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EVALUATION OF AEROBIC AND ANAEROBIC BIODEGRADABILITY AND TOXICITY ASSESSMENT OF REAL PHARMACEUTICAL WASTEWATER FROM INDUSTRIAL PRODUCTION OF ANTIBIOTICS

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Abstract – This study evaluates aerobic and anaerobic biodegradability and toxicity of a real pharmaceutical wastewater, which focuses on antibiotics production. Zahn-Wellens and Organization for Economic Cooperation and Development (OECD) methodologies were applied in order to verify the wastewater's biodegradability and Microtox® analysis was performed for toxicity tests. Tests achieved more than 89% and 63% of Total Organic Carbon reduction, showing 80% and 50% of antibiotic removal, for aerobic and anaerobic processes, respectively. Moreover, acute ecotoxicological tests revealed that both techniques decreased the toxic character of real pharmaceutical wastewater. Desorption tests showed that the antibiotic was not degraded, but, in fact, adsorbed onto the sludge. Since biological treatment is the most widely used method for industrial wastewater treatment, this study indicates that this kind of treatment is probably unable to mineralize antibiotics present in pharmaceutical wastewaters, which may induce the development of resistant pathogens. Therefore, efforts must be taken to elucidate the main mechanisms of biological antibiotic removal from wastewaters since the presence of antibiotics in the environment is considered to be an emerging environmental issue.
Keywords: Pharmaceutical Wastewater; Biodegradability; Antibiotics; Amoxicillin.

INTRODUCTION

Emerging pollutants are groups of compounds which have no specific legal regulations and whose toxic effects to the environment and human health coupled with high occurrence make them subject to future regulations (Miralles-Cuevas *et al*. 2013). This group includes various types of globally widespread organic compounds, such as pesticides, dyes, pharmaceuticals, personal care products, polymers and plastics. Most of the pharmaceutical drugs used worldwide are excreted in an unchanged or only partially metabolized form and disposed of in municipal Wastewater Treatments Plants and in the environment.

Antibiotics are natural or synthetic pharmaceuticals that can eliminate or prevent the multiplication of bacteria. These drugs have been extensively used in human and veterinary medicine to treat bacterial diseases and, in some cases, to prevent bacterial

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infections. They are also used as growth promoters in animals that are included in the food industry and as pesticides for controlling bacterial infections in crop fields, particularly cereals. Therefore, they have become abundant contaminants in the environment (Kim *et al*. 2013). Effluents from antibiotics production are considered to be emerging environmental problems due to their refractory characteristics and toxicity to the environment, even in low concentrations. Moreover, the presence of traces of such pharmaceutical components in the environment may induce the development of antibiotic-resistant pathogens, causing serious problems to human and animal health (Mascolo *et al*. 2010). It is estimated that about half of the pharmaceutical wastewater produced in the world is disposed of into water bodies without any treatment (Deegan 2011).

Biological treatment is the most commonly used and economical method of wastewater treatment. However, available biological techniques present in industrial Wastewater Treatment Plants (WTP) are inefficient for the mineralization of pharmaceutical industrial wastewaters, which contain dangerous compounds (Monteagudo *et al*. 2013, Pérez-Moya *et al*. 2010). Various studies have tested different new technologies for pharmaceutical compound removal from wastewater, such as ozone (Arslan-Alaton and Caglayan 2006), biological and electro-photo-Fenton processes (Mansour *et al*. 2015), membrane bioreactors (MBR) (Cheng *et al*. 2015) and osmosis integrated to electrochemical oxidation (Liu *et al*. 2015). However, most studies have been performed using synthetic antibiotic solutions, indicating the substantial demand for research using real pharmaceutical wastewaters.

Special attention must be given to the biodegradability of the mixture of intermediates generated during advanced oxidation treatment of recalcitrant and dangerous wastewaters based on biodegradability tests (Ballesteros Martín *et al*. 2010). Therefore, biodegradability is a key parameter in the assessment of hazard chemicals and wastewaters since high biodegradability implies a reduced tendency to bioaccumulate or to persist in the environment (Stolte *et al*. 2012).

