

## Evaluation of the aerobic and anaerobic biodegradability of the antibiotic norfloxacin

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### ABSTRACT

The purpose of studying the biodegradability of pharmaceutical compounds is to evaluate their behaviors in relation to the treatment processes generally used in domestic and industrial wastewater treatment plants. The antibiotic norfloxacin was found to be a recalcitrant compound. The studies conducted showed norfloxacin removal rates of 12% and 18% when biomasses from treatments with activated sludge and anaerobic biodigesters, respectively, were used without acclimatization. This suggests that anaerobic digestion shows better performance for norfloxacin removal. Ecotoxicological tests, using the luminescent marine bacteria *Aliivibrio fischeri* as the test organism, show that anaerobic digestion could eliminate the toxicity of the antibiotic norfloxacin, even though total degradation of the drug was not observed. The release of norfloxacin during cell lysis suggests the importance of controlling this phenomenon in biological treatment systems that handle wastewater contaminated with norfloxacin, thus preventing the return of this drug to the environment.

**Key words** | biodegradability, norfloxacin, product degradation, product toxicity

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### INTRODUCTION

Large quantities of different classes of pharmaceuticals are consumed annually, worldwide. These pharmaceutical compounds include anti-inflammatories, antipyretics, analgesics, lipid regulators, antibiotics, antidepressants, chemotherapy agents, contraceptives, and more. Most of these compounds are not completely degraded during treatment processes, and they eventually reach water bodies and generate a series of consequences.

Lajeunesse *et al.* (2012) investigated the behavior of fourteen antidepressants and one anticonvulsive during wastewater treatment using five different biological treatments in sewage treatment plants (STPs) in Canada. The concentrations of the pharmaceuticals were determined in the influents and effluents of the STPs. Of the 15 compounds studied, 13 were detected after biological treatment. The results showed that STPs have moderate capacity for removing compounds, and thus, new treatment technologies should be added to the processes.

Evaluation of the capacity to remove pharmaceuticals using biological treatment processes is fundamentally important, since these treatments are widely used for treating industrial and domestic effluents. Mascolo *et al.* (2010) evaluated the aerobic biodegradability of naproxen (a non-steroidal anti-inflammatory drug), acyclovir (antiviral), and nalidixic acid (antibiotic from the fluoroquinolone family). The tests were conducted according to the methodology proposed by Zahn–Wellens and showed variable results depending on the species studied. Both naproxen and acyclovir were considered to be biodegradable, with total organic carbon (TOC) removal rates of more than 80%. Nalidixic acid proved to be very recalcitrant, with removal rates of less than 40%.

With regard to pharmaceuticals, antibiotics are a special case, because they correspond to the largest category of pharmaceuticals used in human and veterinary medicine. The worldwide annual consumption of antibiotics was

estimated between 100,000 and 200,000 tons (Kümmerer & Henninger 2003). Over 50 million pounds of antibiotics are produced annually in the USA, with approximately 60% for human use and 40% for animal agriculture (Brown *et al.* 2006). Since they are chemically stable compounds, it is estimated that 50–90% of the antibiotics ingested are excreted and released into domestic sewage. Depending on the quantity of antibiotics consumed and the rate of excretion, the antibiotics released in domestic sewage reach STPs, and if they are not degraded, they enter the environment (Kumpel *et al.* 2001).

The discussion about the importance of removing antibiotics in wastewater treatment systems also arises from the fact that they promote bacterial resistance. According to Liu *et al.* (2012a), there is growing concern about long-term exposure to antibiotics in the environment because of the increase in resistant bacteria, as well as potential toxicity for aquatic organisms and humans.

Norfloxacin, an antibiotic in the fluoroquinolone family, is specifically a potent broad-spectrum bactericide used primarily in the treatment of urinary and pulmonary infections. It is a drug that is prescribed excessively and, very often, unnecessarily. Lindberg *et al.* (2005) reported that fluoroquinolones are the antibiotics most frequently detected in wastewater and, in the majority of cases, above the quantification limit. In another study by Lindberg *et al.* (2006), the antibiotics norfloxacin and ciprofloxacin were detected in 97% of the analyzed samples, along with ofloxacin in 50% of those samples. There are studies that indicate some quinolones are not biodegradable. For instance, Mascolo *et al.* (2010), in which the authors concluded that nalidixic acid, a quinolone, could not be considered biodegradable since they observed that only 40% of the compound was removed by aerobic biological treatment.

