removal of amoxicillin and its by-products during wastewater treatment in an MBR is of major importance to avoid the discharge of the former into the environment. Bai et al. (2012) evaluated amoxicillin removal from effluents treated by MBR with various hydraulic residence times (HRT) (25, 20, 15, 10, and 6 hours) and observed a reduction in compound removal (49.1% to 26.3%) with a decrease in HRT. However, as shown by Bai et al. (2012), more than 50% of amoxicillin remained in the final effluent even with an HRT of 25 hours. The potential effects of antibiotic residues were not evaluated.

In this context, the aim of this study is to evaluate amoxicillin and related by-products removal mechanisms in a pre-denitrification membrane bioreactor (A/O-MBR)

operated under a long HRT (40 and 20 hours). Novel results from ecotoxicity tests with Gram-negative bacteria *Escherichia coli* and *Pseudomonas fluorescens* as well as Gram-positive ubiquitous *Bacillus subtilis* to assess potential bacterial resistance to amoxicillin disposal in the environment. These results support the importance of evaluating the fate, persistence, and safety of by-products after wastewater treatment.

Materials and Methods

A/O-MBR Descriptions and Operating Conditions

A pre-denitrification membrane bioreactor (A/O-MBR) pilot system was constructed according to **Figure 1**. The system was gravity fed, with the liquid level regulated by a floating mechanism. Sludge was continuously recirculated (3x permeate flowrate) by pumping it from an aerobic tank to an anoxic tank. During whole experiment, A/O-MBR system exhibited a constant mixed liquor suspended solids concentration at an infinite solids retention time (i.e., no sludge wasting). The anoxic zone volume was 18.5 L and aerobic zone 25.0 L.

Flat sheet ultrafiltration membrane modules (FS-UF) of Polyvinylidene fluoride (PVDF) with a mean pore size of 0.1 microns (SINAP[®], China) were used to separate the solids. The permeate was continuously withdrawn using a peristaltic pump (Tecno[®], DMC 300 L, Brazil) actively operated for 20-minute cycles, with 2 minutes of idle time between cycles.

Figure 1 – Flow chart of A/O-MBR process operating. S1: level floating sensor; S2: pressure sensor; B1, B2: peristaltic pumps; M: membrane module.

Synthetic wastewater was used to reduce the variability of the influent composition and allow the concentration of organic matter, nutrients, salts, and micronutrients, as well as amoxicillin and its by-products, to be monitored.

A/O-MBR operation was divided into three stages (**Table 1**). The first was a control phase; the second and third were for evaluating the levels of amoxicillin and by-products remaining after different HRTs. The amoxicillin concentration chosen was an order of magnitude higher than the values commonly found in the environment (Zucatto et al., 2010; Watkinson et al., 2007) to allow the detection of by-products by liquid chromatography coupled to high resolution mass spectrometry detection (LC-MS).

Table 1 - Characteristics of A/O-MBR operation phases

Phase	Amoxicillin concentration ($\mu\text{g L}^{-1}$)	Flowrate (L h^{-1})	HRT (h)	Operation time (days)
1	0	1	40	40
2	100	1	40	40
3	100	2	20	90

Monitoring Parameters

The reactor sludge was evaluated daily for total and volatile suspended solids (Method 2540, APHA 2016), temperature, dissolved oxygen levels (using an Orion Star A123, Thermo Scientific) and pH (potentiometric method); HRT and food/microorganism (F/M) ratio were calculated.

Transmembrane pressure (TMP) was monitored manometrically (with a vacuum meter) and a membrane flux (J_T ; $\text{L m}^{-2} \text{h}^{-1}$) was calculated (Equation 1) and corrected to a temperature of 20 °C ($J_{20^\circ\text{C}}$, Equation 2) (JUDD, 2011). Permeability (P) was determined using Equation 3.

$$J_T = \frac{Qp}{Am} \quad (1)$$

$$J_{20^\circ\text{C}} = \frac{J_T}{1,025^{T-20}} \quad (2)$$

$$P = \frac{J_{20^\circ\text{C}}}{TMP} \quad (3)$$

Where Q_p is the permeate flowrate ($L\ h^{-1}$), A_m is the membrane area (m^2), P is the permeability ($L\ h^{-1}\ m^{-2}\ bar^{-1}$), and TMP is the transmembrane pressure (bar).