In order to assess wastewater biodegradability several methods such as Zahn-Wellens test, BOD_5/COD ratio (De Bel *et al*. 2009, Ledezma Estrada *et al*. 2012) and the respirometry test, *Pseudomonas putida* bioassay, were proposed by international organizations, such as OECD and the International Organization for Standardization (ISO). These biodegradability assays may be classified into three major groups: tests on ready biodegradability, tests on inherent biodegradability and simulation tests. Zahn-Wellens is an inherent biodegradability test that evaluates the potential biodegradability of water-soluble, non-volatile organic substances exposed to high concentrations of microorganisms and nutrients for 28 days (Ballesteros Martín *et al*. 2010, Pagga 1997).

The Zahn–Wellens test provides the degradation behavior of a wastewater in an activated sludge treatment plant, since the experimental conditions are similar to this process. Nevertheless, low biodegradability results obtained in inherent biodegradability tests are considered adequately indicative of poor biodegradability. Meanwhile, positive results are not necessarily predictive of biodegradability under real environmental conditions, since a significant overestimation of the removal extent has been reported in the literature (Mascolo *et al*. 2010). Ballesteros Martin *et al*. (2010) compared four biodegradability tests (*Pseudomonas putida* bioassay, Zahn–Wellens test, BOD_5/COD and respirometry assay) to determine the biodegradability enhancement of a treated pesticide mixture taking into account repeatability and precision of each biodegradability test. The authors concluded that the *P. putida* and Zahn–Wellens tests showed higher repeatability and precision.

In addition, it is important to investigate the main mechanisms leading to biodegradation of recalcitrant compounds, such as antibiotics, in biological treatment plants since specific interactions between antibiotics and sludge may take place. Some studies performed with sludge from municipal sewage treatment plants have found up to 22 different antibiotics adsorbed to sludge (Gao *et al*. 2012a, Jia *et al*. 2012). This leads to the selection of resistant bacteria which represent a risk to human health (Bouki *et al*. 2013). Desorption tests have also been applied to sludge from a 14-day aerobic biodegradability test using a synthetic antibiotic solution in order to examine antibiotic removal paths, suggesting higher sorption rather than degradation of the compounds (Yang *et al*. 2012).

In this context, this study aims to evaluate aerobic and anaerobic biodegradability and toxicity of a real pharmaceutical wastewater, which focuses on antibiotic production. Also, desorption tests were carried out to analyze the antibiotic fate in biodegradability reactors.

MATERIALS AND METHODS

Biomass

The sludge used as biomass source for the aerobic experiment was collected at the recirculation stage of the biological reactor in an activated sludge system of the municipal Wastewater Treatment Plant (WWTP) in Belo Horizonte, Brazil. For the anaerobic experiments, an adaptation of the method proposed by Owen *et al*. (1979) was performed, using UASB reactor sludge from a research center in Belo Horizonte, Brazil, as inoculum for the anaerobic reactions.

The sludge was used within 6 hours after its sampling and pre-conditioned to the experimental conditions by sedimentation and several washes prior to its use in order to concentrate the biomass and reduce the TOC background. Sludge biomass concentration was determined gravimetrically (APHA *et al*. 2005) by determining volatile suspended solids (VSS).

Wastewater

The wastewater sample used in this work was obtained at an antibiotics production plant in a pharmaceutical industry in Brazil. The effluent is an aqueous mixture of cleaning waters, cleaning products, antibiotics, solvents and intermediates. After collection, the wastewater was characterized for the following parameters: pH (potentiometric method), Conductivity, Dissolved Oxigen, Turbidity, Total Organic Carbon (TOC), Total carbon (TC; mg.L^{-1}) through a Total Organic Carbon Analyzer (Shimadzu), Inorganic Carbon, Chemical Oxygen Demand (COD, mg O_2.L^{-1}) through the colorimetric method (APHA 5220 D), Biological Oxygen Demand (BOD_5), Nitrate, Nitrite, Sulfate, Phosphate, Fluoride, Chloride, Bromide, Alkalinity (potentiometric method), Total Suspended Solids (TSS) and, Volatile Suspended Solids (VSS) (APHA *et al*. 2005).

Chemicals

Sodium hydroxide and sulfuric acid were used in the biological reactor in order to keep a neutral pH (6.5 – 7.5). All the chemicals used for wastewater characterization analysis and for the preparation of the mineral nutrients solutions were at least >98% pure (analytical grade). Amoxicillin standard used in HPLC determinations was provided by the pharmaceutical industry.