This study investigates the aerobic and anaerobic biodegradability of the antibiotic norfloxacin, in a bench scale study, by means of reactors. The antibiotic selected for this study was norfloxacin, which belongs to the fluoroquinolone class. It was selected based on its use and occurrence in the aquatic environment. Furthermore, the toxicity of the gross and treated samples was investigated using ecotoxicological tests with the organism *Vibrio fischeri*, recently reclassified as *Aliivibrio fischeri* (Urbanczyk *et al.* 2007). The objective of this study, in addition to verifying the biodegradability of the analyzed pharmaceutical, was to verify the toxicity and possible formation of intermediate toxic compounds during the biological treatment processes.

## EXPERIMENTAL METHODS

### Chemicals

The purity rate of the acquired norfloxacin is 99.5% (USP), and the pharmaceutical solutions were made in ultrapure Milli-Q water at a concentration of 15 mg/L, which is the maximum solubility of norfloxacin in water. The solutions were prepared immediately prior to each analysis.

Glucose was added to the standard solution of norfloxacin in a concentration of 500 mg/L. This procedure was necessary to provide a second carbon source, and to standardize the concentration of TOC in all the reactors, including the controls.

Other chemicals used for the development of this study, mentioned hereinafter, were acquired from Merck (Germany), Carlo Erba (Italy), Sigma-Aldrich/Fluka Analytical (USA), and SDI (USA), each selected with the highest purity available.

### Sampling and acclimatization of the biomass

The biomasses used in the biodegradability tests were collected from activated sludge reactors and anaerobic biodigesters, all used to treat domestic sewage. Each collected biomass was acclimatized for approximately nine weeks, receiving an increase of 100 mL of antibiotic solution (15 mg/L) and glucose solution (500 mg/L) per week, respectively in these proportions: first week (1:4); second and third week (2:3); fourth, fifth and sixth week (3:2); seventh, eighth and ninth week (4:1). The end of the acclimatization period was defined by the increased biomass in terms of volatile solids.

To acclimatize the aerobic biomass, 2L reactors were assembled using 1,000 mL of aerobic sludge appropriately washed with deionized water, antibiotic/glucose solution according to the week of acclimatization, and 2 mL of each solution of nutrients (CaCl<sub>2</sub>, FeCl<sub>3</sub>·7H<sub>2</sub>O, MgSO<sub>4</sub>, and phosphate buffer) prepared in accordance with the biochemical oxygen demand (BOD) method (APHA 2012). Aeration was promoted in the reactors by using laboratory aerators, and the pH was maintained at 7.

To acclimatize the anaerobic biomass, 2L reactors were assembled using 1,000 mL of each anaerobic sludge appropriately washed with ionized water, antibiotic/glucose solution according to the weeks of acclimatization, and 200 mL of nutrient solution prepared from solutions of macro- and micro-nutrients (Table 1). To prepare the nutrient solution, 2 mL of the micronutrient solution and

**Table 1** | Composition of solutions of macro/micronutrients

Macronutrient solution		Micronutrient solution	
Reagents	Amount (g/L)	Reagents	Amount (g/L)
KH <sub>2</sub> PO <sub>4</sub>	1.5	FeCl <sub>3</sub> ·H <sub>2</sub> O	2
K <sub>2</sub> HPO <sub>4</sub>	6.5	ZnCl <sub>2</sub>	0.05
NH <sub>4</sub> Cl	5	CuCl <sub>2</sub> ·2H <sub>2</sub> O	0.03
Na <sub>2</sub> S <sub>9</sub> H <sub>2</sub> O	0.5	MnCl <sub>2</sub> ·4H <sub>2</sub> O	0.5
CaCl <sub>2</sub> ·2H <sub>2</sub> O	1	(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> ·4H <sub>2</sub> O	0.05
MgCl <sub>2</sub> ·6H <sub>2</sub> O	1	NiCl <sub>2</sub> ·6H <sub>2</sub> O	0.05
		AlCl <sub>3</sub>	0.05
		CaCl <sub>2</sub> ·6H <sub>2</sub> O	2
		H <sub>3</sub> BO <sub>4</sub>	0.01
		HCl	1 mL

200 mL of the macronutrient solution were used. These quantities were transferred to a 1 L flask, which was then filled with deionized water.

#### Description of tests for aerobic biodegradability; Zahn-Wellens method

To evaluate aerobic biodegradability, an adaptation of the methodology proposed by Zahn-Wellens (OECD 1995) was employed.

