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ANALYSIS OF NORFLOXACIN ECOTOXICITY AND THE RELATION WITH ITS DEGRADATION BY MEANS OF ELECTROCHEMICAL OXIDATION USING DIFFERENT ANODES

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Abstract

In this work, ecotoxicological bioassays based on *Lactuca sativa* seeds and bioluminescent bacterium (*Vibrio fischeri*) have been carried out in order to quantify the toxicity of Norfloxacin (NOR) and sodium sulfate solutions, before and after treating them using electrochemical advanced oxidation. The effect of some process variables (anode material, reactor configuration and applied current) on the toxicity evolution of the treated solution has been studied.

A NOR solution shows *an* EC_{50} *(5 days) of 336* $mg \cdot L^{-1}$ *towards Lactuca sativa*. This threshold NOR concentration decreases with sodium sulfate concentration, in solutions that contain simultaneously Norfloxacin and sodium sulfate.

In every case considered in this work, the electrochemical advanced oxidation process increased the toxicity (towards both *Lactuca sativa* and *Vibrio fischeri*) of the solution. This toxicity increase is mainly due to the persulfate formation during the electrochemical treatment. From a final solution toxicity point of view, the best results were obtained using a BDD anode in a divided reactor applying the lowest current intensity.

Keywords: Electrochemical oxidation; *Lactuca sativa*; Norfloxacin; Sodium sulfate; Toxicity; *Vibrio fischeri*.

Highlights

- 5 day EC_{50} of NOR for *Lactuca sativa* is 336 $mg \cdot L^{-1}$.
- Na_2SO_4 diminishes the EC_{50} (5 days) of NOR, but no synergy is observed.
- After electrochemical oxidation, the formation of persulfates increases toxicity.
- The samples treated using a BDD anode show higher toxicity values.
- The higher the applied current the higher the toxicity values obtained

1. Introduction

Since recent years, personal care products and pharmaceuticals are considered emerging contaminants affecting ecosystems and human health. Antibiotics are one of the main types of these emerging pollutants [1]. In fact, antibiotics are toxic, not biodegradable, and capable of accumulating in aquatic organisms. These compounds might also lead to different harmful environmental effects, such as the development of antibiotic resistance in aquatic bacteria, direct toxicity to microorganisms and possible risks to human health through drinking water and/or food-chain [2].

The main sources of antibiotic discharge into the environment are pharmaceutical industries, hospitals and residential buildings. These discharges affect the physical, chemical and biological composition of receptor water bodies [3]. Despite the efforts made to clean up the contaminated effluents by conventional wastewater treatment plants, industry and scientists face a great challenge: to develop methods powerful enough to be able to remove these recalcitrant pollutants from wastewaters, while minimizing the ecotoxicological effects of the water discharged after the treatment.

Despite the efforts made to clean up the contaminated effluents by conventional wastewater treatment plants, industry and scientists face a great challenge: to develop methods powerful enough to be able to remove these recalcitrant pollutants from wastewaters, while minimizing the ecotoxicological effects of the water discharged after the treatment [4]. The electro-oxidation of organic pollutants can be carried out in two different ways: by direct oxidation, in which the pollutant is oxidized by electron transfer directly to the anode material; and by indirect oxidation, where the electron transfer is mediated by an oxidant species, such as the hydroxyl radical. The efficiency of this technique mainly depends on the interaction between the hydroxyl radical and the anode material. Other important factors are the type and concentration of the supporting electrolyte, the applied current, solution pH, the nature of the target contaminant and its initial concentration [4–6].

However, in general, the advanced oxidation of complex organic contaminants does not lead to a fast mineralization (i.e. with the formation of carbon dioxide and inorganic species); instead, intermediate organic products are normally generated. Consequently, an increase of toxicity has sometimes been observed after the treatment, as these products may be more toxic than the parent compounds. In this context, bioassays have been used to assess the toxicity levels of the samples [7]. Some organisms belonging to different trophic levels are commonly used in ecotoxicological biomonitoring: primary producers (algae, i.e. *Pseudokirchneriella subcapitata* [8]), primary consumers (aquatic invertebrates, i.e. *Daphnia magna*, *Gammarus pulex* [9]) or secondary consumers (aquatic vertebrates, i.e. *Gambusia holbrooki* [10]). Although, toxicity studies using higher plants are less frequent than faunal tests, their number has increased in the last years [7,11], Indeed, nowadays, higher plants are recognized as excellent genetic models to detect environmental mutagens and are frequently used in monitoring studies [12].

The most common plant species recommended by the US Environmental Protection Agency or the US Food and Drug Administration are cucumber (*Cucumis sativus* L.), lettuce (*Lactuca sativa* L.), radish (*Raphanus* spp.), red clover (*Trifolium pratense* L.) and wheat (*Triticum aestivum* L.) [13]. Previous studies [13,14] compared some of these species and recommended *Lactuca sativa* as a bioindicator to determine the toxicity of soil and water samples. A. Heberle et al. [15] concluded that the inhibition of root elongation (RE) is a valid and sensitive indicator of environmental toxicity. In this context, several articles [14,16–19] have shown that phytotoxicity tests like seed germination rate (GR) and RE tests present many advantages.

These bioassays are simple, inexpensive and only require a relatively small amount of sample. Moreover, the seeds remain usable for a long time [13].

Luminescent microorganisms, such as the marine bioluminescent bacterium Vibrio fischeri, have also been used in several toxicity test instruments [7]. These tests are based on the change in the bacterial luminescence when exposed to toxic chemicals. Their main advantages are their simplicity and the little time required to perform the test.

A number of studies on toxicity of different fluoroquinolones, a family of antibiotics widely used in human and veterinarian medicine, have been conducted [20–25]. According to the literature, fluoroquinolones are considered highly toxic to bacteria, toxic to algae and plants, and dangerous to fish and crustaceans.

The main goal of this work is to quantify the toxicity of NOR and sodium sulfate solutions, before and after treating them by electrochemical advanced oxidation; and to study the effect of some of the process variables (anode material, reactor configuration and applied current) on the toxicity change during the treatment. The toxicity studies were done using *Lactuca sativa* growth inhibition tests and *Vibrio fischeri* luminescence inhibition tests.

2. Material and methods

2.1. Tested pollutants

NOR ($C_{16}H_{18}FN_3O_3$, Sigma-Aldrich) and sodium sulfate (analytical grade Na_2SO_4, Panreac) solutions were prepared, with concentrations ranging from 0 to 1000 $mg \cdot L^{-1}$ and from 0 to 10 $g \cdot L^{-1}$, respectively. These concentration values have been selected based on those used in the electrochemical reactor. The effect of NOR and sodium sulfate concentrations, and the pH effect, on the phytotoxicity towards *Lactuca sativa* was evaluated. When necessary, the pH was adjusted using 0.1M sodium hydroxide (analytical grade NaOH, Panreac) or 0.1M sulfuric acid (H_2SO4, J.T. Baker). Table 1 presents the experimental conditions used for each experiment.