Analytical Techniques and Procedures

Organic matter degradation was monitored through periodic sampling. Samples were filtered with C40 quantitative filter paper (125mm) to remove suspended solids (sludge) and subsequent analysis of total organic carbon (TOC) in TOC-V CPN equipment (Shimadzu). Specific degradation of amoxicillin after biodegradability experiments was followed by HPLC (Agilent Technologies Model 1260 Infinity) equipped with a reverse-phase Zorbax Eclipse Plus® C18 column (4.6 X 150 mm, 5.0µm). The mobile phase consisted of methanol:water (55:45, v/v) with isocratic elution. Flow was set at 0.750 mL.min^{-1} and the monitoring wavelength was 210 nm. Aliquots were filtered through 0.20 µm syringe filters (Millexs-GN, 25mm, Millipore) before HPLC injection. The injection volume was 20 µL and each run a total of 10 minutes.

Aerobic Biodegradation

Aerobic biodegradability assays were performed according to the Zahn-Welles test methodology (OECD, 1992) using high nutrient and biomass concentrations. Organic matter degradation was monitored through periodic TOC analysis. Biodegradation was also assessed with an adaptation of the method for the use of high organic carbon wastewater (TOC ~2400 mg·L^{-1}), since the original method suggested that the organic charge value must be within the range of 50 a 400 mg·L^{-1}. Thereby, test A (named dilute wastewater) was performed using a diluted wastewater, so that the initial TOC was in the organic matter threshold suggested by Zahn-Wellens method (initial TOC ~ 217 mg·L^{-1}); and test B (named pure wastewater) was performed with the pharmaceutical wastewater without dilution (the mineral nutrients solution added to the reactor represented only a small dilution) and showed initial TOC around 1720 mg·L^{-1}.

A solution composed of micro and macro mineral nutrients was prepared according to the Zahn-Wellens methodology. Then, 500 mL of this nutritive solution were added to each reactor. In order to obtain around 0.6 g·L^{-1} of biomass in the reactors, 50 mL of the sludge (pre-conditioned as previously described) was added to each vessel. The control and blank experiments were prepared using glucose as carbon source, which is highly biodegradable (initial TOC ~ 200 mg·L^{-1}), and distilled water, respectively. Then, mineral nutrients and the activated sludge were also added. Two liter Erlenmeyer vessels were used as reactors and the test was performed under aeration and kept in the dark at room temperature of 23–29 °C for 28 days. Samples were taken at regular time intervals and TOC was determined for each one. For each sampling, loss of volume due to evaporation was mitigated with distilled water and NaOH and H_2SO_4 solutions were used to keep the pH in the reactors at a neutral threshold (6.5 – 7.5).

The percentage of biodegradation (D_t) at time t was determined by Equation (1):

$$D_t = \left[1 - \left(\frac{C_t - C_B}{C_A - C_{BA}}\right)\right] \times 100 \qquad (1)$$

where C_A and C_{BA} are the TOC (mg·L^{-1}) in the sample and in the blank, respectively, measured 3h after the starting time, and C_t and C_B are the TOC (mg·L^{-1}) in the sample and in the blank, respectively, measured at the sampling time t. According to the method definition, wastewater samples are considered biodegradable by the Zahn-Wellens methodology when D_t is higher than 70%.

Anaerobic Biodegradation

Anaerobic biodegradability assays were performed as an adaptation of the methodology proposed by Owen *et al*. (1979). A solution composed of micro and macro mineral nutrients was prepared with high nutrient content (Table 1).

Table 1: Anaerobic biodegradability test nutrient solution: micro and macro minerals.

Nutrients	Concentration (mg·L^{-1})
KH_2PO_4	650
K_2HPO_4	150
NH_4Cl	500
$NaHCO_3$	1000
$MgCl_2$	100
$CaCl_2·2H_2O$	100
$Na_2S·7H_2O$	50
$FeCl_3·6H_2O$	2
$ZnCl_2$	0.05
$CuCl_2·2H_2O$	0.03
$MnCl_2·4H_2O$	0.5
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.05
$AlCl_3·6H_2O$	0.05
$CoCl_2·6H_2O$	2
$NiCl_2·6H_2O$	0.05
H_3BO_3	0.01