Two 1.5 L reactors were employed; one was fed with antibiotic solution and the other with glucose solution (control reactor). Each reactor received 1.5 mL of nutrient solution (CaCl<sub>2</sub>, FeCl<sub>3</sub>·7H<sub>2</sub>O, MgSO<sub>4</sub>, and phosphate buffer) prepared according to the BOD method (APHA 2012), aerobic sludge (properly washed with deionized water) with a concentration of 0.5 g/L of volatile solids, solution containing the antibiotic to be analyzed, and glucose. In the control reactors, the only source of available carbon was glucose with a concentration of 500 mg/L. It is worth noting that the experiment was divided into two stages; the first stage with unacclimatized sludge and the second with acclimatized sludge. Aeration was promoted using laboratory aerators.

#### Description of tests for anaerobic biodegradability, Field *et al.* method

To evaluate anaerobic biodegradability, an adaptation of the methodology proposed by Field *et al.* (1988) was employed.

Two 1.5 L reactors were employed; one was fed with antibiotic solution and the other with glucose solution (control reactor). Each reactor was fed with 200 mL of

micronutrient/macronutrient solution (Table 1), as well as anaerobic sludge collected from anaerobic biodigesters (properly washed with deionized water) with a concentration of 0.5 g/L of volatile solids, the solution of antibiotic (15 mg/L) and glucose (500 mg/L), until completion of reactor volume.

Similar to aerobic biodegradability, glucose was the only source of carbon in the control reactors. For the anaerobic reactors, the experiment was again divided into two stages; the first stage with unacclimatized sludge and the second stage with acclimatized sludge in a concentration of 0.5 g/L of volatile solids.

#### Measuring and monitoring of norfloxacin

Norfloxacin samples were collected every 2 days. The aliquots collected were filtered using 0.45 µm hydrophilic PVDF membranes, and were analyzed immediately after collection. A UV/Vis spectrophotometer (Perkin Elmer, Lambda XL) was used to monitor the norfloxacin. The limit of detection (LOC) of the equipment for norfloxacin is 0.10 mg/L and the limit of quantification (LOQ) is 0.30 mg/L. The molar absorptivity was determined at 273 nm. To evaluate the mineralization of the antibiotic, a TOC Analyzer (Shimadzu, TOC-V<sub>CPN</sub>) with an ASI-V automatic sampler was used. The LOC of the equipment for norfloxacin is 1.00 mg/L and the LOQ is 3.03 mg/L. To evaluate soluble microbial product (SMP) concentrations, a 10 mL sample of sludge was collected and filtered at 0.4 µm, after which the permeate, comprised mainly of SMPs, was characterized in terms of TOC. The concentration of residual norfloxacin in terms of TOC was subtracted.

#### Toxicity biotests

Toxicology tests were conducted using the luminescent marine bacteria *Aliivibrio fischeri*. The gross and treated samples were tested. The treated samples were filtered twice using a semi-qualitative filter before ecotoxicological testing.

The tests were conducted using the MICROTOX<sup>®</sup> Model 500 Analyzer (SDI). All necessary solutions, mentioned below, were also obtained from SDI, including the lyophilized bacteria *Aliivibrio fischeri*, which were stored at -20 °C and reactivated immediately before the tests through hydration with reconstitution solution.

The tests were conducted according to the protocol established for the MICROTOX<sup>®</sup> Model 500 Analyzer software (MICROTOX<sup>®</sup> Omni Software, version 4.1). Changes

in the luminescence of the organisms were measured after 30 minutes of exposure to the contaminant. The analysis was performed on nine dilutions and one control; the latter contained only bacteria in a saline medium (osmotic adjustment solution). These dilutions, made by using a dilution solution, were conducted in series from the original sample, having a 1:2 ratio. It is worth noting that, due to the addition of osmotic adjustment solution at the beginning of the analysis, the most concentrated sample maintained a concentration of 81.9% in relation to the original.

## RESULTS AND DISCUSSION

### Aerobic and anaerobic biodegradability tests

Figure 1 presents the results obtained during the aerobic and anaerobic biodegradation processes by determining the TOC.

The removal of approximately 85% of TOC in the reactors was observed. This reduction of organic material, however, is related to the removal of glucose, which is readily biodegradable, which can be confirmed by the maintenance of the drug concentration after treatment, Figure 2. A series of studies have demonstrated that most antibiotics are not biodegradable under aerobic treatment conditions (Gartiser *et al.* 2007; Li *et al.* 2008). These results suggest that similar situations should occur in STPs, where biological treatment is frequently used and high removal rates of

chemical oxygen demand (COD) and BOD are generally verified. In the STPs, because the techniques for identifying remaining recalcitrant compounds in the effluent are not used, it is not possible to specify which compounds are present in the released effluent.

