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Tolerance and phytoremediation capacity of the *Lemna minor* in an aqueous medium contaminated by the Amoxicillin

Tolerância e capacidade fitorremediadora do *Lemna minor* em meio aquoso contaminado por Amoxicilina

Tolerancia y capacidad fitorremediadora de la *Lemna minor* en medio acuoso contaminado por la Amoxicilina

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Abstract
This study aims to evaluate the behavior and the effectiveness of the aquatic plant *Lemna minor* as a phytoremediation potential about the antibiotic amoxicillin. Experiments were conducted through Central Composite Rotational Design (CCRD) and a kinetic study. The experiments taken by CCRD were performed in culture wells during a contact period of 5 days. The concentration of amoxicillin in the aqueous medium, the quantity of the plant mass, and the solution's pH were studied as independent variables. The dependent variables measured were the remaining amoxicillin in the solution, the plant's tolerance to mortality or fronds cloning and, for stress, chlorophyll-a/chlorophyll-b ratio. A kinetic study determined the rate of antibiotic removal in the aqueous medium. Because the only significant independent variable was the Amoxicillin concentration, the best experimental condition obtained through CCRD was considered the one with the higher level of Amoxicillin removal (92%) - the water medium with the drug concentration at 2.0 mg L^{-1} and pH 7.0. 5 g of wet mass from *L. minor* for each 10 mL of solution was chosen since this independent variable was not significant. There was no pH variation in the solution and the plant stress in the kinetics study. However, there was an amoxicillin removal of 80% after the seventh day. It is considered that, under conditions of low antibiotic load, the aquatic plant *Lemna minor* presents phytoremediation capacity to antibiotic amoxicillin.
Keywords: Antibiotic; Duckweeds; Stress; Tolerance.

Resumo
Este estudo tem como objetivo avaliar o comportamento e a eficácia da planta aquática *Lemna minor* como potencial fitorremediador sobre o antibiótico amoxicilina. Os experimentos foram conduzidos através do Planejamento Composto Central Rotacional (PCCR) e um estudo cinético. Os experimentos realizados pelo PCCR foram realizados em poços de cultura durante um período de contato de 5 dias. A concentração de amoxicilina no meio aquoso, a quantidade de massa vegetal e o pH da solução foram estudados como variáveis independentes. As variáveis dependentes medidas foram a amoxicilina remanescente na solução, a tolerância da planta à mortalidade ou clonagem de frondes e, para estresse, relação clorofila-a/clorofila-b. Um estudo cinético determinou a taxa de remoção do antibiótico no meio aquoso. Como a única variável independente significativa foi a concentração de Amoxicilina, a melhor condição experimental obtida pelo PCCR foi considerada aquela com maior nível de remoção de Amoxicilina (92%) - o meio aquoso com concentração do fármaco em 2,0 mg L^{-1} e pH 7,0. Foram escolhidos 5 g de massa úmida de *L. minor* para cada 10 mL de solução, pois essa variável independente não foi significativa. Não houve variação do pH da solução e do estresse da planta no estudo cinético. No entanto, houve remoção de amoxicilina de 80% após o sétimo dia. Considera-se que, em condições de baixa carga antibiótica, a planta aquática *Lemna minor* apresenta capacidade fitorremediadora para o antibiótico amoxicilina.
Palavras-chave: Antibiótico; Lentilhas; Estresse; Tolerância.

Resumen
Este estudio tiene como objetivo evaluar el comportamiento y eficacia de la planta acuática *Lemna minor* como capacidad fitorremediadora sobre el antibiótico amoxicilina. Los experimentos se realizaron mediante Planificación