The reactors were prepared by the addition of 1 L of wastewater, 500 mL of this nutritive solution and the amount of anaerobic sludge needed to achieve a concentration of 0.6 g·L^{-1} of biomass in the reactors. The test was also conducted for diluted wastewater using the same dissolution used in the aerobic method. Control and blank experiments were prepared using a glucose solution (initial TOC ~ 200 mg·L^{-1}), and distilled water, respectively, instead of 1 L of wastewater. Reactors were then degassed using nitrogen and kept in the dark at room temperature (23–29 °C) for 28 days. Samples were taken at regular time intervals and TOC was determined for each one. After each sampling, the volume loss due to evaporation was mitigated by adding distilled water, and the biodegradation percentage (D_t) at time (t) was determined by Equation (1).

Desorption Test

A desorption test was made in order to verify if the antibiotic removal results were due to antibiotic degradation or if an adsorptive process had taken place. The test was prepared by taking all the sludge used in the aerobic biological reactor and washing it three times with 50 mL of mineral water, in order to separate the wastewater from the sludge. After the washing process, the total volume was completed up to 80 mL by adding mineral water and this solution was aerated for 12 days. Samples were periodically taken and antibiotic concentrations were analyzed by HPCL.

Toxicity Assays

Acute ecotoxicity tests were conducted using the luminescent marine bacteria *Aliivibrio fischeri* according to the methodology presented by Santos and Teixeira (2014). Toxicity results were analyzed using the Ec_{50} (30 minutes) values.

RESULTS AND DISCUSSION

The evaluation of the production of pharmaceutical products at the studied plant revealed that cephalexin and amoxicillin were the most usual antibiotics produced by this industry in the last two years. Figure 1 presents the percentage in mass of these antibiotics produced in 2013 and 2014. As observed in Figure 1, these drugs represent more than 85% of the production. During the sampling period, production data showed that amoxicillin was produced in greater amounts. Based on this fact, since this wastewater was a mixture of various compounds, this study used amoxicillin to investigate antibiotic removal by HPLC analysis.

Figure 1 shows Amoxicillin to be one of the most produced compound and, thus, the most important molecule in this wastewater capable of influencing biological treatment performance and bringing about severe consequences to natural water bodies where it is probably disposed of. However, sampled wastewater includes a variety of other chemicals used in the industrial process and is considered to be a complex effluent. A detailed characterization of the effluent collected at the industry is presented on Table 2.

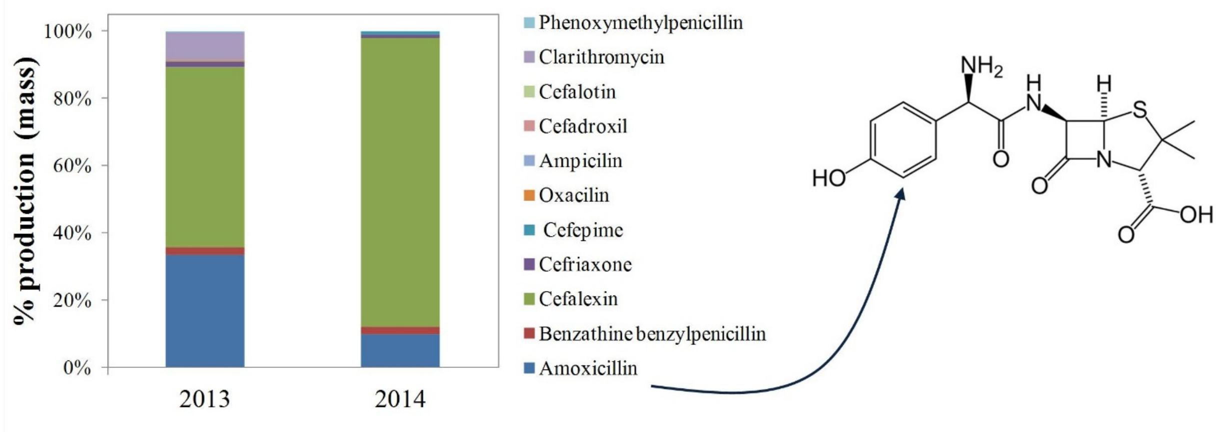

Figure 1: Percentage of mass production for each antibiotic produced by the industry in 2013 and 2014 and molecular structure of the antibiotic used as tracking molecule.