Table 1. Experimental conditions tested for the Lactuca sativa tests

SAMPLE	[NOR] ($mg \cdot L^{-1}$)	Na_2SO_4 ($g \cdot L^{-1}$)	pH
1. pH effect	100	0	1.3, 3.1, 5.7, 6.9, 8.8, 11.3
2. [Na_2SO_4] effect	0	0.5, 1, 2, 4, 6, 8, 10	~ 6.5
3. [NOR] effect	50, 100, 150, 200, 350, 500, 1000	0	~ 6.2
4. [NOR] and [Na_2SO_4] combined effect	50, 100, 150, 200, 350, 500, 1000	2	~ 6.1
	50, 100, 150, 200, 350, 500, 1000	4	~ 6.1
	50, 100, 150, 200, 350, 500, 1000	6	~ 6.1

115 *2.2. Electrochemical advanced oxidation experiments*

116 The toxicity evolution of a contaminated solution after the application of the advanced
117 oxidation method under different experimental conditions was quantified by using both, the
118 *Lactuca sativa* and the *Vibrio fischeri* methods. In this case, 100 $mg \cdot L^{-1}$ NOR solutions, with 2
119 $g \cdot L^{-1}$ or 14 $g \cdot L^{-1}$ of Na_2SO_4, were respectively treated in an undivided electrochemical reactor
120 and in a two-compartment electrochemical reactor divided by a cation-exchange membrane
121 (Nafion 117, Dupont), during 4 hours. A stainless steel sheet and a standard Ag/AgCl electrode
122 were used as counter and reference electrodes, respectively. Two different materials were
123 tested as anode: a Boron-doped diamond (BDD) electrode with a doping level of 2500 $mg \cdot L^{-1}$
124 purchased from NeoCoat SA (Switzerland), and a new microporous Sb-doped SnO_2 ceramic
125 electrode provided by the Institute of Ceramic Technology [26]. Table 2 presents the different
126 experimental conditions evaluated (type of anode, current applied, presence or absence of
127 membrane). The NOR final concentration was determined by UV/Visible spectrophotometry
128 (Unicam UV4-200 UV/Vis Spectrometer) at 277 nm and the final total organic carbon (TOC)
129 concentration was measured using a Shimadzu TNM-L ROHS TOC analyser [27]. The pH of the
130 samples treated by electrochemical oxidation was adjusted to the 6.0-6.3 range before
131 carrying out the toxicity tests.

132 *Table 2. Experimental conditions tested for Lactuca Sativa and Vibrio fischeri tests*

REACTOR TYPE	ANODE MATERIAL	APPLIED CURRENT (A)	pH
Undivided	BDD	0.4	6
		0.6	
		1	
	Ceramic	0.6	
		1	
Divided	BDD	0.4	6.3
		0.6	
		1	
	Ceramic	0.6	
		1	

133

134 *2.3. Ecotoxicological assays with Lactuca sativa*

135 Ecotoxicological tests were carried out using commercial *Lactuca sativa* seeds (Batavia variant,
136 Reina de Mayo). Once received, the seeds were stored at 5°C; and they were used without any
137 pretreatment. Seven experiments, each consisting in four replicates, were carried out. For
138 each replicate, 20 seeds were placed in a Petri dish containing filter paper (90 mm diameter,
139 Whatman 3) impregnated with 4 ml of the solution under study. The Petri dishes were kept at
140 20°C in the dark, for 5 days.

141 After the 5 days, the decrease in seed root elongation was measured and the obtained data set
142 was analyzed using the free software Past 3.20. First, the Shapiro-Wilk test was used in order
143 to assess the normality of the data. After that preliminary analysis, the considered treatments
144 were compared with the negative control (distilled water). This comparison was done using
145 Tukey's pairwise test of means, for normally distributed data; whereas, Mann-Whitney test

was used for non-normally distributed data. A confidence level of 95% was used in both cases. A test was considered valid when at least 65% of the seeds from the negative control germinated [28].

In addition, a statistical analysis was carried out in order to quantify the effect of the experimental factors (i.e. NOR and Na_2SO_4 concentrations) on the response variable (i.e. root elongation decrease with respect to the distilled water control). This statistical analysis was performed using Statgraphics Plus 5.1 (Manugistics, Inc., Rockville, MA, USA). First, an ANOVA analysis was done in order to determine which factors, and which interactions, had a statistically significant effect on the dependent variable. After that, the response surface method (RSM) was used in order to build a black box model relating the relative root decrease (expressed as a percentage) with the factors and interactions that have a statistically significant effect on it (identified by the aforementioned ANOVA analysis). Finally, the built black box model was used to estimate the EC_{50}, defined as the concentration of contaminant that produces 50% inhibition of the root elongation with respect to the control.

Although the RSM is not the standard method used in ecotoxicology to estimate EC_{50} values, Mao and co-workers have recently demonstrated that the RSM is a useful tool in ecotoxicological studies [29]. The main advantage of this methodology is that it requires less experimental points (i.e. fewer experiments) than the traditional methodology [30]. This is the reason why it was preferred for this work.

