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RESEARCH ARTICLE

BIOLOGICAL AND CHEMICAL ANALYSIS IN THE DETECTION OF METHAMIDOPHOS IN SOIL: ECOTOXICOLOGICAL TEST WITH LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY

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ABSTRACT

The development of analytical methods which meet the resolution to quantify pesticides residues in complex environmental matrices still represents a challenge for many laboratories. The integration of two analytical methods, an ecotoxicological and a chemical one, demonstrates the potential for environmental analysis of methamidophos. This study tested two soils: a clayey and a sandy one. Both soils, when in contact with methamidophos, followed the kinetic pseudo-second order model. The clayey soil showed a greater adsorption of methamidophos and followed the Freundlich model, while the sandy, followed the Langmuir model. The technique of LC-MS/MS analytical (Liquid Chromatography/Mass Spectrometry) displayed satisfactory validation parameters, such as linearity, interval, precision, accuracy, and sensitivity. In chronic ecotoxicological tests with *Ceriodaphnia dubia*, the NOEC (No Observed Effect Concentration) were 4.93 and 3.24 ng L^{-1} for the elutriates of sandy and clayey soils, respectively. The ecotoxicological test with *Ceriodaphnia dubia* showed excellent sensitivity for detecting methamidophos in clayey and sandy soils, and it was used for screening the results. However, by decreasing the concentration of the standard analytical methamidophos and adjusting the chemical validation parameters, one could find the limit of quantification (LOQ) in ng L^{-1}, compatible with the established in the ecotoxicological test. The described methods were used as an analytical tool of methamidophos in soils.

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INTRODUCTION

The excessive use of pesticides in the modern agriculture has generated many problems of environmental pollution. Pesticide residues and metabolites are present in foods, air, water, and soil. The concern about controlling the use of these chemicals resides in the losses linked to its usage, which affect the environment and humans. Currently, Brazil is the leading country in the consumption of pesticides in the world. Its production values range between 2.5 to 3 million tons per year.

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The methamidophos, between 2000 and 2008, was a product that maintained a steady growth in imports of several countries (ANVISA, 2012). The methamidophos is an organophosphate used to control the insects. It belongs to the toxicity class I, being extremely toxic. It has a great ability to penetrate the soil and presents a high potential for groundwater contamination (Filizola, 2005). Countries like China, Pakistan and Indonesia, along with the countries members of the European Union, banned the use of this pesticide. In January 2011, during the government's periodical toxicological revaluation of methamidophos, performed by the National Health Surveillance Agency - ANVISA - Brazil, it was determined a gradual withdrawal of the product from the Brazilian market. The ban was set after the companies failed to prove the pesticide's safety. The evaluation report established that the stock of this chemical in factories would remain legal up to the

31st of December of 2011, and in stores until 30 June 2012 (ANVISA, 2012). In the chemical analysis of contaminated soils, some difficulties arise in the literature based on the complexity of the product's matrix. Rissato (2005), while performing recovery experiments, found that the tested concentration varied according to the recovery. He observed that the lower the concentration of soil contamination was, the smaller the percentage of recovery in the final analysis was. The limits of detection (LOD) of several active ingredients of pesticides in soils, showed in literature, are given in milligrams per kilogram (ANVISA; 2003, 2012).The representation of these measures can also be in micrograms per kilogram, which shows the difficulty in obtaining sensitive methods of environmental contamination in complex matrices such as the soil one (BRASIL, 2011; Elnabarawy *et al*., 1986). Effective analytical methods must be developed to perform the analysis of these complex environmental matrices. These methods require more advanced techniques for sample preparation. They also entail the use of equipments with high sensitivity (limit of detection and quantification). The application of these analytical methods represents a higher cost of consumables and accessories for the completion of the analysis (Farias, 2012; Gonçalves *et al*., 2006, Primel *et al*., 2005). The use of ecotoxicological tests with aquatic organisms have been in place to overcome analytical difficulties in determining pollutant concentrations, these tests are able to detect prior concentrations of pollutants in other matrices, such as the solid waste (Ferrari *et. al*., 1999), the leached from the soil (Chelinho *et. al*., 2012), the explosive compounds (Griest *et al*., 1993), the uranium (Kuhne *et al*., 2002), and the mineral oil ones (Van Gestel *et al*., 2001).