Table 2: Physical-chemical characteristics of the investigated pharmaceutical wastewater.

Parameter	Value	Unit
pH	7.0 ± 1.0	
Conductivity	(3.1 ± 0.1) x·10	$\mu S \cdot cm^{-1}$
Dissolved oxygen	3.0 ± 0.5	mg $O_2 \cdot L^{-1}$
Turbidity	(6.6 ± 0.3)·x 10	NTU
TOC	(2.4 ± 0.01)·x 10^3	mg $C \cdot L^{-1}$
Total carbon	(2.4 ± 0.01)·x 10^3	mg $C \cdot L^{-1}$
Inorganic Carbon	(3.7 ± 0.1)·x 10	mg $C \cdot L^{-1}$
COD	(6.0 ± 0.1)·x 10^3	mg $O_2 \cdot L^{-1}$
BOD_5	(3.7 ± 0.1)·x 10^3	mg $O_2 \cdot L^{-1}$
BOD_5/COD	0.6	
Nitrate	1.5± 0.1	mg $NO_3^- \cdot L^{-1}$
Nitrite	0.9± 0.02	mg $NO_2^- \cdot L^{-1}$
Sulfate	(1.5 ± 0.6)·x 10	mg $SO_4^{2-} \cdot L^{-1}$
Phosphate	4.0 ± 0.1	mg $PO_4^{3-} \cdot L^{-1}$
Fluoride	Not detected	mg $F^- \cdot L^{-1}$
Chloride	(4.8± 0.2)·x 10	mg $Cl^- \cdot L^{-1}$
Bromide	2± 0.5	mg $Br^- \cdot L^{-1}$
Alkalinity	(2.3)·x 10^2	mg $CaCO_3 \cdot L^{-1}$
TSS	(3.2 ± 0.3)·x 10^2	mg $\cdot L^{-1}$
VSS	(1.8 ± 0.06)·x 10^2	mg $\cdot L^{-1}$
Amoxicillin	(1.2 ± 0.2)·x 10^2	mg $\cdot L^{-1}$

It is important to emphasize the high amoxicillin concentration, BOD, COD and TOC results in the physical chemical analysis of this wastewater. Moreover, an analysis of the BOD_5/COD ratio value indicates that the effluent is likely to be biodegradable.

Figure 2A shows the average evolution of TOC concentration measured in all of the reactors that were operated during the 28 days of biodegradation by Zahn-Wellens methodology. It is possible to note that the dilute wastewater TOC decreased faster than pure wastewater. The biodegradation percentage (D_t) of diluted wastewater and pure wastewater were determined by Equation (1) and are represented in Figure 2B. As mentioned, wastewater samples are considered biodegradable when D_t is higher than 70%.

According to the Zhan-Wellens test, both wastewaters, dilute and pure, can be considered biodegradable. The results demonstrate that the dilute wastewater biodegradation was higher than 80% as early as in the first day of reaction, while raw wastewater needed 6 days to achieve this biodegradability status. After 28 days, the efficiency of TOC removal was 89% for pure wastewater and 80% for dilute wastewater. These results show that the dilution required for the Zhan-Wellens test may influence the wastewater biodegradability results, since this experimental method may underestimate the time needed for biological acclimation, and neglect the industrial wastewater's toxicity.

Figure 3A presents anaerobic biodegradation TOC results during the 28 days of reaction and Figure 3B shows the percentage of biodegradation during the entire treatment.

As expected, anaerobic biodegradation was slower than the aerobic one due to the slower metabolism of anaerobic microorganisms when compared to aerobic ones. The tests performed show that a 12-day period was necessary for anaerobic treatment to achieve the 70% TOC removal of diluted wastewater, while for pure wastewater only 63% TOC degradation was reached after the proposed 28 days. Therefore, this wastewater cannot be considered to be biodegradable through the anaerobic test, according to the described methodology. However, the acclimation of this kind of anaerobic biomass may take longer; thus, a longer reaction time may also be needed to achieve an increase of TOC removal as observed by Santos and Teixeira (2014).