By evaluating the concentration of norfloxacin using UV/Vis spectrophotometry (Figure 2), it was confirmed that the target compound was not completely biodegraded by the employed biomasses, even after the acclimatization process.

In terms of biodegradability efficiency, a biodegradation rate of 18% with anaerobic biomass and a rate of 13% with aerobic biomass, was observed after 30 days of trial. This difference in efficiency can be explained by the fact that norfloxacin is an antibiotic that has a broad-spectrum activity against aerobic organisms, which suggests its presence cause major toxicity in aerobic reactors.

Laboratory analyses showed an increase in the concentration of norfloxacin in the reactors that used acclimatized biomass, compared to those in which unacclimatized biomass was used. For the reactors that contained biomass obtained from processes with activated sludge and anaerobic biodigesters, increases of 36% and 32%, respectively, were found in the concentration of the antibiotic. These results indicate that, in periods of low availability of organic material, cellular lysis occurs and part of the norfloxacin previously assimilated is released into the reaction medium. These results were confirmed by monitoring the control reactors (Figure 2(b)). In these reactors, the presence

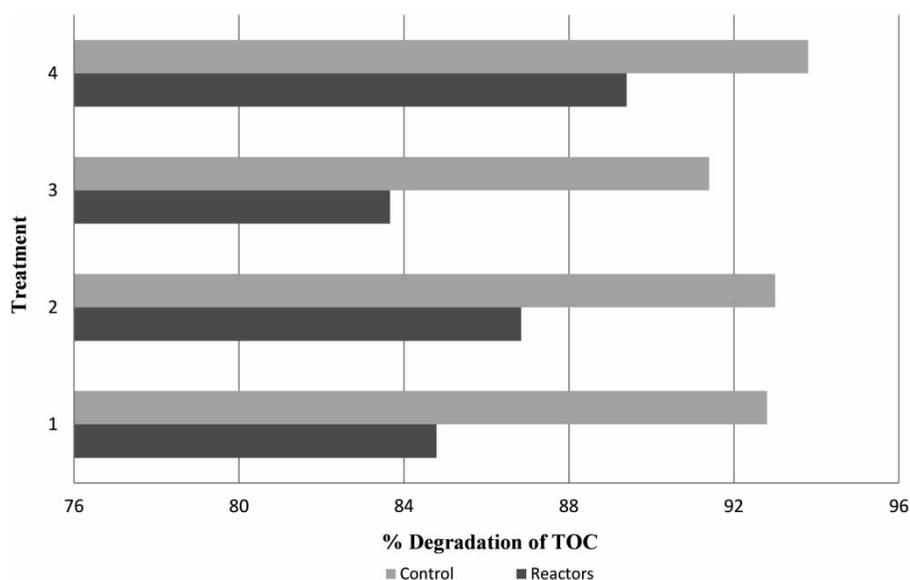
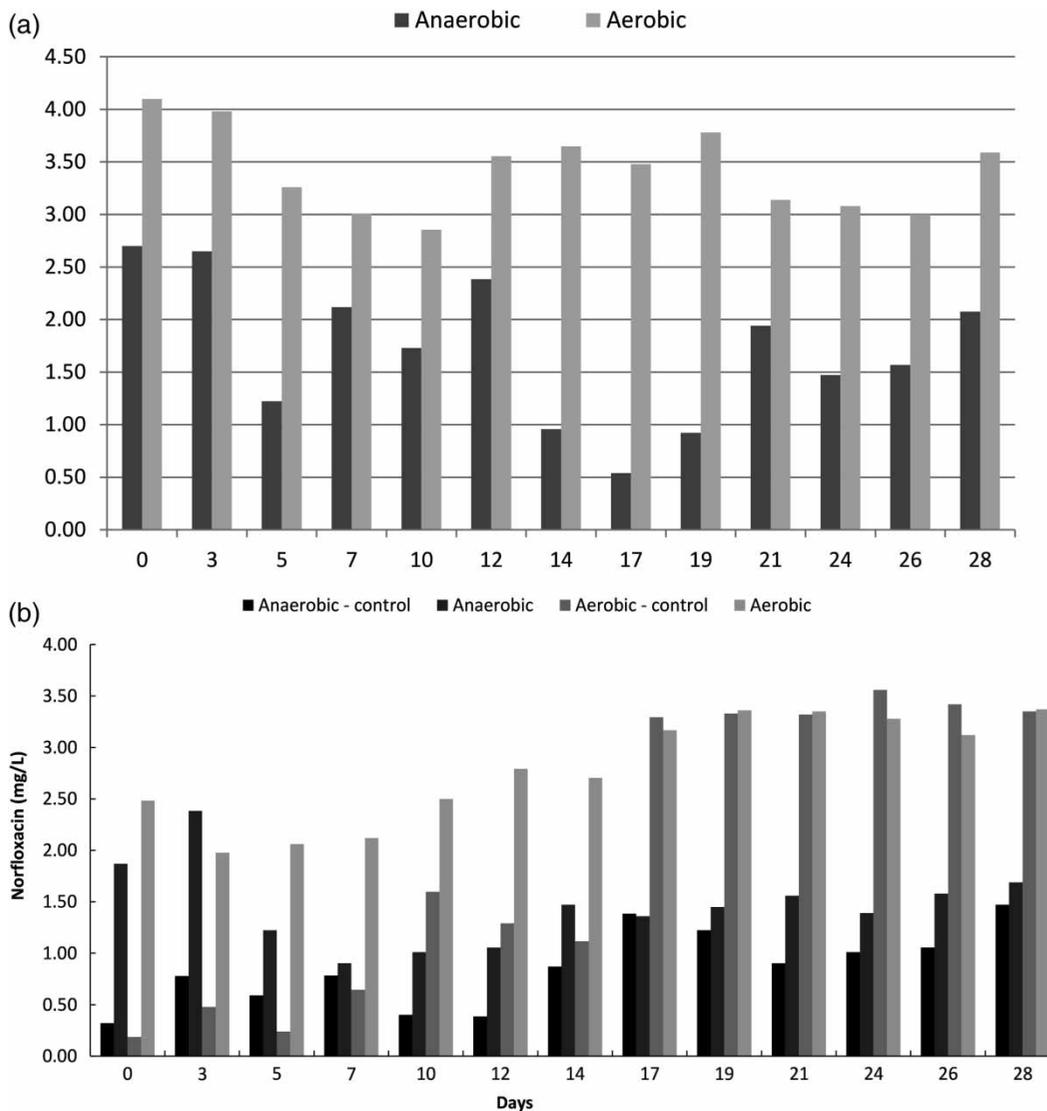