2.4. Ecotoxicological tests with Vibrio fischeri

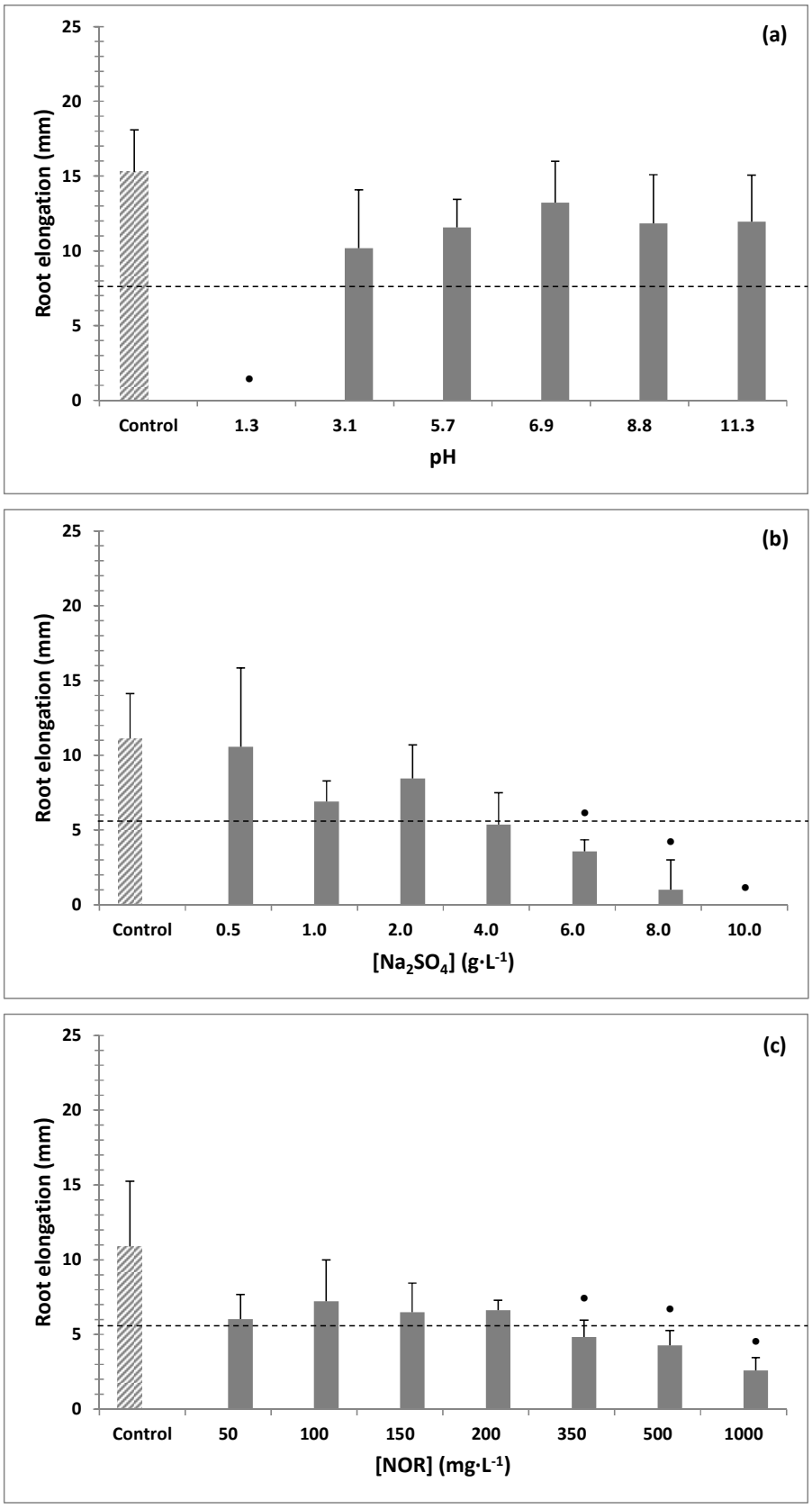
The acute toxicity effects of the different solutions on *Vibrio fischeri* bioluminescence were quantified using a Microtox model M-500 toxicity analyzer (Strategic Diagnostics Inc., USA) following the manufacturer's instructions. The *Vibrio fischeri* bioluminescence inhibition test has been standardized [7] and is commercially available in different versions. The tests were carried out at 15°C, a salinity level of 2% NaCl and a pH in the 6 to 8 range. An exposure time of 15 minutes was used for all the tests. Toxicity can be expressed as *n*-TU (toxicity units), where *n* is the number of times that a sample has to be diluted in order to inhibit the luminescence of 50% of the luminescent microorganisms at 15°C after a 15 min exposure time. Each sample presented in Table 2 was analyzed in triplicate, and values of toxicity (expressed as TU) were obtained by the MicrotoxOmni software supplied by the manufacturer and compared with those obtained using the *Lactuca sativa* method under the same experimental conditions.

3. Results and discussion

3.1. pH effect on ecotoxicity of Norfloxacin towards Lactuca sativa

During an electrochemical process, changes in the pH of the solution may occur due to secondary reactions (eg, water oxidation and reduction reactions). Therefore, pH is an important parameter and its influence on the toxicity of Norfloxacin must be analyzed. This analysis was carried out by the *Lactuca sativa* test using a Norfloxacin solution of 100 $mg \cdot L^{-1}$. Figure 1(a) shows the results of this study, where error bars represent the mean value (arithmetic average) of the root elongation (M) ± the standard deviation (SD) of the four replicates. A sample is considered toxic when it presents a root elongation lower than 50% of the control root elongation (distilled water, represented by a dashed line). According to this definition, Figure 1(a) shows that samples with a pH between 3.1 and 11.3 are not toxic

towards *Lactuca sativa*. The neutral pH sample displayed a root elongation very similar to its corresponding control root elongation. In fact, this sample is the sample for which the root elongation is the closest to its corresponding control. Slightly acidic and basic samples show similar values of root lengths; whereas, the extremely acidic sample (pH = 1.3) turned out to be very toxic towards *Lactuca sativa* since no seed germination was observed. These results agree with the biologically active pH range (i.e. 5.5 to 8.0).



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200 *Figure 1. Effect of (a) pH, (b) Na_2SO_4 concentration and (c) NOR concentration on root*
201 *elongation of Lactuca sativa seeds. Error bars represent the M ± SD of four replicates; black*
202 *points (•) represent statistically significant difference from the negative control (distilled water)*
203 *for a confidence level of 95%*

Therefore, from these results, it can be concluded that the pH for the rest of the tests with *Lactuca sativa* should be comprised between 6 and 11. In this way, the pH will have a low influence on the toxicity of the samples and will not mask the effect of the other variables under study. Specifically, the pH will be adjusted between 6 and 7 in all the samples. This also agrees with the Spanish legislation regarding wastewater discharge, which allows pH values between 5.5 and 9.0.

3.2. Effect of Na_2SO_4 concentration on ecotoxicity towards Lactuca sativa

Sodium sulfate is commonly used as supporting electrolyte, which is required to increase the conductivity of a solution that is going to be treated by an electrochemical process. Therefore, it is important to analyze its effect on the toxicity in order to differentiate its contribution from the one of the pollutant under study. Figure 1(b) shows the results of the *Lactuca sativa* test for different Na_2SO_4 concentrations in the absence of Norfloxacin.

Figure 1(b) shows that, as the concentration of supporting electrolyte increases, the inhibition in the root elongation is higher; and therefore, the toxicity of the samples is greater. Moreover, for 6, 8 and 10 $g \cdot L^{-1}$ of Na_2SO_4 the decrease in the root elongation becomes higher than 50%. Because of this, these samples are considered toxic towards *Lactuca sativa*. The most extreme case was for a Na_2SO_4 concentration of 10 $g \cdot L^{-1}$, which totally inhibits the root growth.

These data were used to estimate the EC_{50}(5 days) of Na_2SO_4 towards *Lactuca sativa* (i.e. the effective concentration that lead to a 50% root elongation inhibition) by linear interpolation, obtaining a value of 4.8 $g \cdot L^{-1}$. According to this result, a supporting electrolyte concentration higher than 6 $g \cdot L^{-1}$ will not be used in the rest of the tests with *Lactuca sativa*, since the samples will be toxic even in the absence of Norfloxacin. Even if 6 $g \cdot L^{-1}$ of Na_2SO_4 is considered toxic for *Lactuca sativa*, at this concentration the seeds can germinate and the root elongation is long enough to be measured. Therefore, this Na_2SO_4 concentration was included in the study, although it is considered toxic for *Lactuca sativa*, in order to observe differences and make further calculations.

