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Mechanistic and Ecotoxicological Studies of Amoxicillin Removal Through Anaerobic Degradation Systems

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ABSTRACT

Many studies have been conducted on the evaluation and monitoring of micropollutants and by-products in wastewater treatment plants. Considering the increase in the production and consumption of emerging contaminants, such as drugs, personal care products, and plasticisers, it is necessary to conduct studies that support the elaboration of laws and regulations that promote the environmentally sustainable use of sludge and effluents. In this work, the biological degradation of amoxicillin was studied under two anaerobic conditions: i) using a 6 L reactor operated under semi-continuous flow; and ii) a batch system with 100 mL sealed glass syringes. According to the statistical analysis, amoxicillin was completely removed from the systems, but biogas production inhibition was observed ($p < 0.05$). Liquid chromatography-high-resolution mass spectrometry analysis identified amoxicillin penicilloic acid, amoxilloic acid, amoxicillin diketopiperazine and phenol hydroxypyrazine as by-products under anaerobic conditions. Ecotoxicity tests on effluent treated under the batch conditions showed that the addition of higher amounts of amoxicillin inhibited the target species *Aliivibrio fischeri* and *Raphidocelis subcapitata*, causing functional decreases of 28.5% and 22.2% when the antibiotic concentration was 2,500 $\mu\text{g L}^{-1}$. *A. fischeri* was the most sensitive organism to effluent treated under semi-continuous flow conditions; a continuous reduction in bioluminescence of up to 88.8% was observed after 39 days of feeding, which was associated with by-products accumulation due to unbalanced conditions during anaerobic digestion. Changes in the physico-chemical characteristics of the effluent

caused the accumulation and removal of AMX-DKP IV and modified the toxicity to *Lactuca sativa* and *R. subcapitata*.

Keywords: Pharmaceuticals; Anaerobic Biodegradation; LC-HRMS; *Aliivibrio fischeri*; *Lactuca sativa*; *Raphidocelis subcapitata*

1 INTRODUCTION

Urban lifestyles, characterised by the high consumption of drugs (such as analgesics, antibiotics, lipid regulators, anti-inflammatories, and synthetic hormones), personal care products, surfactants, and plasticisers, render domestic wastewater one of the main routes for the transfer of emerging contaminants to the environment. The main characteristics of these pollutants are their persistence and non-biodegradability (refractory substances). These compounds have been the subject of many monitoring studies since they were first detected in treated water, effluent, sediments, surface water, and even underground water, indicating the low removal efficiency of conventional wastewater treatment plants (WWTPs) for these compounds (Petrović et al., 2014; Salgado et al., 2010; Simazaki et al., 2015).

One class of micropollutants, i.e., antibiotics, has received special attention from the scientific community as they may disturb the physiological functions of WWTP microbiota and agricultural soils, and risk promoting the development of resistant microorganisms (Aydin, 2015). Among the antibiotics, amoxicillin is widely consumed worldwide. It is a semi-synthetic penicillin class (β -lactam antibiotics) drug used in the treatment and prevention of bacterial respiratory, gastrointestinal, urinary, and skin infections in humans and animals (Bush, 2003; Rao et al., 2011). In the excretion of amoxicillin, over 80% of the drug is eliminated as the parent compound (Hirsch, 1999). Although its consumption is widespread, little information about the levels of amoxicillin found in waste and surface water has been published in the literature. This can be explained by the instability of the β -lactam ring in amoxicillin, which readily undergoes hydrolysis after excretion, producing by-products that are not always evaluated in the monitoring of this compound.

Usually, anaerobic digesters are widely applied in the treatment of solid wastes, including sludge from WWTPs, however, the application becomes advantageous in the treatment of sanitary sewage specially in countries of tropical climate when using

UASB type reactors. This kind of technology not only generates biogas that can be used as energy supply but also allows the reduction of the effluent treatment plant total size, the energy consumption and sludge production. In this sense, anaerobic degradation technologies are a cost-effective alternative for wastewater treatment. However, the process may be affected by the presence of toxic compounds, such as antibiotics, reducing the production of biogas or modify the production and conversion of volatile fatty acids (Fountoulakis et al., 2008; Meng et al., 2015), which would then cause the accumulation of several intermediates that inhibit the microbial communities responsible for pollutant degradation.

It should also be noted that international legislation on the disposal of pollutants in treated effluent provides maximum concentration limits for few potential pollutants compounds. Furthermore, there is no concern regarding the quantification of by-products derived from physico-chemical and biological processes that occur before, during, or after wastewater treatment, which can be as toxic as the original drug. Amoxicillin has only recently been included in the European Union Watch List as a target compound for monitoring its risk to the aquatic environment (Loos et al., 2018). However, the inclusion of amoxicillin and other compounds that are produced and consumed by the population is limited when conventional analytical methods (such as liquid chromatography) are followed. Thus, further information regarding the effluent treatment efficiency and composition is required.