Rotacional Compuesta Central (PRCC) y un estudio cinético. Los experimentos realizados por PRCC se realizaron en pocillos de cultivo durante un periodo de contacto de 5 días. Se estudiaron como variables independientes la concentración de amoxicilina en el medio acuoso, la cantidad de masa vegetal y el pH de la solución. Las variables dependientes medidas fueron amoxicilina remanente en solución, tolerancia de las plantas a la mortalidad o clonación de frondas y, para el estrés, relación clorofila-a/clorofila-b. Un estudio cinético determinó la velocidad de eliminación del antibiótico en el medio acuoso. Como la única variable independiente significativa fue la concentración de Amoxicilina, la mejor condición experimental obtenida por PRCC se consideró aquella con mayor nivel de remoción de Amoxicilina (92%) - el medio acuoso con concentración de fármaco a 2,0 mg L^{-1} y pH 7,0. Se escogieron 5 g de masa húmeda de *L. minor* por cada 10 mL de solución, ya que esta variable independiente no fue significativa. No hubo variación en el pH de la solución y el estrés de la planta en el estudio cinético. Sin embargo, hubo una eliminación de amoxicilina del 80% después del séptimo día. Se considera que, en condiciones de baja carga antibiótica, la planta acuática *Lemna minor* presenta capacidad fitorremediadora para el antibiótico amoxicilina.
Palabras clave: Antibiótico; Lentejas; Estrés; Tolerancia.

1. Introduction

Good quality water is a human right, and it is necessary for people's health and well-being. However, potable water is not a benefit that is favorable to everyone, especially in the developing countries where the quantity of polluted water supplied is larger than the quantity of potable water (Jiao et al., 2020).

A contaminated site is caused by the compounds or residues which were introduced into that environment by accident or in a planned way. They are carried in different ways, modifying their original characteristics and causing negative impacts on the environment (J. F. da Silva, 2012). One of the main problems is the organic pollutants because they consume the water oxygen during the biodegradation, which affects its quality and destroys the ecological balance (Jiao et al., 2020). An example of that is drugs.

The compounds present in the drugs are excreted in the urine and feces, and part of them contains their unchanged and active form (Ul-Ain et al., 2018). It also happens that these compounds come into contact with surface and groundwater. Thus, regardless of the concentration of the compound, it causes pathogens to become resistant, which can harm the environment and human health (Shakak et al., 2020), especially when it comes to antibiotics (Lima et al., 2020; Zhao et al., 2020).

Antibiotics are frequently used in the treatment/prevention of diseases, as well as in promoting animal growth. Their overuse boosts the resistance of the microorganisms, which can minimize the control of the bacterial disease and make the bacteria resistant and also increase the health and environmental risks. Antibiotic resistance genes were found in several aquatic environments, such as lakes, rivers, even glaciers and groundwater (Zhang et al., 2020).

Over time, antibiotics on the ground have been registered, in concentrations of ng, kg, and mg, besides the ones found in aquatic environments at higher levels, in waters during the cold and dry seasons (Zhao et al., 2019). One of the reasons for that is attributed to the development of bacterial strains that are resistant to antibiotics, which disturb the natural bacterial ecosystems. Bacterial populations answer to imposed environmental conditions; they adapt themselves and proliferate, becoming versions of the original ones and able to survive in new conditions (Feng et al., 2020).

Amoxicillin is a beta-lactam antibiotic (responsible for the inhibition of the bacterial cell wall) which is a large class of antibiotics that includes penicillin and its derivatives; also very popular in treating several infections caused by bacteria in both humans and animals. The largest issue with this drug is the improper disposal of it, which contributes to bacterial resistance. Therefore, it is necessary that its compounds be eliminated completely. However, the sewage treatment stations lack this step (Moreira et al., 2015).

Because drugs are a great risk to the environment, the concern about their removal increases each day. As a result, some methods have been developed so this treatment can be efficiently done, such as the phytoremediation (Li et al., 2020). Phytoremediation is the process that uses plants capable of removing, stabilizing, or degrading the pollutants in the water or on

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the ground (J. F. da Silva, 2012). It is used to extract, isolate or detoxify pollutants, which can be a cost-effective technology. It has advantages such as plants growth, nutrients absorption – like ammonia and phosphate – as well as the production of proteins and amides, among other substances used to generate biofuels and supplies for animals (Hu et al., 2019).

In this research, the amoxicillin antibiotic was chosen as the contaminant of the aqueous medium for being frequently used in treatments of different infections both in humans and in animals. To this end, the behavior and effectiveness of the aquatic plant *Lemna minor* as a phytoremediation potential was evaluated.

2. Methodology

2.1 *L. minor*

The duckweeds were cultured in tanks at constant temperature (25 ± 3 ºC) and light/dark cycle: 14 h / 10 h. *L. minor* with at least two fronds with green color and no clones were selected for the experiments.