3.3. Effect of NOR concentration on ecotoxicity towards Lactuca sativa

Figure 1(c) shows the results obtained with *Lactuca sativa* seeds for different NOR concentrations, which is the pollutant under study, in the absence of supporting electrolyte. The decrease of the root elongation of the seeds increases with the concentration of NOR and, therefore, the toxicity of the samples becomes higher. The samples are not toxic towards *Lactuca sativa* up to 200 $mg \cdot L^{-1}$ of Norfloxacin; however, from 350 $mg \cdot L^{-1}$ of NOR all the samples can be considered toxic, as indicated by both parameters (p-value < 0.05 and inhibition of root growth greater than 50%).

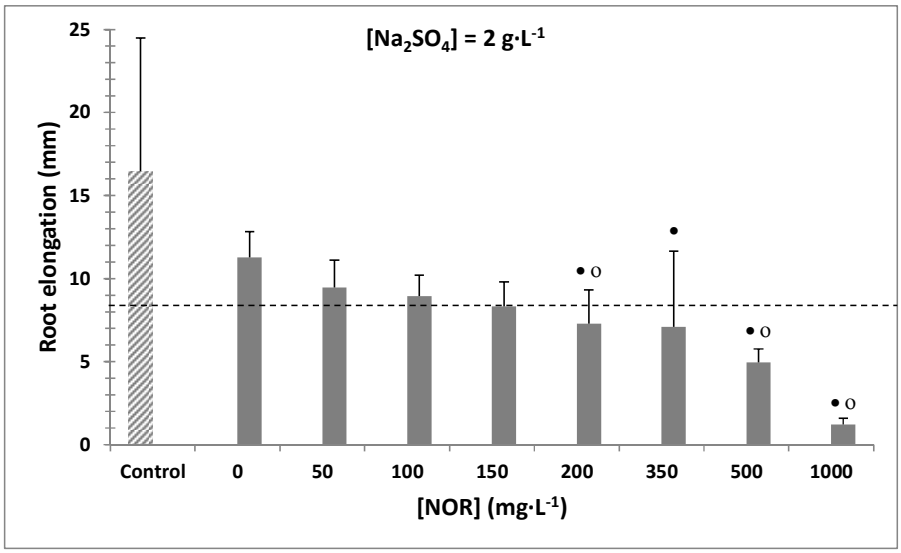
Applying linear interpolation to these data, the EC_{50}(5 days) of NOR towards *Lactuca sativa* was estimated, obtaining a value of 336 $mg \cdot L^{-1}$ in absence of supporting electrolyte. Comparing this value with that obtained for Na_2SO_4 (4.8 $g \cdot L^{-1}$), NOR is considerably more toxic than the supporting electrolyte and the ratio between both EC_{50}(5 days) values is approximately 14:1 (Na_2SO_4:NOR). Similar results were reported in other works [31,32] which obtained an EC_{50}(48 hours) value for NOR of 180 $mg \cdot L^{-1}$ and an EC_{50}(48 hours) value for Na_2SO_4 of 2564 $mg \cdot L^{-1}$ [33], using the *Daphnia magna* test. These values lead as well to the 14:1 ratio (Na_2SO_4:NOR)

obtained in this work. Therefore, the results obtained with *Lactuca sativa* are consistent with those obtained with *Daphnia magna*.

3.4. Combined effect of NOR and Na_2SO_4 concentrations on ecotoxicity towards Lactuca sativa

Figure 2 shows the results obtained with the *Lactuca sativa* test for three concentrations of supporting electrolyte (2, 4 and 6 $g \cdot L^{-1}$).

Comparing the data presented in Figure 2 with Figure 1(c) (in the absence of supporting electrolyte), it is inferred that the addition of Na_2SO_4 to the Norfloxacin samples, leads to an increase in the toxicity of the sample towards *Lactuca sativa*. In addition, as the concentration of Na_2SO_4 increases, the value of the NOR concentration that inhibits more than 50% the elongation of the root with respect to the control decreases. Specifically, for a concentration of 2 $g \cdot L^{-1}$ of Na_2SO_4, both the root elongation and the p-value show that, beyond 200 $mg \cdot L^{-1}$ of NOR, samples are toxic towards *Lactuca sativa*; for 4 $g \cdot L^{-1}$ of Na_2SO_4 this value decreases down to 150 $mg \cdot L^{-1}$; and for 6 $g \cdot L^{-1}$ of Na_2SO_4 a decrease in root elongation higher than 50% is reached for all NOR concentrations, as it could be expected according to the results of Figure 1(b). Applying linear interpolation to these data, the EC_{50}(5 days) of NOR towards *Lactuca sativa* in the presence of supporting electrolyte was estimated, obtaining values of 178, 138 and 0 $mg \cdot L^{-1}$ for 2, 4 and 6 $g \cdot L^{-1}$ of Na_2SO_4, respectively.



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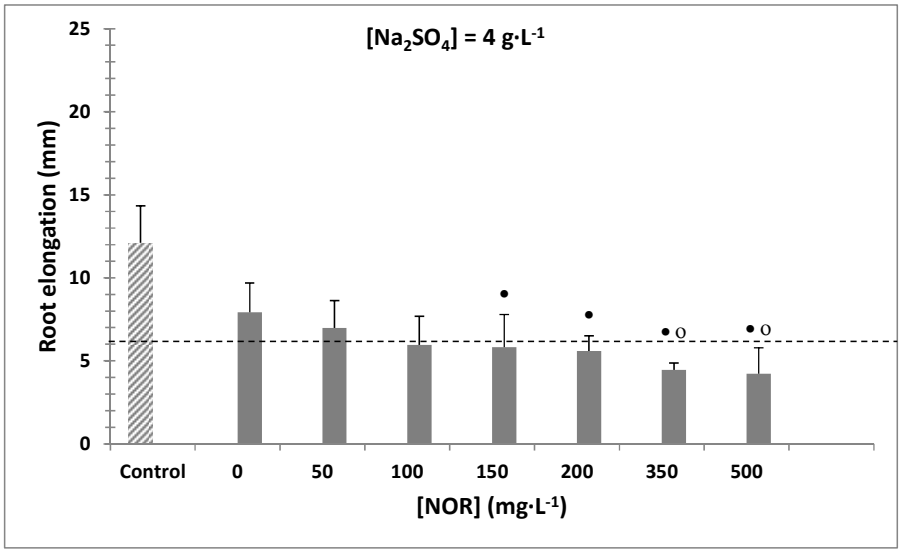