The novelty of this work lies in the evaluation of amoxicillin degradation under anaerobic conditions and by-products formation and accumulation, whose estimated concentration were confronted to ecotoxicity analyzes performed with species from three different trophic levels. Usually studies concerning on removal of pollutants do not give attention to the formation of by-products (Çelebi and Sponza, 2012; Meng et al., 2015; Rizzo et al., 2009) which can be responsible for more pronounced toxic effects than those of the parent compound. Within this, ecotoxicological assays come as an advantageous technique in order to monitor the performance of WWTPs, providing information about trouble-shooting in case of failures in biological treatment and evaluating environmental risk regarding treated effluent discharge or even in subsequent/ tertiary treatments.

2 MATERIAL AND METHODS

2.1 Source of Seed Sludge

Mesophilic seed sludge was collected in plastic containers from a mesophilic anaerobic digestion treatment plant (Dunns Wood Road, Wardpark South Industrial Estate, Cumbernauld, Scotland). The facility is jointly operated by Paragon-e and Shanks Waste Management Ltd., and treats animal by-products, general food waste, and waste produced by the food processing industry in an anaerobic digester (AD).

2.2 Synthetic Wastewater

Synthetic wastewater created following Souza and Foresti (2013), Torres (1992) and Van Loosdrecht et al. (2016) was used in the experiments. A synthetic matrix was selected to control the physico-chemical characteristics throughout the test period with a chemical composition similar to that of domestic wastewater, ensuring the presence of macro- and micronutrients for the anaerobic microbiota. Table S1 presented in the Supplementary Material shows the typical composition of synthetic wastewater used in this work. All reagents used were analytical grade from Aldrich, Acros or Fisher Scientific brands.

2.3 Batch Tests for Determining the Anaerobic Degradability

Tests were conducted to determine the anaerobic biodegradation of amoxicillin (Acros, 96%) according to ISO 11734 (1995) with 100 mL glass syringes filled with synthetic effluent containing the target compound AMX. Each syringe was filled with 120 mg of organic carbon (synthetic wastewater) and 500 mg of volatile solids (inoculum) to produce a final ratio of 0.2. For that, synthetic wastewater and AMX concentrations chosen in this study (250, 2,500, and 25,000 $\mu\text{g L}^{-1}$) were prepared 10 times concentrated (i.e., final concentrations of AMX ranged from 2,500 to 250,000 $\mu\text{g L}^{-1}$). These concentrations were chosen based on previous ecotoxicity results of Di Nica et al. (2017) and Magdaleno et al. (2015). Nitrogen was later bubbled into the system for 30 s to create anaerobic conditions. The syringes were then sealed with a plunger (greased with Vaseline to facilitate movement and prevent gas leakage) and incubated at $37 \pm 1^\circ\text{C}$ for 28 days. The syringes were mixed to homogenise the contents before each measurement.

Two control experiments were conducted: one containing only the inoculum (blank), and the other containing polyethylene glycol (7 g L^{-1}) as a reference

substrate. This compound is easily biodegradable, and the test is considered valid if the reference is degraded over 60%. The biodegradability was estimated by means of Equation 1:

$$\text{Biodegradability (\%)} = \frac{(V_t - V_c) \times 0.039}{\text{mmol C added}} \times 100 \quad (1)$$

where V_t is the volume of biogas measured at day t and V_c is the measured volume of control at day t and 0.039 is the conversion factor obtained from ideal gas equation.

2.4 Anaerobic Digester (BioStat)

The AD was operated at $37.0 \pm 0.1^\circ\text{C}$ throughout the study period under semi-continuous flow. The system was filled with the inoculum (1.05 L) and synthetic wastewater (4.95 L) to obtain a final volatile solids content of 4 g L^{-1} . After 18 days, the reactor was fed with a hydraulic retention time (HRT) of six days (start-up/ first phase, data not shown). After the stabilisation phase (22 days), the reactor was fed with synthetic wastewater at a HRT of three days, followed by the control/ second phase, which was maintained for 32 days prior to the addition of amoxicillin (third phase). An amoxicillin concentration of 1 mg L^{-1} was selected to improve the detectability of by-products in LC-HRMS analysis and allows correlation with ecotoxicological studies. It must be stated that interpretation of results are quite different from that expected in real WWTP considering that usual concentrations of amoxicillin in wastewater are in the order of $10 \text{ } \mu\text{g L}^{-1}$ (Mutyar and Mittal, 2013; Verlicchi et al., 2012). On the 58th feeding day, pH dropped to 6.5 and NaHCO_3 was used to increase the value to approximately 7.2.

The physico-chemical variables of pH, alkalinity, total ammonia nitrogen (TAN), chemical oxygendemand (COD), total and volatile solids (TS and VS), and biogas production were monitored during the experiments following the Standard Methods for the Examination of Water and Wastewater (APHA, 2012).

2.5 Ecotoxicological Tests

For these assays, batch syringe samples were diluted at a ratio of 1:10 prior to analysis while the samples from biostat were used as collected, without any dilution.