Analytical Methods

During operation, influent and effluent samples were collected in duplicate, five times a week, to check the following treatment parameters: pH, apparent colour, turbidity, electrical conductivity, alkalinity, COD, dissolved organic carbon (DOC), total phosphorus (PT), nitrogen series (total nitrogen – NT; Kjeldahl nitrogen – NKT; NO_3^- ; NO_2^-), and the concentration of amoxicillin and by-products. All analyses were performed according to APHA (2016) methods. The concentration of amoxicillin and its by-products were evaluated using the solid phase extraction (SPE) method, followed by LC-MS analysis, as described below.

Analysis of amoxicillin and its by-products

SPE operation was optimized by using extractions of amoxicillin-spiked samples. The mass balance of the by-products' molar concentrations was used to estimate the best extraction method. Samples were extracted using HLB cartridges (Waters, Oasis[®], USA) with a peristaltic pump under $3.0\ mL\ min^{-1}$ flowrate. The sample volume breakthrough level was determined experimentally using a frontal chromatography approach (Bielicka-Daszkiewicz and Voelkel, 2009) in which synthetic effluent containing amoxicillin was continually applied to the SPE cartridge until some analyte eluted from the column. The volume of 200 mL was selected for influent samples and 300 mL for effluent samples.

After extraction, cartridges were refrigerated in the dark for up to seven days until elution and LC-MS analysis. The extracted samples were eluted with 12 mL acetonitrile (HPLC grade, Fisher Scientific, USA) and evaporated in an inert atmosphere to pre-concentrate the analytes (Gozlan et al., 2013). Samples were re-suspended in 1 mL acetonitrile prior to LC-MS analysis (pre-concentration factor 200x for influent and 300x for effluent).

Amoxicillin and its by-products were analysed using a liquid chromatograph coupled to a high-resolution mass spectrometer (Thermo Scientific Q-Exactive Orbitrap, USA) fitted with a pump and an autosampler (Dionex Ultimate 3000 RS, USA). The mass spectrometer was fitted with electrospray ionization (ESI) and operated in positive ion mode. The nitrogen sheath and auxiliary gas levels were set at 45 and 10 arbitrary units, respectively. The spray voltage was +3.5 kV and the ion source temperature was

300 °C. The full MS scan range was $m/z = 150$ to 1500 , with the resolution set at $17,500$. The product ion (MS2) determination was conducted using a mass resolution of $17,500$. The isolation window for the product ion was $2.0 m/z$ with a normalised collision energy of $35 eV$.

A C_{18} column (150×2.1 mm, particle size $3 \mu m$, Waters) was used for LC separation. The mobile phase (A) was methanol (LC-MS grade, Fisher Scientific) and the aqueous phase (B) was $18 M\Omega$ purity water containing 0.1% (v/v) formic acid (98% , Fisher Scientific). The eluent flowrate was $0.2 mL min^{-1}$ and a gradient elution mode was used: 99% B for 1 minute, 30% B over 12 minutes, and then 1% B over 1 minute. The gradient profile was maintained at 1% B for 6 minutes before returning to 99% B for 1 minute and being equilibrated for a further 9 minutes.

The calibration curve was in the range of $25-1,000 ng mL^{-1}$ of amoxicillin (in molar concentration, $68-2.7 mmol L^{-1}$) fitting the criteria of $\pm 20\%$ of the best fit line with a $1/X^2$ weighting. The MS was calibrated in positive ion mode using a Pierce LTQ Velos ESI Positive Ion Calibration Solution (Fisher Scientific).