Figure 1 | Monitoring of the aerobic and anaerobic biodegradability of the reactors, which contain the antibiotic norfloxacin, and control reactors (1) anaerobic biodigester, (2) anaerobic biodigester acclimatized, (3) activated sludge and (4) activated sludge acclimatized.



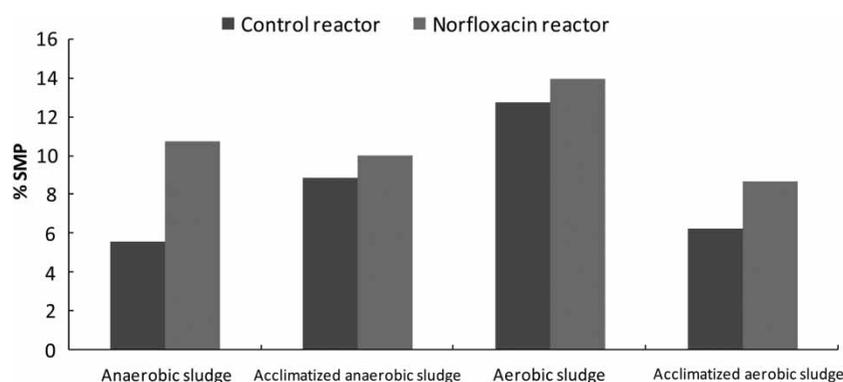
**Figure 2** | Monitoring of the aerobic and anaerobic biodegradability of the antibiotic norfloxacin. (a) Without biomass acclimatization and (b) with biomass acclimatization, by UV/Vis spectrophotometry.

of norfloxacin arises exclusively from those bacteria which previously absorbed the antibiotic during their acclimatization. The antibiotic is released in the reaction medium due to the cellular lysis process. These control reactors were assembled solely with a solution of nutrients, glucose, and acclimatized biomass.

These results indicate that a portion of the drug, previously absorbed, is released by the bacteria into the reaction medium after the reduction in the concentration of available organic carbon. This is shown in Figure 3, where it is possible to verify the presence of SMPs in the medium. It shows the increase of cell lysis in those reactors fed by norfloxacin, once the concentration of SMPs in these

reactors is higher than the control reactors. The soluble microbial products generated by microbial populations can adversely affect the efficiency of the biological wastewater treatment systems and secondary effluent quality (Xie *et al.* 2012).