Three species from different trophic levels were select to evaluate ecotoxicity of wastewater before and after anaerobic treatment. Seed Germination test, *Aliivibrio fischeri* and *Raphidocelis subcapitata* were chosen due its advantages in terms of simplicity, cost effectively, reproducibility and low time to obtain the results. *A. fischeri* is a very sensitivity specie that has been widely used for wastewater monitoring and can be applied for a large number of compounds, including amoxicillin (Çelebi and Sponza, 2012; Di Nica et al., 2017).

2.5.1 Seed Germination and Root Growth Method

The analysis was performed according to USEPA (1996). For each test sample, a circle of blotting paper (150 g m⁻²) was placed into the lids of 90 mm Petri dishes with 2 mL of the sample (analysis was conducted in triplicate). Between 10 and 12 seeds of lettuce (*Lactuca sativa*), which is commonly cultivated worldwide, were placed in a line three-quarters from the bottom of the blotting paper in each Petri dish. A positive control sample with reconstituted water (294.0 mg L⁻¹ CaCl₂.2H₂O, 122.8 mg L⁻¹ MgSO₄.7H₂O, 64.8 mg L⁻¹ NaHCO₃ and 5.8 mg L⁻¹ KCl) (OECD, 2004) was tested in duplicate. Each Petri dish was wrapped in cling film and placed in a semi-vertical position in a 25 ± 1°C incubator. After four days of incubation, the Petri dishes were examined, and the number and individual root length of germinated seeds were measured using a vernier calliper (resolution of 0.05 mm).

2.5.2 Marine bacteria *Aliivibrio fischeri*

Acute toxicity tests were conducted using *Aliivibrio fischeri* according to ISO 11348-3 (2007). Prior to conducting the ecotoxicological assays, samples were diluted (1:10) with NaCl solution to obtain a 2% (w/v)-salinity medium. The reference substance (negative control), potassium dichromate, was prepared by adding 0.653 g of K₂Cr₂O₇ to 100 mL of deionised water and, from that, a 1:100 dilution was obtained using a 2% (w/v) NaCl solution. Reconstituted water was used as the positive control. Freeze-dried *A. fischeri* was activated by rehydration using the “BioFixLumi Medium” (both from Macherey-Nagel GmbH & Co. KG). During the test, vials were put into an incubator at 15 ± 1°C. Approximately 100 µL of the bacterial suspension and 900 µL of each sample were added to the vials, and the samples

were analysed after 30 min (I_{30}) using a portable luminometer (BioFix Lumi-10, Macherey-Nagel GmbH & Co. KG). The gamma value (Γ , measured as a loss of bioluminescence in relation to positive control) was calculated by means of Equation 2:

$$\Gamma = \frac{I_{30}}{(I_0 f_{kt})} \times 100 \quad (2)$$

Where f_{kt} is the correction ratio for positive control and I_{30}/I_0 is the bioluminescence ratio measured at 30 and 0 min.

2.5.3 Algae *Raphidocelis subcapitata*

Algae assays were conducted following OECD (2011). A culture containing approximately 10^7 cells mL^{-1} of *Raphidocelis subcapitata* (from Culture Collection of Algae and Protozoa - CCAP 278/4, Scotland, U.K.) was prepared in Jaworski medium and used after three days by 1:10 dilution in the samples to obtain a final concentration of 10^6 cells mL^{-1} . During the tests (conducted in quadruplicate), samples were constantly shaken at 120 rpm to ensure that the algae remained in suspension and facilitate CO_2 transfer. The temperature was set at $21 \pm 1^\circ\text{C}$, and the light intensity was 3500 ± 350 lux. Measurements were taken after 96 h and the cells were counted using a microscope (Brunel Microscopes, Model N-300M) and a cytometer (Celeromics).

2.6 LC-HRMS Analysis

2.6.1 LC-HRMS Conditions

The presence of amoxicillin and its by-products were analyzed using liquid chromatography coupled to a high resolution mass spectrometer (LC-HRMS) Thermo Scientific Q-Exactive Orbitrap fitted with a Dionex Ultimate 3000 RS Pump, Dionex Ultimate 3000 RS autosampler (temperature controlled at 10°C) and Dionex Ultimate 3000 RS column compartment (temperature controlled at 30°C) (all from Thermo Fisher Scientific, Hemel Hempstead, England). The mass spectrometer was fitted with an electrospray ion source (ESI) operated in positive ion mode. The nitrogen sheath and auxiliary gas were set at 45 and 10 arbitrary units, respectively. The spray voltage was +3.5 kV and the ion source temperature 300°C . The full MS

experiment scan range was m/z 150 to 1500, with the resolution set at 17500. The product ion experiment (MS^2) was conducted using a mass resolution of 17500. The isolation window for the product ion experiment was 2.0 m/z with a normalised collision energy (NCE) of 35 eV. A Waters (Elstree, UK) C_{18} column 150 × 2.1 mm, particle size 3 μm was used for chromatographic separation.