2.2 Amoxicillin

Antibiotic was purchased from a manipulation pharmacy with a purity of 98%. A stock solution of amoxicillin (AMX) was produced with 10 mg L^{-1}. Then, the AMX solution was scanned by UV-VIS spectrophotometer (Thermo Scientific Evolution 60S) in the range 190–500 nm using deionized water as blank. The wavelength corresponding to maximum absorbance (λmax) was 223 nm. The stock solution was diluted for the calibration curve to obtain a concentration ranging from 1 to 10 mg L^{-1}. The relation between AMX concentration and absorbance presented linearity (determination coefficient – R^2: 0.9992).

2.3 Central Composite Rotational Design (CCRD)

To evaluate the influence of some parameters in the drug removal by the duckweeds, a CCRD was conducted (2^3 – 6 axial points and 4 center points). The independent variables were the concentration of antibiotic in the medium (AMX), the wet mass of lentils (WM), and the pH of the solution (pH). For the evaluation of the process, the dependent variables were amoxicillin removal (%R), plant tolerance (mortality or frond cloning) (FN), and plant stress through the chlorophyll-a/chlorophyll-b ratio (C_a/C_b).

The experiments were carried out on culture plates. 10 mL of contaminated medium and duckweeds were added to each well by CCRD (Table 1) for five days. HCl 0.1 mol L^{-1} and KOH 0.1 mol L^{-1} were used for the pH adjustment. Samples of deionized water were controls in the experiments.

Table 1. Central Composite Rotational Design (CCRD).

Variables	Symbol	Coded and real levels				
		-1.68	**-1**	**0**	**1**	**1.68**
Amoxicillin concentration (mg L^{-1})	AMX	2.0	3.8	6.0	8.2	10.0
Wet mass (g)	WM	5.0	7.3	10.0	12.7	15.0
pH of the solution	pH	5.0	5.9	7.0	8.1	9.0

Source: Authors.

The amoxicillin removal (%R) was calculated based on the determination of the drug remaining in the aqueous medium, according to section 2.2. The duckweed tolerance was defined by fronds number (FN), considering the frond's mortality with white-yellowish coloration.

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For chlorophylls, although both are green, the adsorption spectra are different, so that for the human eye, chlorophyll-a has a bluish-green hue, and chlorophyll-b yellowish-green (Steet & Tong, 2006). The quantification of C_a and C_b (plant stress) was determined by the extraction methodology described by Lichtenthaler & Buschmann (2001). The plant extract was obtained by macerating 0.5 g of the duckweed dry mass in 6 mL of the hexane:ethanol mixture (1:1). After 10 minutes of steeping, the extract was filtered. The C_a e C_b concentration was calculated using the quantitative method described by Silva et al. (2021) and Miner et al. (1995), which is based on spectrophotometry and the equations (1) and (2), $A_{664.1}$ is the value of the absorbance in 664.1 nm and $A_{648.6}$ in 648.6 nm, and the ratio for C_a/C_b.

C_a (mg g^{-1})	$C_a = 13.36(A_{664.1}) – 5.19(A_{648.6})$	(1)
C_b (mg g^{-1})	$C_a = 27.43(A_{648.6}) – 8.12(A_{664.1})$	(2)

2.4 kinetic study

The kinetics study was conducted from the optimized point of the CCRD, having the definitive parameter the significant independent variable (AMX) that presented the best drug removal. For the experiments, 125 g of duckweeds wet mass were used in 250 mL of aqueous medium with 2.0 mg L^{-1} and pH 7.0. Aliquots (10 mL) were taken in predetermined time intervals for ten days to analyze the remaining solution concentration and the plant stress.

3. Results and Discussion

3.1 CCRD results

Through the evaluation of the independent variables of amoxicillin concentration in the medium (AMX), wet mass plant (WM), and pH of the solution (pH) during five days of contact, the results presented in Table 2 were obtained for the drug removal (R%), chlorophyll-a/chlorophyll-b ratio (C_a/C_b) and fronds number (FN).

Depending on the conditions used in the experimental study, the percentage of drug removal (R%) varied from 18.2% to 92.0% (run 10 and 9, respectively). To plant stress was observed variance between 1.16 and 2.22 (run 3 and 14, respectively) to the chlorophyll-a/chlorophyll-b ratio (C_a/C_b) and related to tolerance (FN) from -15 to 35 (run 15 and 3, respectively).