It was also found that the bacteria from the aerobic reactors, compared to the bacteria from the anaerobic reactors, release larger quantities of norfloxacin into the reaction medium, suggesting that these bacteria have a lower degradation capacity for the drug. These results indicate the importance of controlling cell lysis in biological treatment systems that handle wastewater contaminated with norfloxacin.



**Figure 3** | Percentage of soluble microbial products (SMP) in the medium of activated sludge and anaerobic digesters.

### Ecotoxicological tests

The ecotoxicological analyses conducted demonstrated that norfloxacin at 15 mg/L is toxic, with the EC<sub>50</sub> of 26.25% after 30 minutes of exposure. The value found for EC<sub>50</sub> corresponds, therefore, to a 3.9 mg/L concentration. This value differs from previous studies (Backhaus *et al.* 2000) conducted at different time intervals, which indicate the toxicity of the drug for the same organism, *Aliivibrio fischeri*, to be equal to 22 µg/L in tests with an exposure time of 24 hours. In fact, it is true that a lower EC<sub>50</sub> is expected in longer analyses. Studies with the algae *P. subcapitata* and *Chlorella vulgaris* indicate, respectively, an EC<sub>50</sub> for norfloxacin equal to 16.6 mg/L and 10.4 mg/L (Eguchi *et al.* 2004).

In tests conducted on effluent from the anaerobic digesters, there was evidence of toxicity. That is, the reaction medium was not toxic (EC<sub>50</sub> > 81.9%), but there was an effect of light inhibition on the bacteria. In this case, the results are expressed as 'highest percentage of effect', which means the largest effect of light inhibition detected. In the reactor with unacclimatized biomass, the largest observed effect of light inhibition was 12.99% in 30 minutes. In the reactor with acclimatized biomass, this effect was 7.88% in 30 minutes, less than that observed in the presence of unacclimatized bacteria, which are, in fact, less efficient.

It is important to clarify that the samples whose toxicity results are expressed in 'highest percentage of effect' generally are toxic in longer acute toxicity tests or in chronic toxicity tests. A series of studies indicates that the fact a chemical substance does not produce toxic effects on aquatic organisms in acute toxicity tests does not indicate that the substance is not toxic for the organisms. This verification has been made many times through chronic toxicity tests (Costa *et al.* 2008; Magalhães & Filho 2008).

The results found on the effluent from the activated sludge process reactors were the most toxic among those evaluated, an occurrence that could be justified by the lower efficiency of this treatment in comparison with the others. The results of toxicity in the aforementioned effluent elucidate that those derived from reactors with acclimatized biomass present higher toxicity (EC<sub>50</sub> (30 min) 2.14% for the reactor with norfloxacin; EC<sub>50</sub> (30 min) 1.31% for the control reactor) than the reactors with unacclimatized biomass (EC<sub>50</sub> (30 min) 25.94%). This situation is due to the cellular lysis process (discussed above) that caused an additional concentration of norfloxacin, of endogenous origin, to be added to the reaction medium after the cellular lysis process, especially in the activated sludge process. Another possible hypothesis could be that the bacteria derived from the acclimatized biomass generate a larger quantity of toxic sub-products during the degradation of the drug.

### CONCLUSIONS

Among the different biomasses used, the most efficient for the removal of norfloxacin came from the anaerobic biodigester, suggesting that the anaerobic process shows better performance for norfloxacin removal.

The biomass acclimatization step is important for the improvement of various removal processes. However, even with acclimatized sludge, the biological process was not efficient for the complete removal of norfloxacin.

The release of norfloxacin during cell lysis suggests the importance of controlling this phenomenon in biological treatment systems that handle wastewater contaminated with norfloxacin, in preventing the return of the drug to the environment. The cell lysis phenomenon was demonstrated by the increase in the concentration of norfloxacin

in the reactors in which acclimatized biomass was used, in comparison with those in which unacclimatized biomass was used.

As for the ecotoxicological tests, it is possible to say that biological treatment with anaerobic bioreactors is sufficient to eliminate the toxicity of the antibiotic norfloxacin, even though significant rates of degradation of the drug were not shown. However, in relation to the process with activated sludge, all of the effluents generated were toxic, especially those from reactors with acclimatized biomass. It can be assumed that this is due to the process of cellular lysis, which caused the addition of a concentration of norfloxacin to the reaction medium after the lysis process.

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