The organic solvent was methanol (A) (Optima LCMS grade, Fisher Scientific) and the aqueous (B) was deionized water containing 0.1% formic acid (98%, Fisher Scientific). A gradient elution technique was used: the initial conditions were 99% B for 1 min, dropping to 30% B over 12 min then 1% B over 1 min. The gradient was maintained at 1% B for 6 min before returning to 99% B over 1 min and equilibrating for a further 9 min. The eluent flow rate was 0.2 mL min⁻¹.

The software to operate the LC-HRMS system was Tracefinder and for MS interpretation Xcalibur. Prior to analysis the instrument was calibrated in positive ion mode using Pierce LTQ Velos ESI Positive ion calibration solution from Fisher Scientific (Loughborough, England).

A 17-point calibration line for amoxicillin (Sigma Aldrich) was prepared in the range 0.01 – 2,500 ng mL⁻¹. The calibration points fitting the criteria of $\pm 20\%$ of the best fit line with a $1/X^2$ weighting was in the range 25 – 1,000 ng mL⁻¹.

2.6.2 Determination of unknown by-products

An amoxicillin solution was subjected to degradation under acidic conditions and then analysed by LC-HRMS to obtain the retention time and precursor ions of the by-products. Once the precursor ions were established, product ion data were determined for each compound. The concentration of by-products in the samples was estimated from the amoxicillin calibration curve as the standards for these by-products are not commercially available, and the unknown compounds could ionise differently to amoxicillin. A matrix effect study was conducted (not shown in this work), and found that the compounds present in the synthetic effluent had little influence on the detectability and quantification of amoxicillin. Considering the highly consistent effluent composition and the use of a high-resolution mass spectrometer, isotopically labelled amoxicillin was not used in this study.

2.7 Statistical Analysis of Results

The parameters were compared using Tukey's and one-way ANOVA tests with a 5% significance level ($p < 0.05$). All statistical analyses were conducted in OriginPro 9.0 software. The different letters on the tops of each box in the boxplot indicate statistically different data.

3 RESULTS AND DISCUSSION

3.1 Identification of by-products by HRMS

Figure 1 presents the by-products identified in the anaerobic degradation of amoxicillin. According to Nägele and Moritz (2005), the hydrolysis of the β -lactam ring of amoxicillin produces amoxicillin penicilloic acid (AMXC), which can be further degraded through two different pathways. The first generates amoxilloic acid (AMXO) after the decarboxylation of the free carboxylic acid group, and the second occurs by the formation of a new, stable, six-membered ring, generating amoxicillin diketopiperazine (AMX-DKP). The mechanism of 3-(4-hydroxyphenyl)-2(1H)-pyrazinone (phenol hydroxypyrazine) formation has not been reported in the literature. Figure 1 shows the precursor and product ions of amoxicillin and the by-products identified by LC-HRMS analysis.

Figure 1: Precursor and product ions obtained for amoxicillin and its by-products identified by LC-HRMS analysis.

These results agree with those of Gozlan et al. (2013), Hirte et al. (2016) and Pérez-Parada et al. (2011). The authors observed two different peaks for AMXO, AMX-DKP, and AMXC, which were associated with the corresponding stereoisomers

by the fragmentation pattern. Several by-products with the same precursor ions as amoxicillin were detected in the samples. The amoxicillin retention time was 4.7 min, and the main ion products were observed at 114.0014 and 160.0429 m/z. The by-products with the same ion-precursor as amoxicillin had LC retention times of 6.8, 7.1, 7.5, 8.0, 8.2, and 8.5 min. These are likely AMX-DKP or compounds with similar structures. The products also had different ion-product ratios of 160.0428 and 114.0372 m/z. Thus, it is possible to distinguish between the AMX and AMX-DKP owing to the high accuracy of the HRMS detector, which produces ion-products with different mass values (accurate and exact), such as the m/z ion-product of 114.0008 for AMX and 114.0372 for AMX-DKP.

3.2 Batch (Syringe) Tests

The first set of experiments was conducted with support of glass syringes to estimate the effect of the amoxicillin concentration on the anaerobic degradability and biogas production (Figure S1, see Supplementary Material). The experiment was considered valid once the biodegradability of polyethylene glycol reached $69 \pm 9\%$ after 21 days.

The ANOVA analysis for $p < 0.05$ showed that amoxicillin concentrations of 2,500 and 25,000 $\mu\text{g L}^{-1}$ in the systems only affected the anaerobic process in the first three days of exposure, reducing the biogas production, microbial activity, and effluent biodegradability. Following this, the residual AMX concentration is no more significant than that in the control system (without any pollutant). However, an amoxicillin concentration of 250,000 $\mu\text{g L}^{-1}$ exerted significant effects for up to 16 days of exposure, even in comparison to other systems containing amoxicillin. Even with the inhibition of anaerobic digestion caused by high concentrations of amoxicillin, the degradation ability of microorganisms can recover and produce similar quantities of biogas to systems with low amounts or without antibiotics ($p > 0.05$). Lallai et al. (2002) obtained similar results from their fermentation batch tests up to 250 h: the higher the initial concentration of amoxicillin, the lower the methane production. They attributed this result to the inhibitory effects on one or more metabolic bacterial groups active in methane conversion.