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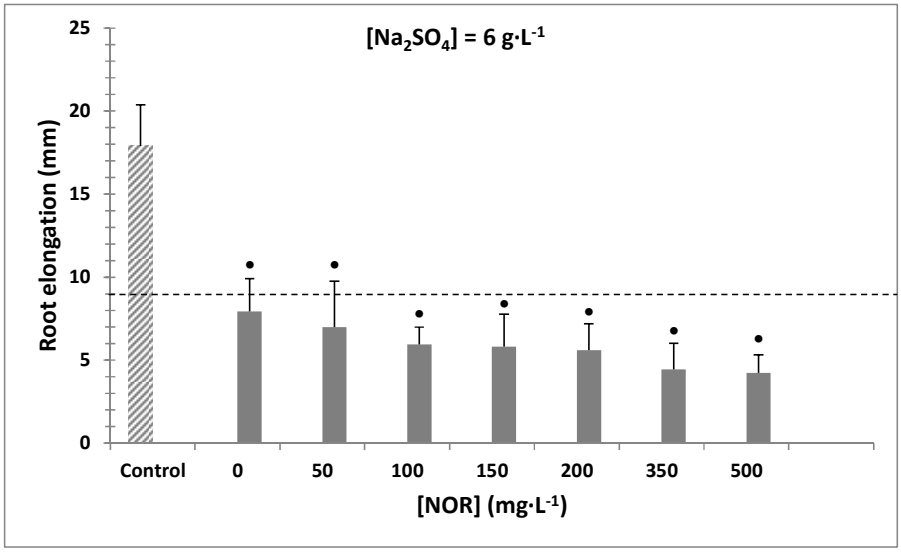

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269 *Figure 2. Combined effect of NOR and Na_2SO_4 concentrations on root elongation of Lactuca*
270 *sativa. Error bars represent the M ± SD of four replicates; black points (•) represent statistically*
271 *significant difference from the negative control (distilled water) for a confidence level of 95%*

and white points (○) represent statistically significant difference from 0 NOR concentration for a confidence level of 95%

In order to analyze a possible synergy phenomenon between Na_2SO_4 and NOR on toxicity, the decrease in root elongation (*DRE*) (Equation (1)) with respect to distilled water has been calculated.

$$DRE\ (\%) = ((Lc - L) / Lc) \cdot 100 \quad (1)$$

where *Lc* is the average root length for the control solution (distilled water) and *L* is the average root length for the test solution.

Figure 3 shows the DRE results for NOR in the presence of 2, 4 and 6 $g \cdot L^{-1}$ of Na_2SO_4. The grey bar corresponds to the decrease in root elongation observed for the different concentrations of NOR in the absence of supporting electrolyte; black bar represents the decrease in root elongation observed for the different concentrations of NOR in the presence of Na_2SO_4; and the bar with stripes corresponds to the sum of the individual effects (decrease in root elongation for NOR and Na_2SO_4 separately).

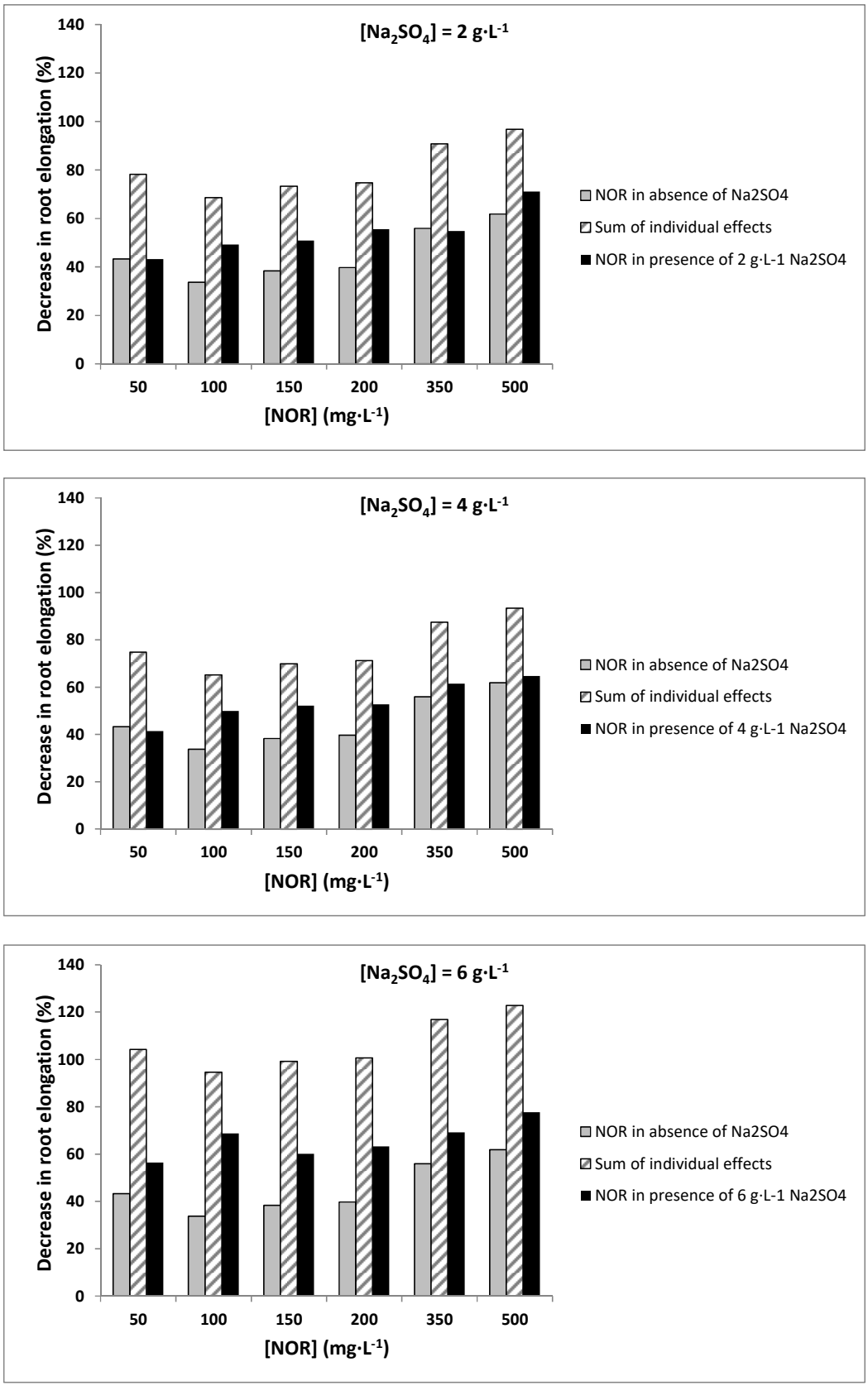

Figure 3. Analysis of synergy phenomenon between NOR and Na_2SO_4 on toxicity determined with the Lactuca sativa test.