Table 3 shows the values of the estimated effects, the interactions with significant and non-significant parameters, the error associated with the effects, and the p-value for the dependent variables. In the analysis of effect estimates, significant factors for the 95% confidence interval ($p<0.05$) were considered. Factors that were significant at this confidence interval are shown in bold.

The linear and quadratic terms of the AMX factor could produce variations in removal percentage (R%). A high determination coefficient was shown, 90.88%, indicating that data variability may be explained by the proposed model, which suggests this model is suitable to evaluate the behavior of the antibiotic removal percentage compared to the variation of all evaluated parameters. C_a/C_b ratio was influenced by the quadratic term of pH facto and the interaction between factors AMX x WM, producing significant variations in the plant stress (R^2: 0.6800). The linear term of the AMX factor could produce variations in fronds number (FN), with a coefficient determination of 0.9070.

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Table 2. CCRD results: independent and dependent variables.

Run	Independent variable			Dependent variable		
	AMX (mg L^{-1})	**WM (g)**	**pH**	**%R**	**C_a/ C_b**	**FN**
Control	0.0	10.0	7.0	-	1.87	30
1	3.8	7.3	5.9	53.2	1.89	14
2	3.8	7.3	8.1	39.2	1.89	9
3	3.8	12.7	5.9	47.3	1.16	35
4	3.8	12.7	8.1	42.8	1.22	5
5	8.2	7.3	5.9	24.8	1.25	-6
6	8.2	7.3	8.1	21.5	2.01	-4
7	8.2	12.7	5.9	24.5	2.01	-7
8	8.2	12.7	8.1	25.4	1.99	-10
9	2.0	10.0	7.0	92.0	1.94	9
10	10.0	10.0	7.0	18.2	2.02	-10
11	6.0	5.0	7.0	29.0	2.04	-8
12	6.0	15.0	7.0	28.2	1.42	3
13	6.0	10.0	5.0	27.0	1.49	-8
14	6.0	10.0	9.0	34.0	1.43	-1
15	6.0	10.0	7.0	28.2	2.22	-5
16	6.0	10.0	7.0	25.3	2.18	-4
17	6.0	10.0	7.0	23.5	2.15	-5
18	6.0	10.0	7.0	31.0	1.87	-5

Source: Authors.

Table 3: CCRD results: main effects, interaction effects and interactions for the dependent variables.

Factor	R%			C_a/ C_b			FN		
	Effect	**Effects Errors**	**p-value**	**Effect**	**Effects Errors**	**p-value**	**Effect**	**Effects Errors**	**p-value**
Mean	**26.783**	**3.758**	**0.000**	**1.982**	**0.145**	**0.000**	**-14.167**	**4.158**	**0.009**
AMX (L)	**-30.179**	**3.953**	**0.000**	-0.056	0.152	0.722	**-15.952**	**4.374**	**0.006**
AMX (Q)	**16.366**	**3.796**	**0.002**	-0.069	0.146	0.648	4.281	4.201	0.337
WM (L)	-0.024	3.920	0.995	0.117	0.151	0.459	-0.977	4.337	0.827
WM (Q)	0.348	3.673	0.926	-0.026	0.142	0.857	7.554	4.064	0.100
pH (L)	-1.118	3.953	0.784	-0.078	0.152	0.619	-2.090	4.374	0.645
pH (Q)	1.483	3.796	0.706	**-0.376**	**0.146**	**0.033**	1.861	4.201	0.669
AMX x WM	1.475	5.342	0.789	**0.561**	**0.206**	**0.026**	-8.000	5.911	0.212
AMX x pH	4.025	5.342	0.472	0.184	0.206	0.397	10.500	5.911	0.113
WM x pH	3.425	5.342	0.539	-0.196	0.206	0.369	-9.500	5.911	0.146

Statistical significant factors ($p<0.05$). Source: Authors.

Figure 1 shows the contour curve graphs for the antibiotic removal percentage response variable (Figure 1a) as a function of AMX and WM parameters; in this case, pH was held constant at the central point. It could be observed that the removal percentage (%R) increases with low amoxicillin concentration (AMX). As a function of pH and WM parameters (Figure 1b), AMX was held constant at the central point. It could be observed that the %R is significant with extreme pH.