The LC-HRMS analysis showed that while no amoxicillin was detected after 28 days, proportional concentrations of the by-products were obtained in the syringes;

the higher the initial concentration of amoxicillin, the higher the final concentration of the by-products. The sum of all by-products corresponded to $26.3 \pm 1.4\%$, $28.1 \pm 3.7\%$, and $31.2 \pm 2.9\%$ of the initial pollutant concentration in the systems containing 2,500, 25,000, and 250,000 $\mu\text{g L}^{-1}$ of amoxicillin, respectively (Figure S2, see Supplementary Material). Andreozzi et al. (2004) demonstrated that both adsorption on sludge and biodegradation play important roles in the transformation and removal of amoxicillin under aerobic conditions, with the latter being more important and rapid in this process. Hirte et al. (2016) showed that hydrolysis plays an important role in the transformation of amoxicillin. After 20 days, under conditions of neutral pH and temperature of 30°C , compound AMXC corresponded to approximately 45%, whereas AMX-DKP and AMXO corresponded to less than 10% each in terms of relative intensity of the peaks detected by liquid chromatography. In fact, these results corroborate the idea that, although AMX is highly susceptible to hydrolysis, its degradation by-products are recalcitrant to biodegradation, possibly being accumulated in reactors from real WWTPs along time.

It is also important to draw attention to the fact that the conditions used in this test do not correspond to the optimal conditions of biodegradation, since a dilute sludge is used with a relatively high concentration of test chemical. The results obtained, however, are important for analysis in conjunction with other studies in the literature that assess bacterial resistance to antibiotics and changes in the microbial community. The accumulation of by-products derived from long treatment periods can cause changes in the abundance and diversity of the bacterial population as exposed in the work of Meng et al. (2017) and thus, affect treatment efficiency and effluent toxicity.

The ecotoxicological tests with *L. sativa* (Figure 2) exhibited no inhibition under the increased amounts of amoxicillin in the raw effluent, according to the ANOVA results (Tukey test, p values ranging from 0.54 to 0.99 for seed germination and from 0.17 to 0.97 for root length). Hillis et al. (2011) also concluded that amoxicillin is not toxic to *L. sativa* with an $\text{EC}_{25} > 10,000 \mu\text{g L}^{-1}$ (effective concentration that causes 25 % inhibition) while *Daucus carota* (carrot) is more sensitive to the compound, with an EC_{25} of $9,342 \mu\text{g L}^{-1}$ for root length. Following treatment, decreases in seed germination and average root length were expected as the initial concentration of amoxicillin and the formation of by-products in the system

increased. However, higher initial concentrations of the antibiotic stimulated the growth of lettuce seeds. A lower initial concentration of amoxicillin ($250 \mu\text{g L}^{-1}$) caused inhibition when compared to the blank, with reductions in seed germination of 38.5% ($p = 0.0033$) and average root length of 33% ($p = 0.12$). An amoxicillin concentration of $2,500 \mu\text{g L}^{-1}$ also led to reductions in seed germination of 33% ($p = 0.016$) and average root length of 37.2% ($p = 0.053$). This indicates that amoxicillin by-products may be more toxic to this target organism than the amoxicillin itself.

Figure 2: Results for Seed Germination test (specie *Lactuca sativa*) in the syringes with amoxicillin (28 days). Graphics show Seed Germination for raw (A) and treated (B) effluents and Average Root Length for raw (C) and treated (D) effluents. The different letters on the tops of each boxplot indicate statistically different data according to ANOVA for $p < 0.05$.

The results for *A. Fischeri* are shown in Figure 3. With the raw effluent, inhibition of bioluminescence was observed in all systems, including the blank, when

compared to the test substance ($\Gamma = 100\%$). However, no correlation between bioluminescence and concentrations of amoxicillin was observed in the ANOVA test. An average reduction in luminescence from 41.1% (blank) to 29.9% was observed with the addition of antibiotic.

For treated samples, an increase in the bioluminescence from $6.9 \pm 0.9\%$ (blank) to $20.3 \pm 4.2\%$ was observed with an initial amoxicillin concentration of $25,000 \mu\text{g L}^{-1}$ as the amount of by-products increased. The results of the ANOVA test showed that samples from the blank and the system containing $250 \mu\text{g L}^{-1}$ of amoxicillin exhibited the same level of toxicity to *A. Fischeri* ($p = 0.98$), while the other samples were less toxic ($p = 2.41 \times 10^{-4}$ and 4.19×10^{-6} for 2,500 and 25,000 $\mu\text{g L}^{-1}$ of amoxicillin compared to the blank, respectively), indicating that amoxicillin by-products are less toxic to this organism. In the study conducted by Çelebi and Sponza (2012), wastewater treatment using AMCBR (anaerobic multi-chamber bed reactor) and containing 150 mg L^{-1} of amoxicillin led to a reduction in the EC_{50} (effective concentration that causes 50% inhibition) values of *A. fischeri* from 143 (influent) to 215 mg L^{-1} (effluent) with a HRT of 5.5 days. The lower Γ than that of the raw effluent could be ascribed to dispersion of luminescent by particles or by the presence of bacteria from the inoculums used in the test.