On the one hand, as it can be observed in Figure 3, the decrease in root elongation is greater in the presence of supporting electrolyte (black bar) than in the absence of it (gray bar), for the three analyzed Na_2SO_4 concentrations. This confirms that the presence of Na_2SO_4 increases the toxicity of the samples towards *Lactuca sativa*, as it was discussed previously. On the other hand, the phenomenon of synergy occurs when the real effect (black bar) is greater than the theoretical sum of the two effects separately (bar with stripes); if the real effect is equal to the theoretical sum, an additive effect is observed; and if the real effect is lower than the theoretical sum, there is an antagonistic response. According to the results presented in Figure 3, the latter occurs. Therefore, there is no synergy between NOR and the supporting electrolyte; as the real effect is lower than the theoretical sum. Other authors have analyzed the combined toxic effects of heavy metals and NOR towards *Pseudomonas fluorescens Strain ZY2* [34] and the toxicity of mixtures of NOR and other pharmaceutical products towards aquatic organisms [35,36] and found an antagonistic effect in the first case, and in the latter one, a synergistic or antagonistic response depending on the other organic compounds present together with NOR in the solution.

An analysis of variance (ANOVA) was performed to evaluate the effects of the factors (NOR and Na_2SO_4 concentrations) and their interactions on the response variable (the decrease in root elongation respect to distilled water control). The Pareto chart was obtained once the non-statistically significant effects have been discarded, showing that both factors, NOR concentration and Na_2SO_4 concentration, have a statistically significant effect on the decrease of root elongation, for a confidence level of 95%. Moreover, the Pareto chart also indicates that the effect of the Na_2SO_4 concentration on the decrease in root elongation is somewhat greater than the effect of the NOR concentration. On the other hand, the positive sign of the effects indicates that both NOR and Na_2SO_4 concentrations produce an increase of the response variable (i.e. higher decrease in root elongation), as it was expected according to the previous results.

Furthermore, the RSM was applied in order to obtain the second order regression model relating the DRE (%) with both, NOR and Na_2SO_4 concentrations. The residuals defined as the difference between the experimental and the predicted values were analyzed. The outliers were not considered in the analysis, and the normality of the residues was confirmed using the standardized asymmetry and kurtosis coefficients, the normal probability plot and the Shapiro-Wilk normality test. In addition, only the statistically significant coefficients (i.e. effects that cross the significance threshold in the Pareto chart) were taken into account. The obtained quadratic model (with an R-squared value of 81.5%) is presented in Equation (2):

$$DRE\ (\%) = 30.3904 + 0.0584804 \cdot [NOR] + 1.02074 \cdot [Na_2SO_4] + 0.586096 \cdot [Na_2SO_4]^2 \quad (2)$$

where *[NOR]* is the NOR concentration expressed in $mg \cdot L^{-1}$ and *[Na_2SO_4]* is the sodium sulfate concentration expressed in $g \cdot L^{-1}$.

Equation (2) allows the calculation of the values of NOR and sodium sulfate concentrations beyond which a solution would be considered toxic towards *Lactuca sativa*, by substituting DRE by 50% and, reordering the terms. The following function of a parabola is obtained:

$$[NOR] = -10.0221 \cdot [Na_2SO_4]^2 - 17.4544 \cdot [Na_2SO_4] + 335.3192 \quad (3)$$

Figure 4 shows the sketch of Equation (3) in the first quadrant, since it is the only one with physical sense (positive values of the concentrations of the substances). The black line represents EC_{50}(5 days) defined as the combined concentrations of pollutants (NOR and Na_2SO_4) that produce inhibition of the root elongation of 50% with respect to distilled water.

All the points located above the black line correspond to toxic solutions towards *Lactuca sativa*. As observed in Figure 4, the predicted EC_{50}(5 days) values (expressed as a pair of concentrations) are similar to those obtained experimentally.

EC_{100}(5 days) values can also be calculated from Equation (2). These values are defined as the combined concentrations of pollutants (NOR and Na_2SO_4) that produce inhibition of the root elongation of 100% with respect to distilled water. In other words, the combined concentrations for which the *Lactuca sativa* seeds do not germinate. In this case, introducing the value 100% in Equation (2), and, after reordering the terms, this other parabola is obtained:

$$[NOR] = -10.0221 \cdot [Na_2SO_4]^2 - 17.4544 \cdot [Na_2SO_4] + 1190.3065 \quad (4)$$

The dotted line in Figure 4 represents the EC_{100}(5 days) values according to Equation (4). All the points located above the dotted line correspond to the most toxic solutions, which do not allow the *Lactuca sativa* seeds to germinate at all. The predicted EC_{100} values (expressed as a pair of NOR and Na_2SO_4 concentrations) are consistent with the experimental results.

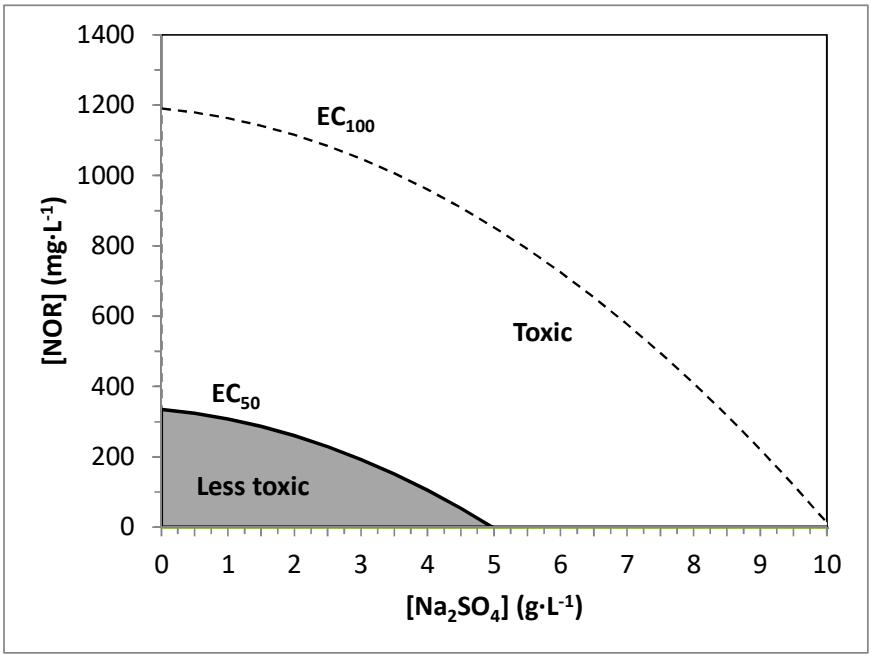

Figure 4. Predicted EC_{50}(5 days) and EC_{100}(5 days) values towards Lactuca sativa for solutions with the simultaneous presence of NOR and Na_2SO_4.