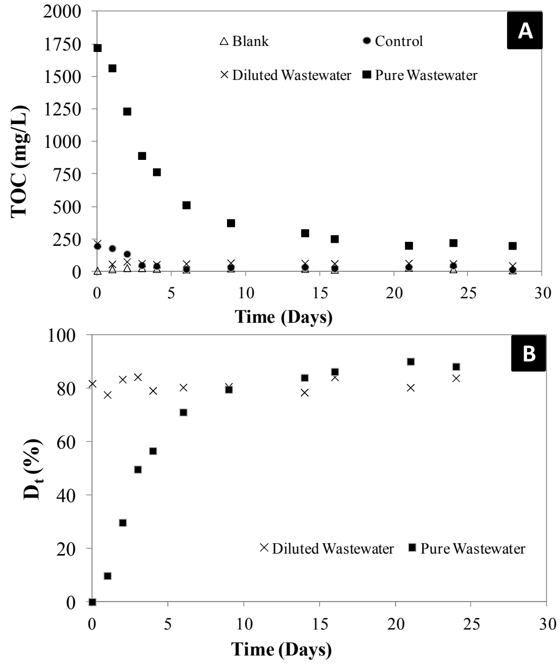

Figure 2: TOC evolution in the blank, control, dilute wastewater and pure wastewater reactors during the 28 days of biodegradation by Zahn-Wellens methodology (A) and Zahn-Wellens percentage of biodegradation (D_t) for the diluted wastewater and for the pure wastewater (B).

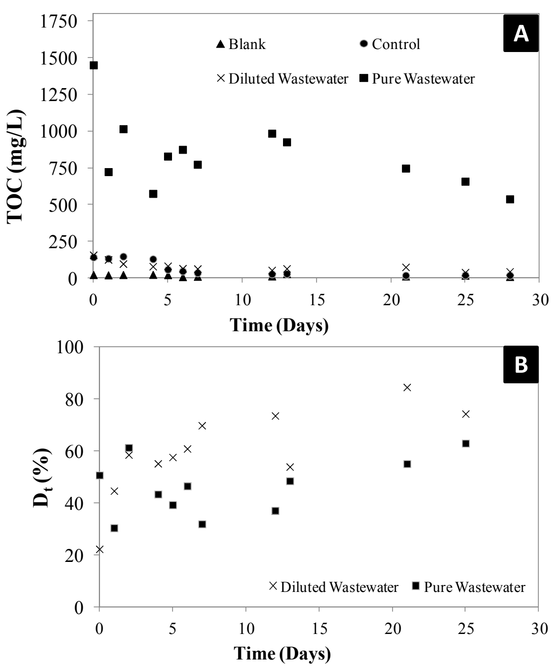

Figure 3: TOC evolution of blank, control, dilute wastewater and pure wastewater reactors during the 28 days of anaerobic biodegradation (A) and percentage of biodegradation (D_t) for the diluted wastewater and for pure wastewater (B).

Although the method classifies this wastewater as biodegradable, positive results are not necessarily predictive of biodegradability under real scale treatment conditions. Some studies (Mascolo *et al*., 2010), reported that a significant overestimation of the removal extent can be presumed by the Zahn-Wellens method. A real wastewater treatment plant may not receive dilute effluents, may not supply all nutrients added to reactors in these experiments and/or may not hold effluents for the same residence time proposed by the Zahn-Wellens methodology. Also, removal rates do not necessarily mean that the antibiotic was biodegraded since it could only have been adsorbed by sludge (Yang *et al*. 2012).

In order to verify the occurrence of antibiotic removal, HPLC analyses were performed using aerobic biodegradability final samples (sampled on the 28th day) (Figure 4).

HPLC monitoring showed that more than 80% of the amoxicillin present in the pure wastewater was removed after 28-days of aerobic biodegradation and, for the dilute wastewater this value was even higher, 98%. Regarding the anaerobic process, the amoxicillin removal rate achieved 50% for the pure wastewater and 70% for the dilute one (data no shown). Another concern regarding high concentrations of antibiotic in wastewaters is its toxicity and possible effects on the bacterial community present in the biological treatment. Toxicity tests provide interesting information, especially when acute toxicity values of raw and treated wastewater are compared. Figure 5 presents toxicity test results performed using the luminescent marine bacteria *Aliivibrio fischeri*.