Figure 3: Effect of amoxicillin and its by-products in the *Aliivibrio fischeri* ecotoxicity after 30 min of exposure for raw (A) and treated (B) effluents after 28 days experiment. The different letters on the tops of each boxplot indicate statistically different data according to ANOVA for $p < 0.05$.

The presence of amoxicillin in the effluent was toxic to *R. subcapitata* (Figure 4). The ANOVA showed statistically different results for systems with amoxicillin concentrations that were two or three orders of magnitude different from each other. In contrast to the study by Andreozzi et al. (2004), where *R. subcapitata* was insensitive to amoxicillin within the range of 50 ng L⁻¹ to 50 mg L⁻¹, the concentration of 25,000 µg L⁻¹ used in this study caused a mean reduction in the number of cells of 30.9%. The authors utilised AMX dilutions in distilled water, while this work presents the effect of antibiotics in a more complex matrix, similar to that of wastewater, demonstrating the matrix effect in this study. However, the presence of by-products in the treated effluent did not appear to affect the algae growth (Figure 4B) unless higher concentrations of by-products are compared (2,500 and 25,000 µg L⁻¹, p = 0.047).

Figure 4: Effect of amoxicillin and its by-products in the *Raphidocelis subcapitata* cells counting after 96 h of experiment for (A) raw and (B) treated effluent after 28 days of test. The different letters on the tops of each boxplot indicate statistically different data according to ANOVA for p < 0.05.

3.3 Anaerobic Digester (BioStat)

Figure 5 shows the profiles for pH, alkalinity, biogas production, COD, and TAN in the anaerobic reactor in phases two (control phase) and three (addition of amoxicillin). The influent composition is that described on Table S1 (see Supplementary Material), corresponding to the following characterization: pH = 7.4, COD = 890 mg L⁻¹ and TAN = 36.4 mg L⁻¹. For alkalinity, the titration was executed in two parts: i) titration until pH 5.7 and named bicarbonate/ partial alkalinity (0.26 g L⁻¹);

ii) titration between pHs 5.7 and 4.3, giving an estimative of volatile fatty acids and thus named VFA alkalinity (0.07 g L^{-1}).

Figure 5: Physico-chemical variables monitored along anaerobic digester operation. (a) pH, (b) Alkalinity, (c) Total Ammonium Nitrogen, (d) Chemical Oxygen Demand, (e) Biogas production and (f) Total and Volatile Solids.

The pH values varied between 7.0 and 7.8 in both systems, but generally remained near pH 7.2. A decrease to pH 6.5 was observed in the reactor on day 58 (as mentioned in section 2.4), but no significant modifications occurred because of

amoxicillin addition according to ANOVA ($p = 0.155$), although a peak at $\text{pH} = 7.6$ was observed 10 days after the initial addition of AMX to the system (day 43).

The alkalinity decreased from day one until sixteen, and then remained stable until day fifty-eight. This reduction was attributed to the loss of solids at the reactor exit. During the first days, lighter solids, which contribute to the alkalinity of the sample (as ammonium bicarbonate), are drawn out of the reactor. Therefore, at the end of the 16th day, only the denser solids that precipitate in the reactor remain. On day 58, due to great reduction of pH (0.7 units), the total alkalinity decreased from 0.3 to 0.2 g L^{-1} , and NaHCO_3 was added to balance the pH of the system, which caused an increase in total alkalinity to 0.47 g L^{-1} on day 61. Statistical analysis revealed no difference in the total alkalinity between the phases of operation (p values ranging from 0.102 to 0.897). However, an individual evaluation of the bicarbonate alkalinity ($\text{pH} 5.7$) and VFA alkalinity ($\text{pH} 4.3$) showed that the addition of AMX caused a mean reduction in the bicarbonate alkalinity of 20.1% ($p = 0.021$), while after pH reduction there was an increase in the VFA alkalinity of 42.0% from that of the system with amoxicillin ($p = 0.0012$). These results indicate that the additions of the antibiotic and pH reduction (even after correction with NaHCO_3) created unbalanced conditions during anaerobic digestion.

Analysis showed that AMX caused a mean reduction in COD from $560 \pm 74\%$ to $456 \pm 28\%$ in relation to that in the control phase, demonstrating that AMX stimulated the biodegradation of the effluent ($p = 9.52 \times 10^{-4}$) in a first moment possibly by supplying easily degradable carbon sources like formic acid through the decarboxylation of AMXC to form AMXO. The average biogas production did not change ($p = 0.371$) for second and third phases: from day one to thirty-three (control phase) mean production was $148 \pm 49 \text{ mL day}^{-1}$, while that in the period from day 33 to 58 was $107 \pm 33 \text{ mL day}^{-1}$. After day 58, reduction of pH was followed by the increase in COD values (with mean COD removal of only 27.8%), which culminated with a reduction in biogas production until no biogas was released. This can be attributed to the accumulation of volatile fatty acids, represented by a rapid decrease in total alkalinity from 0.47 to 0.30 g L^{-1} and an increase in the VFA alkalinity, as shown previously.