On the one hand, Figure 4 allows to select the maximum Na_2SO_4 concentration that can be used as supporting electrolyte for the electrochemical treatment of a solution containing NOR, so that the final solution is less toxic (i.e. below the EC_{50} line) towards *Lactuca sativa* as a consequence of the presence of the electrolyte. On the other hand, figure 4 gives the range of NOR and Na_2SO_4 combined working concentrations that can be used in order to carry out the measurements of toxicity towards *Lactuca sativa* without requiring to dilute the samples (i.e. below the EC_{100} line).

3.5. Ecotoxicity of solutions towards both Lactuca sativa and Vibrio fisheri after applying electrochemical oxidation

In this point, the toxicity evolution of a contaminated solution containing NOR and sodium sulfate was investigated after applying the electrochemical oxidation method under different experimental conditions (Table 2) by using both, the *Lactuca sativa* and the *Vibrio fischeri* methods. Figure 5 shows the results obtained with the *Lactuca sativa* test for the undivided reactor and Table 3 shows the results obtained with the *Vibrio fischeri* test for both the divided and undivided reactors, where the achieved degree of NOR degradation ([NOR]/[NOR_0]) and mineralization (TOC/TOC_0) is also presented. Based on the results presented previously (Figure 4), the divided reactor samples were not analyzed using the *Lactuca sativa* test, due to the high supporting electrolyte concentration used in this type of reactor.

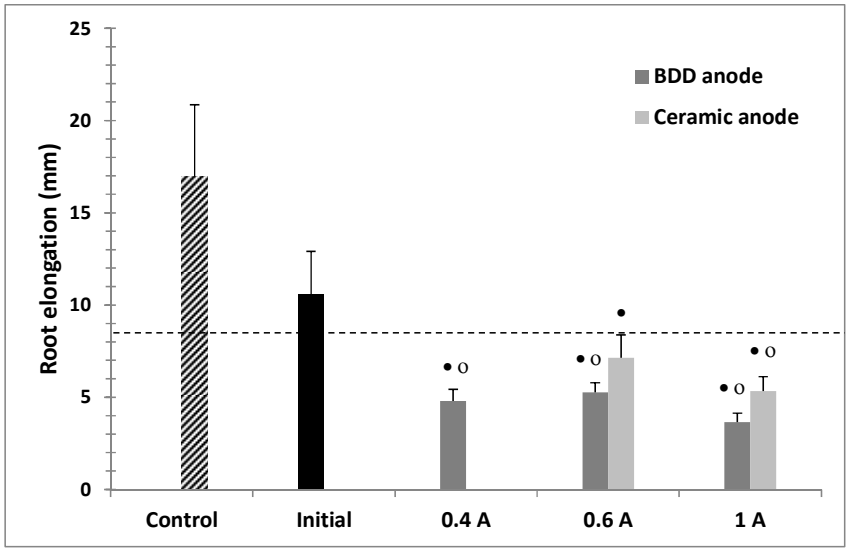

Figure 5. Ecotoxicity analysis (Lactuca sativa test) of solutions initially containing 100 mg·L^{-1} of NOR and 2g·L^{-1} of Na_2SO_4 and treated by an undivided electrochemical reactor. Error bars represent the M ± SD of four replicates; black points (•) represent statistically significant difference from the negative control for a confidence level of 95% and white points (o) represent statistically significant difference from 0 NOR concentration for a confidence level of 95%

Figure 5 shows that the initial solution, containing 100 mg·L^{-1} of NOR and 2 g·L^{-1} of Na_2SO_4, is not toxic towards *Lactuca sativa*, as seen previously (Figures 1(c) and 4). This is supported by the statistical analysis that shows that there is no statistically significant difference (with a 95% confidence level) in the root elongation means between the control and the initial samples. On the contrary, after electrochemical oxidation, all samples become toxic towards *Lactuca sativa* because a root growth decrease greater than 50% and p-values lesser than 0.05, are obtained regardless of the experimental conditions applied. These results agree with those obtained with the *Vibrio fischeri* test presented in Table 3, which also shows that even though the initial solution for the undivided reactor is not toxic towards *Vibrio fischeri*, in general all the treated samples show a certain degree of toxicity (although some authors [37,38] suggest a limit value of 10 TU to consider a sample toxic towards *Vibrio fischeri*). For the divided reactor, results also indicate that the treated samples are more toxic towards *Vibrio fischeri* than the

respective initial solution. This fact implies that, even though during the electrochemical oxidation process the Norfloxacin is degraded (reflected by the low values of $[NOR]/[NOR_0]$), other products are being formed that increase the toxicity of the solution.

On the other hand, Figure 5 shows that, in general, when the applied current intensity is higher, the final samples seem to be more toxic towards *Lactuca sativa, specially for the BDD*. In fact, statistical analysis revealed that there is a significant difference (with a 95% confidence level) in the root elongation means when working at the highest applied current in the presence of the BDD anode. In addition, for a given applied intensity, samples treated with the BDD anode become more toxic that those treated with the ceramic one. This fact is also supported by the statistical analysis that shows that there is a significant difference (p-value < 0.05) in the root elongation means between the samples treated with the different anodes independently of the applied current. These results agree with those obtained with the *Vibrio fischeri* method for the undivided reactor, as seen in Table 3. For the divided reactor, Table 3 also shows that, in general, for the highest applied intensities, the toxicity of the solution towards *Vibrio fischeri* is greater than the toxicity of the final solutions obtained for lower intensity values, and the samples treated with the BDD anode are more toxic than those treated with the ceramic one. In addition, the toxicity of the final solution (towards both *Lactuca sativa* and *Vibrio fischeri)* is much greater when a divided reactor is used, due to the higher concentration of supporting electrolyte.