An amoxicillin solution ($1 mmol L^{-1}$) was subjected to degradation under acidic and basic conditions and then analysed by LC-MS to obtain the retention time and precursor ions of by-products. Once the precursor ions were recognized, product ion data were determined for each by-product. Their concentrations in the samples were estimated from the molar concentration calibration curve for amoxicillin as standards were not available for these by-products.

Amoxicillin and By-Products Removal Mechanisms

A/O-MBR removal capacity was determined according to Equation 4 below, where the brackets represent compound molar concentration:

$$\begin{aligned} [affluent] = & [adsorbed\ in\ the\ sludge\ and\ available\ for\ biodegradation] + \\ & [fouled\ membrane\ performance] + [hydrolysis] + [photolysis] + \\ & [permeate] + [biodegraded\ amount + unquantified\ contributions] \end{aligned} \quad (4)$$

Concentrations of amoxicillin and by-products contained in the influent were determined by LC-MS as described above. The amount of amoxicillin adsorbed on sludge was determined by extracting $200 mL$ of mixed liquor into SPE cartridges and analysing them using LC-MS. To calculate the amount of compound adsorbed into the

sludge, the average Total Suspended Solids (TSS) along the experiment was considered to amoxicillin mass balance.

Membrane retention was evaluated by filtering an amoxicillin solution in two steps. In the first, the solution was filtered by a fouled membrane; in the second, the solution was filtered after the membrane was chemically cleaned. For each step, 500 mL of permeate was collected and analysed. The difference between the amoxicillin concentration of permeate from the fouled membrane and that from the cleaned filter indicates the portion retained on the biofilm.

Hydrolysis by-products were quantified after exposing 1000 mL of synthetic effluent (pH = 7) to 100 µg L⁻¹ of amoxicillin for 150 hours in a beaker under light. Samples were collected every 24 h and analysed using LC-MS to evaluate the development of by-products. The same experiment was conducted under ambient light conditions to evaluate photolysis.

The amount of biodegraded amoxicillin was not determined analytically; it was derived by calculating the difference between the initial molar concentrations and transformation/removal processes described above. The biodegradation contribution, along with volatilization and (bio)chemical transformation into other by-products not quantified by LC-MS methodology, and uncertainties regarding the analytical methods, were incorporated into the term “unquantified contributions” in Equation 4.

Effect of Amoxicillin on Heterotrophic Bacteria

Respirometry open system assays with intermittent aeration were performed to evaluate the effect of amoxicillin on the metabolism of A/O-MBR heterotrophic bacteria. A sodium acetate solution (100 mg L⁻¹ C₂H₃NaO₂) was used as the substrate. The dissolved oxygen (DO) concentration was measured to calculate the oxygen uptake rate (OUR; mg L⁻¹ h⁻¹) according to Equation 5.

$$OUR = \frac{DO_{max} - DO_{min}}{\Delta t} \quad (5)$$

where DO_{max} is the upper reference dissolved oxygen content (mg L⁻¹), DO_{min} is lower dissolved oxygen reference (mg L⁻¹), and Δt is time variation (h).

The endogenous rate is identified when the OUR becomes constant. The acetate substrate was added and DO was monitored until this value was reached. The amoxicillin effect in the biomass was evaluated from 1 µg L⁻¹ to 100 mg L⁻¹ by

comparing the OUR with a reference system containing only sodium acetate substrate (Basnyat, 2011).

Ecotoxicity Bacteria Resistance Assay

Bacteria ecotoxicity tests were carried out with influent and effluent samples from the A/O-MBR during phases 2 and 3. Three target bacteria species were tested for antibiotic performance: *Escherichia coli*, *Pseudomonas fluorescens*, and *Bacillus subtilis*. Assays began with preparation of the bacteria using axenic cultures grown for 72 h, separating them from the medium and re-suspending them in a phosphate-buffered saline (PBS) solution. The bacteria were then exposed to influent and effluent samples and to positive controls (50 mg L⁻¹ K₂Cr₂O₇) and negative controls (ISO water, composed of 2 mmol L⁻¹ of CaCl₂, 0.5 mmol L⁻¹ of MgSO₄, 0.8 mmol L⁻¹ of NaHCO₃, and 0.08 mmol L⁻¹ of KCl) for 48 h.