TAN values were also modified in third phase when AMX was present. The mean value was reduced from $68.9 \pm 11.2 \text{ mg L}^{-1}$ to $52.0 \pm 8.6 \text{ mg L}^{-1}$ ($p = 9.36 \times$

10⁻⁷), but this result is more related to pH changes and equilibrium between NH₄⁺/NH₃ and not AMX presence. The pH reduction on day 58, on the other hand (and its subsequent correction with NaHCO₃), did not modify ammonia concentration (p < 0.05). Considering that influent TAN value was 36.4 ± 3.9 mg L⁻¹, which is lower than that after treatment, it is verified an increase in the amount of ammonia occurred during anaerobic digestion through the breakdown of organic nitrogen and its subsequent conversion into ammonia, which confirms that the system was anaerobic. The high HRT associated with the low amount of carbohydrates added to the system, also culminated in a reduction in the solid content of the anaerobic digester. Average reductions in the total and volatile solid contents of 54.2% and 49.8% were observed after 72 days of operation, respectively.

The samples analysed by HRMS showed the accumulation of several by-products identified in the degradation of amoxicillin (Figure S3, see Supplementary Material). An accumulation followed by removal was observed only for AMX-DKP IV. Amoxicillin was observed in the first two days after its initial addition, with a maximum estimated concentration of 0.0068 µmol L⁻¹ confirming its rapid hydrolysis. The main observed by-product was amoxicillin penicilloic acid II (AMXC II). After 18 days, its concentration reached 1.58 µmol L⁻¹ (604.4 µg L⁻¹), corresponding to 57.6% of AMX influent concentration (2.74 µmol L⁻¹). AMXC was also found in a higher relative proportion than other by-products in monitored WWTPs and laboratory scale studies (Gozlan et al., 2013; Hirte et al., 2016). For AMX-DKP IV its maximum concentration (0.0264 µmol L⁻¹ or 9.6 µg L⁻¹) was observed on the ninth day after the addition of amoxicillin, and then decreased to almost 0.0055 µmol L⁻¹. After 40 days of feeding the sum of all by-products corresponded to 76% of the total AMX added to biostat. The formation of AMX-DKP IV was associated with changes in COD, TAN, and pH, as its maximum observed concentration in BioStat coincided with the maximum pH value, maximum removal of COD but minimum concentration of ammonia nitrogen due to increased pH in this period (see Figure S4 from Supplementary Material).

The presence of phenol hydroxypyrazine as a by-product of amoxicillin degradation was not observed during the syringe tests. On the other hand, in the studies conducted with the semi-continuous flow reactor, the compound was observed in concentrations lower than 0.011 µmol L⁻¹ when then, after the pH drop on the 25th day of feeding with AMX, its concentration rapidly raised from 0.0073 to

0.011 $\mu\text{mol L}^{-1}$ and then to more than 0.089 $\mu\text{mol L}^{-1}$ in the subsequent days (Figure S3e). The appearance of phenol hydroxypyrazine after pH drop is in agreement with the study of Hirte et al. (2016) where it was observed the preferential formation of compound at acidic conditions. Gozlan et al. (2013) associated the appearance of phenol hydroxypyrazine with the major abundance of AMXC.

The accumulation of by-products can be explained due to unbalanced conditions observed in physico-chemical parameters. The low HRT used in this study was not enough for the complete mineralization of the compounds generated during the anaerobic treatment. Besides the reduction in COD after addition of amoxicillin, this value did not exceed 50%, indicating the low removal efficiency of the system. Çelebi and Sponza (2012) observed a reduction in the removal of amoxicillin and COD when HRTs as low as 1.13 days were used. They attributed this to the insufficient time available for the mineralisation of COD, amoxicillin, and its metabolites owing to the inadequate conditions for mass transfer into the granules of the AMCBR. Another reason for the decrease in the treatment efficiency was the partial inhibition of microorganisms by amoxicillin, resulting in lower methanogenic activity and deficiencies in the metabolization of volatile fatty acids. During this study, there was an increase in the VFA/ bicarbonate alkalinity ratio of 0.30 (system without AMX) to 0.39 (after addition of AMX) and then to 0.64 after 58 days of reactor operation, indicating in fact the occurrence of anaerobic reactor disturbances. Meng et al. (2015) also observed an increase in the concentration of volatile acids as the concentration of AMX in the influent was increased. This result may be related to the formation and accumulation of more recalcitrant molecules of amoxicillin by-products, as they require longer periods for biodegradation. Although this work has employed high concentrations of drug, the results point to the fact that long periods of operation with effluents containing this type of compound can cause system failure and consequent death of the biomass.