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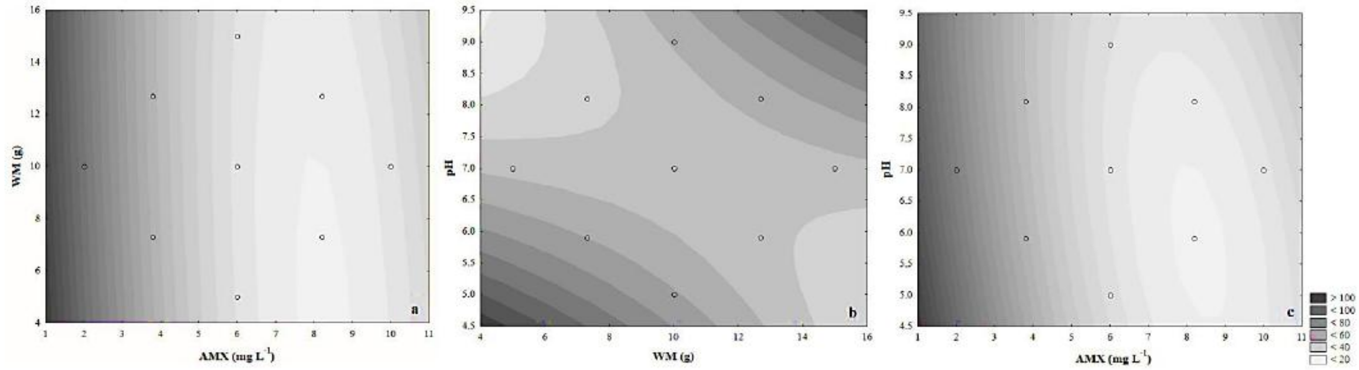
According to AMX and pH parameters (Figure 1c), the WM variable was constant at the central point. Indicating the response is strongly influenced by low amoxicillin concentration values.

Figure 1: Contour plot of percentage antibiotic removal (a) as a function of AMX and WM, (b) as a function of pH and WM (c) as a function of pH and AMX.

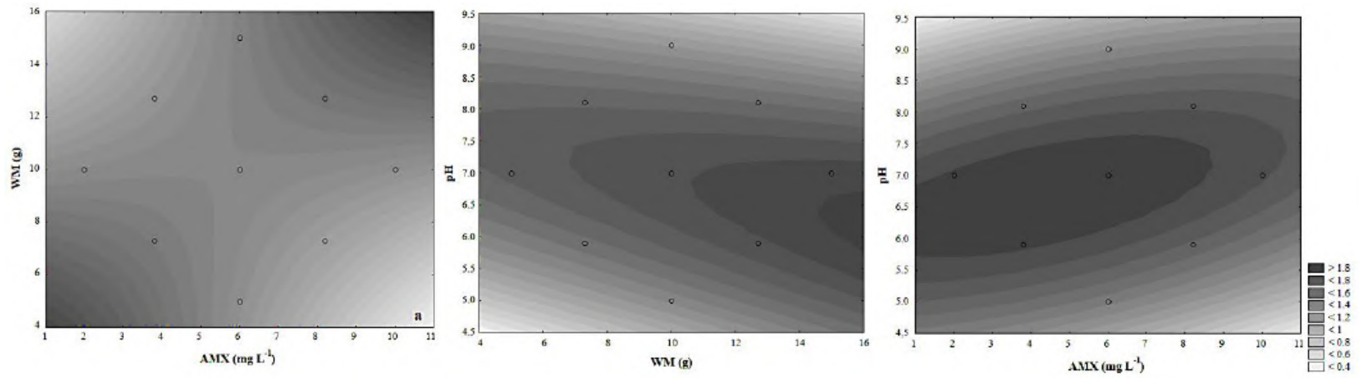


Source: Authors.

Research reports the use of *L. minor* to remove various pollutants in wastewater: reduction of organic matter and pollutant removal (drugs, microplastics and metals heavy) (Alkimin et al., 2019; Nunes et al., 2019; Sasmaz et al., 2019; Mateos-Cárdenas et al., 2019; Xiao et al., 2022). *L minor* has demonstrated the ability to transform some persistent organic pollutants by phytoremediation.