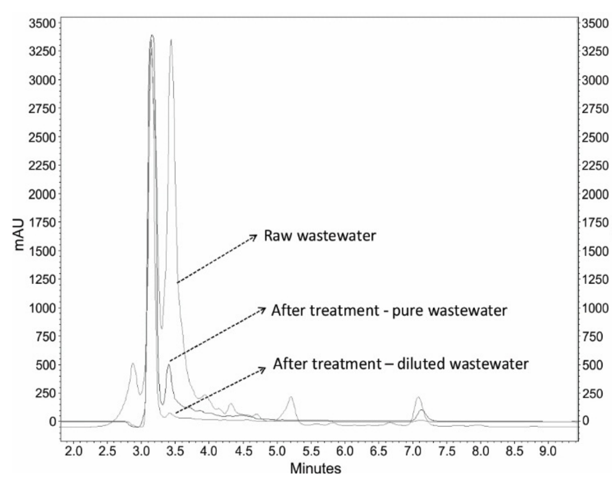

Figure 4: HPLC chromatograms of wastewater samples taken during aerobic biodegradability test on day 0 and on day 28 for dilute wastewater and for pure wastewater.

Toxicity test results show that raw wastewater is toxic (2.1% EC_{50}), and that anaerobic (6.3% EC_{50}) and aerobic (21.8% EC_{50}) treatments have decreased the wastewater's toxicity. However, it is noteworthy that the bacteria used in this kind of test are extremely sensitive, especially if compared to bacteria present in wastewater biological treatment sludge.

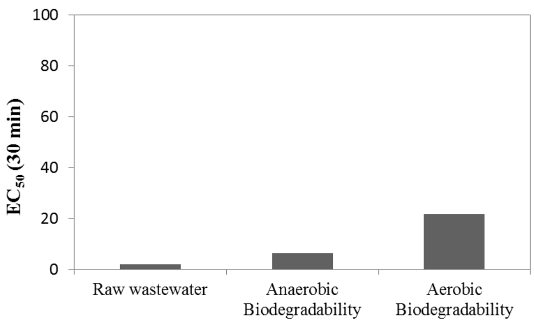

Figure 5: Acute toxicity test using the luminescent marine bacterium *Aliivibrio fischeri* for raw wastewater and waste water treated by aerobic and anaerobic biological treatment.

The fact that the biological treatment decreases wastewater toxicity indicates that there is a need to understand the antibiotic removal mechanism. Biodegradation and adsorption have been reported to play important roles in antibiotic elimination (Gao *et al*. 2012b, Yang *et al*. 2012). In order to better understand the main removal mechanism involved in this process, desorption tests were carried out using the aerobic sludge applied in the treatment presented here. The results showed that, after 12 days, 68 mg of amoxicillin were desorbed from the sludge. This indicates that at least 54% of the initial amoxicillin present in the reactor was absorbed onto the sludge, but not degraded by microorganisms.

The adsorption of the antibiotic onto the sludge may lead to drug accumulation in real wastewater treatment plants and, on a long term basis, may induce the development of antibiotic-resistant pathogenic microorganisms (Bouki *et al*. 2013, Xu *et al*. 2015), whose resistance genes can be horizontally passed on to many generations, causing serious problems for human and animal health.

CONCLUSIONS

Results presented in this work indicate that the wastewater derived from antibiotic production investigated in this work can be considered to be biodegradable according to the Zahn-Wells test (both dilute and raw wastewater) and that biomass was able to remove 89% TOC and 80% of the antibiotics present in the raw wastewater. Anaerobic biodegradability test using pure effluent achieved 63% biodegradation and 50% antibiotic removal. However, it is crucial to mention that the aerobic and anaerobic treatments decreased the wastewater acute toxicity, and that the desorption test showed that more than 54% of the antibiotic present in raw wastewater was actually adsorbed onto the aerobic sludge, rather than mineralized, as presented in the scheme in Figure 6.

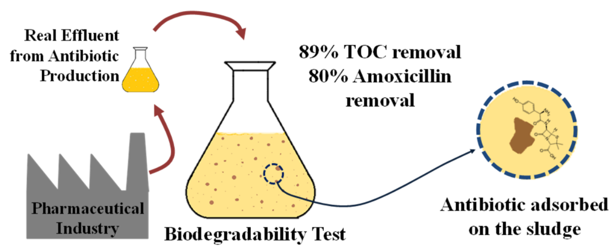

Figure 6: Representative scheme of the highlights of this research.

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