On the other hand, the process of adsorption of these compounds to the biomass cannot be ignored. Marcelino et al. (2016) observed a removal of 80% of amoxicillin present in a pharmaceutical wastewater after 28 days of aerobic biodegradation, however desorption tests were carried out and the results showed that after 12 days at least 54 % of amoxicillin were desorbed from the sludge. With

this, it is clear the possibility of drug accumulation in real WWTPs and attention should be paid to the use of sludge as a source of biofertilizer in agriculture.

Among the target organisms used in the ecotoxicity assays, *Aliivibrio fischeri* was the most sensitive to the degradation by-products of amoxicillin in the treated effluent samples (Figure S5, see Supplementary Material). The effluent collected after 39 days was 89% more toxic than that obtained before the addition of amoxicillin. This result disagrees with that observed for the syringe samples, in which higher by-product concentrations were less toxic to *A. fischeri*. In this case, the presence of AMXO II and the formation of AMX-DKP I, II, III, V, and VI were not observed, which could positively stimulate the bioluminescence. The preferential formation of AMX-DKP in the syringes could be attributed to the higher pH (approximately 7.8 at the end of the experiment) in comparison to pH values observed in BioStat (average of 7.3). At higher pH values, the amine group is deprotonated ($pK_a=7.56$), thus it is available for a nucleophilic attack on the carbonyl group to create a diketopiperazine ring as observed by Gozlan et al. (2013). *A. fischeri* was also the most sensitive species in the study by Çelebi and Sponza (2012), who observed an increase in the toxicity when the HRT was reduced to 0.9 days, which resulted in a reduction in the biogas production and an increase in the volatile fatty acids concentration that could enhance the effluent toxicity.

The ecotoxicological assays conducted using lettuce seeds (Figure 6) showed that the seeds are sensitive to the presence of AMX-DKP IV. While the toxicity of the samples increased from the first day to day 11 (observed by the reduction in the calculated indexes), as the concentration of amoxicillin by-products increased in the effluent, the toxicity was lower on day 18 and the concentration of AMX-DKP IV was also reduced, even with the successive accumulation of other by-products.

Figure 6: Results Seed Germination tests for samples after anaerobic treatment. Time zero represents the treated effluent before the addition of compounds. The different letters on the tops of each bar indicate statistically different data according to ANOVA for $p < 0.05$.

The opposite behaviour was observed in the test with *R. subcapitata*. The formation of AMX-DKP IV coincided with the stimulation of algae cells until day 11, and then decreased until day 25. On days 32 and 39, further stimulation was observed, which coincided with the formation of phenol hydroxypyrazine (Figure 7).

Figure 7: Effect of anaerobic treatment in *Raphidocelis subcapitata* cells counting after 96 h of experiment. Time zero represents the treated effluent before the addition of amoxicillin. Grey shadows identify where it was observed a correlation between formation of AMX-DKP IV and Phenol hydroxypyrazine and modifications in counting of algae cells. The different letters on the tops of each bar indicate statistically different data according to ANOVA for $p < 0.05$.

4 CONCLUSIONS

This study has demonstrated the importance of determining degradation by-products generated during sewage treatment and their correlation with ecotoxicological assays. Although 100% removal of the antibiotic amoxicillin was observed in both systems studied (syringes and semi-continuous flow reactor), the formation and accumulation of various by-products was observed, including AMXC, AMXO and AMX-DKP. The rapid hydrolysis of the antibiotic and consequent formation of these compounds led to inhibitions in the production of biogas in the first days of experiment using glass syringes, whereas the effect of the accumulation of recalcitrant by-products to the anaerobic treatment only appeared in the semi-continuous flow reactor 25 days after the first addition of the pollutant when an abrupt drop in pH and alkalinity was observed.

The presence of compound AMX-DKP IV in the BioStat was related to changes in physico-chemical variables. The increase in its concentration led to a growth stimulation of algae *R. subcapitata* but to a reduction in the number of germinated lettuce (*L. sativa*) seeds and its average root length. On the other hand, studies involving the species *A. fischeri* indicated successive increases in the toxicity of the treated effluent, regardless by-products accumulation and/ or elimination that were formed in the reactor during its operation. The high inhibition observed for this species was related to unbalanced conditions in AD which culminated in a substantial reduction in COD removal, estimated by-product accumulation of 76% (in relation to the influent amoxicillin concentration) and a two-fold increase in the VFA/ bicarbonate alkalinity ratio in relation to the system without addition of amoxicillin, indicating the accumulation of volatile fatty acids, and therefore, the occurrence of disturbances in the process of anaerobic digestion.

It is thus noted the importance and advantages of performing ecotoxicological assays using organisms of different trophic levels as a way to classify effluents for proper disposal, reuse or design of tertiary treatment systems. Although still an incipient field in many countries, the assessment of effects on aquatic organisms is often a quick, simple and inexpensive way to provide information about environmental effects of treated effluents and should be considered as an important parameter in regulatory frameworks.

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