Table 3. Toxicity towards Vibrio fischeri of solutions containing NOR and Na_2SO_4 before and after the treatment by electrochemical oxidation

REACTOR TYPE	ANODE MATERIAL	APPLIED CURRENT (A)	$[NOR]/[NOR]_0$	TOC/TOC_0	Toxicity (TU)
Undivided	Initial solution		1	1	0
	BDD	0.4	0.01	0.42	1
		0.6	0	0.50	5
		1	0	0.59	3
	Ceramic	0.6	0.04	0.61	0
		1	0.02	0.52	1
Divided	Initial solution		1	1	3
	BDD	0.4	0	0.08	5
		0.6	0	0.06	390
		1	0	0.09	230
	Ceramic	0.6	0	0.43	200
		1	0	0.38	20

Table 3 shows the degradation of Norfloxacin ($[NOR]/[NOR]_0$) achieved by electrochemical oxidation in the different experimental conditions, as well as the achieved degree of mineralization (TOC/TOC_0). In the undivided reactor case, when a BDD anode is used, a zero NOR concentration is reached (i.e. NOR is completely eliminated); however, the entire TOC is not eliminated. This could suggest that the higher toxicity of the final samples may be due to the intermediate organic compounds formed during the electrochemical oxidation. However, in the divided reactor case, when a BDD anode is used, although virtually zero TOC values are achieved, the toxicity of the final samples towards *Vibrio fischeri* is much higher than in the

undivided reactor. This means that the higher toxicity of the final samples cannot be due to these intermediate organic compounds formed during the oxidation of NOR, but it is due to other compounds. Moreover, this fact is also observed when the ceramic anode is used: the degree of mineralization achieved in the divided reactor is also greater than the one achieved in the undivided reactor, but the toxicity towards *Vibrio fischeri* is also higher.

The total oxidation of NOR involves the following reaction [6,39]:

$$C_{16}H_{18}FN_3O_3 + 29\ H_2O \rightarrow 16\ CO_2 + 3\ NH_4^+ + F^- + 64\ H^+ + 66\ e^- \quad (5)$$

The inorganic products obtained according to Equation (5) are fluorides and ammonium. The EC_{50}(48 hours) values of sodium fluoride and ammonium sulfate for *Daphnia magna* are 338 $mg{\cdot}L^{-1}$ and 121.7 $mg{\cdot}L^{-1}$, respectively [40,41], and the corresponding EC_{50}(48 hours) value of NOR is 180 $mg{\cdot}L^{-1}$ [31,42]. Therefore, fluorides are less toxic towards *Daphnia magna* than NOR, but this does not happen for ammonium. However, the molar ratio between NOR and ammonium calculated from their EC_{50}(48 hours) values towards *Daphnia magna* is approximately 1:3.3 (NOR:NH_4^+). Assuming a total oxidation of NOR according to Equation (5), the molar ratio of these species is 1:3. This fact indicates that even though the total oxidation of NOR took place, the final toxicity of the samples cannot be due to the formed inorganic compounds, nor to changes in pH of the solution, since the pH of the samples was adjusted between 6 and 7 before carrying out the toxicity tests.

In summary, the final toxicity of the samples towards *Vibrio fischeri* seems to be higher as the achieved oxidation of the organic compounds is greater. In other words, the toxicity values are higher for those experimental conditions with greater oxidation power. However, these values cannot be attributed to the oxidation products formed from NOR. In addition, the final toxicity is also greater for the system with higher initial concentration of supporting electrolyte. This fact suggests that the greater toxicity of the final solution may be due to the oxidation products formed from the supporting electrolyte. For instance, sulfates (SO_4^{2-}) can be oxidized to persulfates ($S_2O_8^{2-}$), which are much more toxic (the EC_{50}(48 hours) value of sodium persulfate for *Daphnia magna* is equal to 133 $mg{\cdot}L^{-1}$ [43] while the corresponding value of sodium sulfate is 2564 $mg{\cdot}L^{-1}$ [33]). These results seem to suggest that higher oxidizing power electrochemical processes generate larger amounts of these compounds.

Other authors [44–46] have noticed the formation of persulfates from sulfates during an electrochemical oxidation process. In this way, the organic pollutant could be oxidized by direct electron transfer, and indirectly, by persulfate ions, sulfate radicals and/or hydroxyl radicals electrogenerated at the anode surface. Therefore, when sodium sulfate is added to the solution to be treated, an increase in the ionic force and conductivity of the solution takes place, favoring the movement and transport of generated persulfate ions, from the surface of the anode to the bulk of the solution, and leading to further degradation of the organic pollutant. In addition, the electrode material may have an influence on the oxidation mechanism of the organic pollutant, favoring the direct oxidation and/or the mediated one by persulfate ions and/or hydroxyl radicals. The duration of an electrochemical process is another variable that should be taken into account to reduce the formation of undesired by-products [47].

Therefore, in an electrochemical process, it is important to carefully select the supporting electrolyte concentration, and the operation parameters, in order to achieve a high degradation and mineralization of the pollutant while minimizing the formation of by-products which may increase the toxicity of the final solution. According to these goals, among the different experimental conditions tested in this work, the best results were obtained using a

BDD anode in a divided reactor and applying a current intensity of 0.4 A. For these operational parameters, a complete degradation and mineralization of NOR is achieved and the final solution can be considered less toxic towards *Vibrio fischeri* (toxicity value < 10 TU), although it is toxic towards *Lactuca sativa*.

4. Conclusions

The main conclusions extracted from this work are:

- A solution containing Norfloxacin shows an EC_{50} (5 days) of 336 $mg \cdot L^{-1}$ towards *Lactuca sativa*.

- The presence of sodium sulfate in the solution affects the threshold Norfloxacin concentration above which the solution can be considered toxic towards *Lactuca sativa*. The aforementioned threshold concentration decreases as the concentration of sodium sulfate increases. However, there is no synergy between both compounds.

- The toxicity of solutions contaminated with Norfloxacin and sodium sulfate have been determined using statistical tools. A quadratic model was fitted to the experimental data. The model allowed to identify the regions of the NOR concentration-sodium sulfate concentration plane in which the solution can be considered toxic towards *Lactuca sativa*.

- The *Lactuca sativa* test is recommended for assessing the toxicity of low toxicity solutions; since its higher sensitivity increases the resolution of the toxicity quantification. On the contrary, the bioluminescence test is recommended for high toxicity solutions; since it is less laborious (multiple dilutions would have to be prepared with the seeds test).

- The electrochemical treatment increases the toxicity of the Norfloxacin and sodium sulfate solution, in every configuration considered in this work. This is due to the persulfate formation by sulfate oxidation on the anode. This hypothesis also explains why the toxicity of the final solution (towards both *Lactuca sativa* and *Vibrio fischeri*) is much greater when a divided reactor is used (due to the higher supporting electrolyte concentration).

- For the undivided reactor, when the applied intensity is greater, the final samples are more toxic towards *Lactuca sativa* and *Vibrio fischeri;* and for a given applied current, samples treated with the BDD anode become more toxic that those treated with the ceramic one. The same trend is observed for the divided reactor towards *Vibrio fischeri.*

- In an electrochemical oxidation process, it is very important to correctly select the supporting electrolyte concentration and the operation conditions, so that the final solution's toxicity is minimized, while high degradation and mineralization ratios of the organic pollutant are achieved. According to these goals, in this case, the best results were obtained using a BDD anode in a divided reactor applying a current intensity of 0.4 A, since a complete degradation and mineralization of NOR is achieved and the final solution can be considered less toxic towards *Vibrio fischeri (*although it is toxic towards *Lactuca sativa*).

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