Samples were placed in a 96-well microplate and read on a spectrophotometer (BioTek Instruments[®], ELx808). The plates were incubated for 24 hours at 25 °C. After the incubation period, 10 µL of nutrient solution and 10 µL of ISO water were added to half the samples; 10 µL of nutrient solution and 10 µL of resazurin dye (0.2 mg mL⁻¹) were added to the other half. The addition of a nutrient solution prevents the inhibition of microorganism growth due to a lack of nutrients, and resazurin dye is an indicator of aerobic bacteria respiration. The colour of the solution changes from blue to pink in the presence of resorufin (González-Pinzón et al., 2012).

Spectrophotometric readings were carried out at three wavelengths: at 405 nm without the dye (related to bacteria growth), 570 nm associated with resorufin (pink), and 630 nm with resazurin fluorescence (blue). Sample results were later compared to the positive and negative control assays to evaluate the influence of bacterial growth and the oxidation/reduction state of the indicator dye.

Statistical Analysis

Statistical analysis was performed using Origin Pro version 8.0. Concentrations found for the studied parameters at the different A/O-MBR phases were compared through variance analysis (one-way ANOVA) with significance level of 0.05. Significant differences were tested by Tukey's post-hoc multiple test. To investigate antibiotic resistance, 15 samples were randomly collected and analysed for each A/O-MBR phase.

Results and Discussion

A/O-MBR Operational Condition and Performance

An effective flowrate control (1.0 L h^{-1} in phase 1 and 2, and 2.0 L h^{-1} in phase 3), resulted in two different HRT conditions (40 h and 20 h, respectively). During the experiment, the recirculation flowrate (Q_r) maintained was at least three times higher than the permeate flowrate (see supplementary material).

Temperature, pH, and DO were adequate to maintain the biomass and assure an anoxic condition for denitrification (Zoppas et al., 2016). The A/O MBR performed as expected for removing organic matter and nitrogen when operated with a formal anoxic pre-denitrification zone. In all phases, the average COD removal was over 94% while total nitrogen removal reached up to 79% in phase 2 and remained high, at 77%, in phase 3 (see supplementary material).

Effect of Amoxicillin on Heterotrophic Bacteria

The kinetic behaviour of heterotrophic bacteria exposed to different amoxicillin concentrations ($1\text{--}100 \text{ mg L}^{-1}$) is shown in the respirograms in **Figure 2**. They were normalised to the initial endogenous OUR of the control system so that it would be possible to observe the variation in the maximum OUR in different scenarios.

Figure 2 - Respirograms of heterotrophic bacteria from A/O-MBR system exposed to different amoxicillin concentrations.

Respirograms presented two different behaviours related to the amoxicillin concentration ranges. At concentrations up to $100 \mu\text{g L}^{-1}$, the microbial respiration rate rapidly increased the OUR and reached a single maximum value (above $40 \text{ mg L}^{-1}\text{h}^{-1}$) before returning to the endogenous condition. At concentrations above 1 mg L^{-1} of amoxicillin, the OUR also increased when sodium acetate was added, remained at the maximum value for some time, and then gradually returned to the endogenous value.

Concentrations in the $1\text{--}100 \mu\text{g L}^{-1}$ range achieved a higher OUR value than the control system, indicating that low levels of amoxicillin do not inhibit bacterial respiration. However, the maximum OUR for concentrations in the mg L^{-1} range were lower than the control and the reduction in the maximum oxygen consumption rate indicates the effects of toxicity caused by chemicals in the biomass. The highest tested concentration (100 mg L^{-1}) had the lowest OUR (about $22 \text{ mg L}^{-1} \text{ h}^{-1}$) and remained at this maximum value for 20 minutes. This indicates that toxicity has some effect on the properties of sludge and respiration rate due to the presence of the micropollutant (Besha et al., 2017).