Figure 2 presents the contour plot for the plant stress response - chlorophyll-a/chlorophyll-b ratio (C_a/C_b), in the function of the different parameters. As seen in Figure 2a, it is possible to observe that the Ca/Cb ratio decreases at a high concentration of amoxicillin (AMX) observed in the AMX x WM interaction. The pH produces a variation in plant stress, being significant at lower values (Figure 2b and c) at the two extremes of pH (high and low).

Figure 2: Contour plot of plant stress - chlorophyll-a/chlorophyll-b ratio (a) as a function of AMX and WM, (b) as a function of pH and WM (c) as a function of pH and AMX.

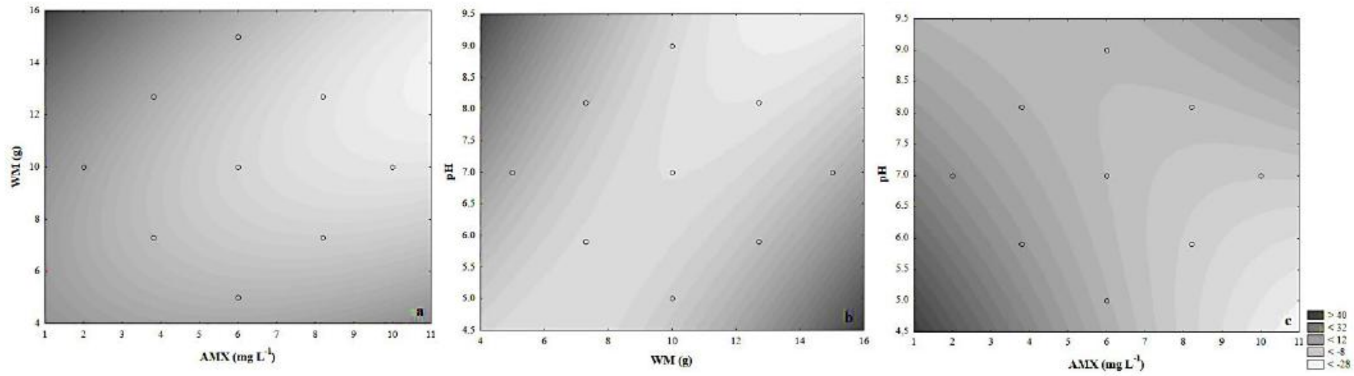


Source: Authors.

Chlorophyll-a and chlorophyll-b are found in an average ratio of 3:1, respectively. However, this proportion varies with species, leafage, and leaf location. This ratio is lower in plants located in the shade than in plants with high irradiance (Taiz & Zeiger, 2004). When the plants are subjected to phytoremediation, stress can occur, which can be seen by the impact on the plant's ability to overcome through the chlorophyll composition. Driscoll et al. (2006) reports that quantifying chlorophylls a and b is vital to understanding plants' activity photosynthesis when they are found in different environments. As a result, the correlation shows that chlorophylls are dependent on each other, and that photosynthetic activity will depend on the degree of association and the environmental conditions in which the plants are subjected.

Figure 3 shows the contour curve graphs for the plant tolerance response – FN variable (Figure 3a) as a function of AMX and WM parameters; it can be observed that L. minor has greater tolerance at low concentrations of amoxicillin (AMX) with a production of clones. As a function of pH and WM parameters (Figure 3b), one can observe the non-influence of pH and WM in the process. According to AMX and pH parameters (Figure 3c), the indication of the response is strongly influenced by the amoxicillin concentration values. Observe plant mortality at high antibiotic concentrations.

Figure 3: Contour plot of plant tolerance - mortality or fronds cloning (FN) (a) as a function of AMX and WM, (b) as a function of pH and WM (c) as a function of pH and AMX.



Source: Authors.