These results confirm that adding amoxicillin at a concentration of $100 \mu\text{g L}^{-1}$ to the influent would not affect the performance of heterotrophic bacteria to aerobically biodegrade organic matter, resulting in COD removal efficiency above 94% in all A/O-MBR phases.

Amoxicillin By-Products

The limit of detection (LOD) and limit of quantification (LOQ) values for amoxicillin were 2.0 and $6.6 \mu\text{g L}^{-1}$ (5.5 and 18 nmol L^{-1}), respectively, for the calibration curve in the aqueous phase and 4.0 and $12.0 \mu\text{g L}^{-1}$ (10 and 32 nmol L^{-1}) for the same curve in the effluent, indicating a decrease in sensitivity due to the sample matrix effect. Thus, calibration curves were prepared for synthetic wastewater to guarantee the analytical reliability of the results.

Figure 3 shows the results of by-product hydrolysis analysis, as determined by LC-MS.

The kinetic results of amoxicillin hydrolysis showed a linear time increase in by-product concentration at around 150 hours, with a maximum amoxicillin conversion rate of 45%. For times below 50 h (the HRT of the A/O-MBR system), less than 5% of amoxicillin was hydrolysed into by-products. **Table 2** shows the mean concentrations of

the by-products found in influent and effluent samples from phases 2 and 3. The amoxicillin by-products were only quantified in the A/O-MBR influent sample, indicating that the amoxicillin molecule had already hydrolysed in the feed tank. Gozlan et al. (2013) state that the presence of bivalent ions enhances amoxicillin hydrolysis. They found a 16.0% decrease in amoxicillin concentration at a pH of 7 with the addition of 25 mg L⁻¹ Mg²⁺ and 80 mg L⁻¹ Ca²⁺ to 100 µg L⁻¹ amoxicillin after 36 hours. The concentration of bivalent ions in this synthetic effluent was lower reflecting the minor influence that this process has on by-product formation. No by-products were quantified in the A/O-MBR-treated effluents, possibly due to concentrations being above the analytical LOQ. Gozlan et al. (2013) detected amoxicillin penicilloic acid (0.15 µg L⁻¹) and *diketopiperazine* amoxicillin (0.5 µg L⁻¹) degradation products in the secondary effluent. Amoxicillin penicilloic acid and pyrazinone were also detected but not quantified; however, no information was given about amoxicillin levels in the influent.

Figure 3 - Chromatogram of amoxicillin and by-products. (a) Total chromatogram; (b) Amoxicillin penicilloic acid; (c) Diketopiperazine amoxicillin; (d) Pyrazinone.

Table 2 - Amoxicillin by-products mass concentration in affluent and effluent A/O-MBR at operation Phases 2 and 3.

Degradation products	Concentration (µg L ⁻¹)			MS transition (m/z)	Retention time (min)
	Effluent	Effluent			
		Phase 2	Phase 3		
Amoxicillin penilloic acid I	0.74 ± 0.45	n.d.	n.d.	189.0693	5.0
Amoxicillin penilloic acid II	0.27 ± 0.17	n.d.	n.d.	189.0693	5.5
Diketopiperazine amoxicillin	0.46 ± 0.07	n.d.	n.d.	160.0428	7.1
Pyrazinone	0.19 ± 0.03	n.d.	n.d.	145.0760	7.7

*n.d. = not detected, below the limit of quantification.

Amoxicillin can be transformed by two pathways, as described in **Figure 4**: (1) hydrolysis of β -lactam ring cleavages, which produces amoxicillin penicilloic acid; and (2) a nucleophilic attack of the amino group of benzyl carbonyl, forming phenol hydroxypyrazine. In addition, amoxicillin penicilloic acid may also exhibit two degradation routes: (1) the decarboxylation of the free carboxylic acid, forming the stereoisomers of amoxicillin penicilloic acid I and II; and (2) the formation of a new stable ring resulting in diketopiperazine amoxicillin (Nägele and Moritz, 2005). Other products can still be formed as a result of nucleophilic attack by sample matrix components, generating numerous amoxicillin degradation products (Deschamps et al., 2012).

Figure 4: Suggested degradation pathway of amoxicillin in aqueous medium.

381

382 A/O-MBR Amoxicillin Removal Mechanism

383 **Figure 5** shows the combined concentration of amoxicillin and by-products in
 384 the synthetic effluent before and after A/O-MBR treatment. Statistical analysis showed
 385 that compound removal was different for each evaluated phase.

386

387

Figure 5- Amoxicillin concentration and removal in A/O-MBR operation Phases 2 and 3. Error bar stands for the standard error of overall antibiotic concentrations in each phase.

The amoxicillin removal average in phase 2 (HRT = 40 h) was approximately 80%; in phase 3 (HRT = 20 hours) it was 54%. A relationship was observed between amoxicillin removal and HRT with use of A/O-MBR. This proportionality was also reported by Tambosi et al. (2010), for the antibiotics roxithromycin, sulfamethoxazole, and trimethoprim, presenting 57%, 55%, and 86% removal, respectively. These results

were achieved by treatment with the MBR operating with an HRT of 9 h. Treatment at an HRT of 13 hours showed a removal rate of 81%, 64%, and 94%, respectively, demonstrating a tendency for higher removals with the enhanced HRT.

Prasertkulsak et al. (2016) investigated the degradation of 11 pharmaceutical compounds in the MBR system operating with an HRT of 3 hours and a Total Suspended Solids (TSS) of 13 g L⁻¹, i.e. a low HRT and a high biomass concentration, and obtained high removal percentages of the antibiotics sulfamethoxazole (78%) and trimethoprim (80%). While this tendency was not seen in this study, the reduction of HRT in phase 3 and the consequent increase in TSS enhanced the amoxicillin concentration in the effluent and reinforced the necessity of improving contact between biomass and the compound.

Labinghisa and Rollon (2014) used an MBR to evaluate β -lactam ampicillin removal and observed higher removal under nitrification conditions (87.6%) than in the absence of the nitrification (78.1%). Since amoxicillin is in the same group, nitrification in the aerobic zone might have contributed to its biodegradation.

Figure 6 shows the percentages related to total amoxicillin by-products that were available in each process and involved in treatment by the A/O-MBR system; it is considered a closed system. Hydrolysis is responsible for only 2% of the decrease in amoxicillin concentration conversion. Xu et al. (2011) also investigated amoxicillin hydrolysis and found that it is responsible for a 5–18% loss in the compound in solutions with different DOC concentrations.

Figure 6 – Amoxicillin distribution at A/O-MBR system.

Most of the amoxicillin entering the system (more than 68%) is available in the sludge through adsorption or biodegradation, which indicates biomass action as the main removal mechanism for amoxicillin by A/O-MBR. This explains the proportional decrease in removal as HRT increases. Although it has a low solubility in water, the amoxicillin molecule is polar and, according to Besha et al. (2017) and Fan et al. (2014), tends to favour removal by biodegradation. The efficiency of pollutant biodegradation based on MBR biomass depends on several system operating variables, such as HRT, solids retention time (SRT), temperature, pH, biomass concentration, and compound physical chemical characteristics (Boonnorat et al., 2016). HRT influences the bacterial community in the bioreactor and, consequently, the degradation of micropollutants and the wastewater treatment (Win et al., 2016). Moreover, a high partition coefficient ($\log K_{oc} = 2.94$) indicates that amoxicillin affinity in the MBR biota is higher than in the aqueous phase, because it is more available for adsorption on the biomass and thus for subsequent biodegradation. Non-quantified contributions (18%) include biodegraded amoxicillin, analytical errors, volatilization, and other mechanisms not evaluated in this study.