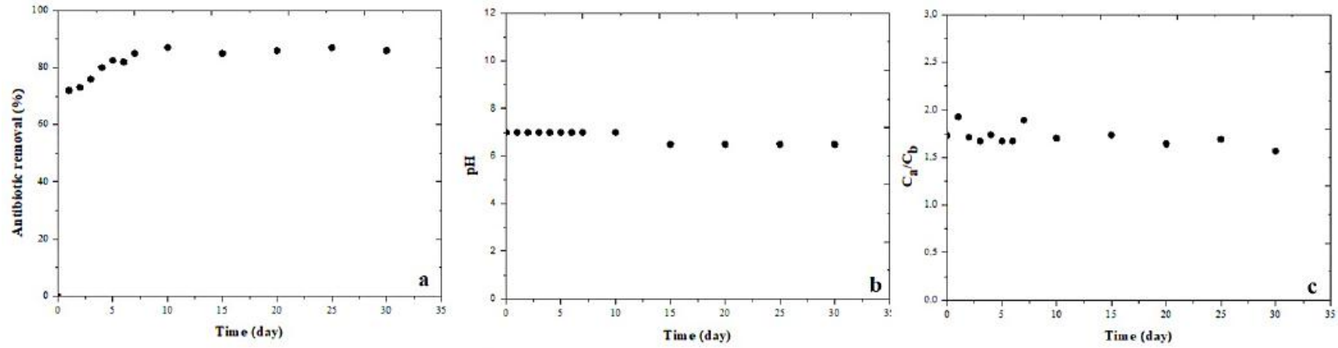
In experiments with higher AMX concentration, there was a reduction in chlorophyll-a and an increase in chlorophyll-b, which provided a reduction in the C_a/C_b ratio, as seen in Figure 2. This variation in the amount of chlorophyll can be associated with the verification of frond mortality (FN < 0). AMX interfered with chlorophyll-a production, causing the fronds to undergo chlorosis and subsequent necrosis.

According to Streit et al. (2005), the plant does not carry out photosynthesis, a fundamental activity for its development with chlorosis. Furthermore, with the reduction of chlorophyll-a, there is an increase in the production of chlorophyll-b (accessory pigment) so that it is converted into chlorophyll-a through an oxygenase enzyme that catalyzes the conversion of the methyl group to the aldehyde group. This process is a defense of the plant when subjected to contaminated environments.

3.2 Kinetics Study

Since the results obtained from CCRD is possible to optimize the phytoremediation process by *L. minor* holding constant those variables with significant influence in the process: AMX (2.0 mg g^{-1}), pH (7.0) and WM (5 g per 10 mL). The analyses were done for 30 days for the kinetics, and the results are in Figure 4.

Figure 4: Kinetics of the phytoremediation process with *L. minor* in an amoxicillin-contaminated medium: (a) antibiotic removal; (b) pH (c) chlorophyll-a/chlorophyll-b ratio.



Source: Authors.

The pH of the solution remained neutral (6.7-7.0), and there was a stress on L. minor, mainly at the beginning of the contact time (1.56-1.92). However, there was no chlorosis and necrosis in the fronds with the observed stress. Thus, it can be said that stress on the plant ends up causing damage to the photosynthetic process (Driscoll et al., 2006). However, the *L. minor* used in the study showed that its defense system was effective enough to prevent deleterious effects.

There was a removal of 70% of antibiotics within 24 hours of phytoremediation and final constant removal of 87% from the 10th day, demonstrating efficiency in the treatment. Satisfactory results using *L. minor* were also observed by Reinhold et al. (2010), Shi et al. (2010) and Garcia-Rodríguez et al. (2015) in drug removal studies such as diclofenac (41%), ibuprofen (88-100%), and estrone (95%). The results obtained showed that phytoremediation has an excellent ability to remove amoxicillin when compared to the results obtained from high-cost tertiary treatments, such as adsorption (20-98%), photocatalysis (69-88%), photocatalysis-biodegradation (40-65%) and adsorption-photocatalysis (18-92%) (Abd & Mohammed-Ridha, 2020; Kanakaraju et al., 2015; Wang et al., 2019; Yahia et al., 2022).

4. Conclusion

In this work, we report the efficiency of *L. minor* in phytoremediation of the antibiotic AMX. Statistically evaluating the process by CCRD, processes with low concentrations of the antibiotic (<3.8 mg L^{-1}) and neutral pH showed more effective removal of AMX, with less stress and greater plant tolerance. At extreme pH and higher AMX concentration, a reduction in chlorophyll-a production and an increase in chlorophyll-b were observed, indicating damage to the photosynthesis process, chlorosis, and frond necrosis (high stress and low plant tolerance). In kinetic studies with neutral pH and low AMX concentration, *L. minor* showed an efficiency of 87% in the removal of AMX in 10 days of contact, however with low levels of stress and an effective defense system, which makes it excellent in phytoremediation of areas contaminated with the antibiotic.

The study group has developed research involving histological analyses on *L. minor* and studies with other emerging contaminants.

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