Analysis of Bacterial Resistance

Figure 7 shows the growth in *Escherichia coli*, *Bacillus subtilis*, and *Pseudomonas fluorescens* cells in ISO water, deionized water, and A/O-MBR samples (influent and effluent). Although the cell growth seems similar in the ISO and deionized water, statistical analysis indicated that the bacterial growth was significantly different ($p = 0.017$), due to the absence of nutrients in pure water. This proved the accuracy of the test.

Figure 7 - Bacterial cell growth in ISO water, deionized water, A/O-MBR influent and effluent ($n = 20$ replicates). Error bar stands for the standard error of overall cells growth in each phase.

Antibiotic concentration interfered with *E. coli* growth, with a 47% inhibition in influent samples. The higher growth in the effluent samples from both phases indicates that the residual levels of the antibiotics did not affect target metabolism. Tests performed in the 1980s showed the sensitivity of *E. coli* to $10 \mu\text{g L}^{-1}$ of amoxicillin (Rolinson, 1980); however, the selective pressures caused by the exposure of the bacteria to antibacterial compounds over the years resulted in it becoming more resistant to antibiotics (Jiménez-belenguer et al., 2016; Kibret and Abera, 2011; Reinthaler et al., 2003). Thus, the non-inhibition of growth in the permeate samples indicated that it is resistant to concentrations of at least $50 \mu\text{g L}^{-1}$ (phase 3).

Bacillus subtilis also presented inhibited growth with the influent sample comparing to ISO water. However, its level of inhibition (27%) was lower than that for *E. coli*, indicating that the former species shows some antibiotic resistance. This was reported by Luo and Helmann (2012), who evaluated the extra cytoplasmic function of *B. subtilis* with various antibiotics including amoxicillin.

Pseudomonas fluorescens had a statistically similar increase in the control samples ($p = 0.2$), which could be related to resistance to amoxicillin, even at concentrations of $100 \mu\text{g L}^{-1}$. *P. fluorescens* was considered to be resistant to amoxicillin and tetracycline and to have intermediate resistance to cephradine and norfloxacin in antibiogram tests performed by Zhou et al. (2015).

Within effluent samples, the behaviour of *B. subtilis* and *P. fluorescens* was similar, with a growth increase in phase 3. These results are related to the presence of favourable conditions for the growth of the target organisms, particularly in terms of the availability of regarding carbon sources. Araújo et al., (2006) and Perotti et al. (2005) demonstrated that the presence of organic matter in the environment favours the survival and growth of *B. subtilis* and *P. fluorescens*.

The A/O-MBR system removed a large amount of organic matter such that effluent quality, particularly during phase 2, caused bacterial cells to grow more slowly due to low nutrient availability (Lanna Filho et al., 2010). With a reduction in HRT, the higher levels of amoxicillin and DOC were detected in the permeate. Thus, the elevation of DOC concentration in the effluent (from 5.5 to 26.0 mg L⁻¹) allowed higher bacterial growth in phase 3 effluent samples.

Although *B. subtilis* concentrations grew in the phase 3 samples, the profile indicated a slight inhibition of its metabolism (4%) relative to deionized water, possibly due to residual amoxicillin concentration (around 50 µg L⁻¹). In general, the results of bacterial growth indicate different resistance levels for each bacterium studied, indicating that *P. fluorescens* is more resistant and *E. coli* is the less resistant to the effluent sample matrix.

Conclusion

This study showed HRT has an important role in the removal of amoxicillin and by-products through an MBR system. Amoxicillin removal increased with an increase in A/O-MBR HRT, with a large fraction of amoxicillin adsorbed in the sludge and available for biodegradation. This suggests that main total amoxicillin removal mechanism in the system is biomass removal. Hydrolysis contributed less than 5% to the removal efficiency and was quantified using five different by-products in the influent sample. This finding indicates that when MBR technology is considered for removing non-conventional pollutants, such as amoxicillin, operating them at higher-than-typical HRTs (e.g. 4–6 hours for an MBR) should be considered. Our analysis of the performance of the A/O-MBR system during phase 2 showed a lower inhibition of the metabolic rates of the target bacterial organisms, emphasizing the effect of the sample matrix on ecotoxicity assays.

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