The results of ecotoxicological aquatic tests are not applied directly to evaluate the environmental impact on the soil. Nevertheless, it is possible to use these results to screen the presence or absence of methamidophos in the sample, confirming the analytes which present some difficulties in analytical chemistry in complex matrices. Thus, this paper conducts an exploratory analysis using ecotoxicological tests to verify the toxic effects to *Ceriodaphnia dubia* in the presence of methamidophos in sandy and clay soils. The Liquid Chromatography coupled with mass spectrometry is a particular analytical technique which specifies the molecular mass of the compound, in this case, the methamidophos one. During the process, the methamidophos is selected in the first quadrupole and its fragments are detected in the third quadrupole (Lambropolou and Albanis, 2004). Therefore, the LC-MS/MS is a confirmatory chemical analysis of the methamidophos molecule.

MATERIALS AND METHODS

Soil contaminations

Two types of soils, with different physical-chemical properties and grain sizes, were contaminated: a sandy, classified as light sand (11.1% clay, 1.6% silt, and 87.5% sand); and a clayey, or sandy-clayey, (41.8% clay, 8.9% silt, and 49.3% sand). A 400 mL of solution was added to 0.39 µg mL^{-1} of methamidophos PILARQUIM (99%) with Milli-Q water, and this mixture was added to every 100g of soil. The minimum contact time was 48 hours.

Preparation of elutriate soil

First, all the contaminating water was separated from the soil. Then, 100g of methamidophos-contaminated soil, 400 mL of natural water (Jundiaí river water – Macaíba / Rio Grande do Norte, Brazil) and pond Boqueirão – Touros / Rio Grande do Norte, Brazil), Figure 1, used in *Ceriodaphnia dubia* rearing and ecotoxicological tests were added to the experiment as described in ABNT (2007). The pH, conductivity or hardness, source of natural water are shown in Table 1. After the addition of natural water, the experiment was placed under horizontal agitation, type Kline, at 170 x g for 24 hours, in a 1000 mL erlenmeyer flask. After that time, the soils were centrifuged at 12000 x g, in a refrigerated centrifuge, at 4ºC for 40 minutes. Following, the vacuum was filtered in a membrane millex millipore of 0.45 micrometers.

Table 1. pH, conductivity or hardness, source of natural water

Water	pH	Conductivity (µS cm^{-1})	Hardness (mg L^{-1})
Touros	7.55	497	110.79
Jundiaí	7.10	140.4	19.44
70% Jundiaí e 30 % Touros	7.64	264	43.73

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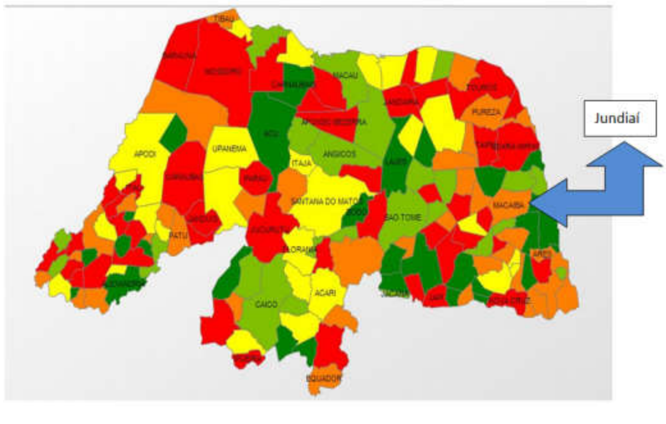

Figure 1. Location: Macaíba (a) and Touros (b) - Rio Grande do Norte, Brazil

The ecotoxicological test was performed using a supernatant. An analytic curve of methamidophos was prepared at concentrations of 4.93; 6.48; 8.30; 10.37; and 12.97 ng L^{-1} for the sandy soil. The clayey soil had the concentrations of 3.24; 4.27; 5.46; 6.82; and 8.53 ng L^{-1}. The curve was created from this elutriate free of solid particles for ecotoxicological test and chemical analysis. The dilutions were carried out in series.

Eco toxicological analysis

The ecotoxicological test used to determine the chronic toxicity with *Ceriodaphnia dubia* in the elutriate soils was the ABNT NBR 13373 (2005) (Primel *et al*., 2005). In short, a neonate was transferred into non-toxic plastic cups containing 15 ml of test solutions or control. The experiments were prepared using ten replicates for each treatment. The animals were neonates aged between 6 and 24h, who were exposed to concentrations of methamidophos from 4 to 12 ng L^{-1} (sandy soil), and from 3 to 8 ng L^{-1} (clayey soil) for a period of 7 days. The feed (*P. Subcaptata* and solubilized feed) was administered daily. The animals were incubated at 25±2°C, with a photo-period of 16 hours/day. The solution renewal took place every 2 to 3 days. At the end of the tests, data were recorded on the survival and reproduction of organisms. The NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) of the ecotoxicological tests were calculated by ANOVA (Dunnett's test), using the software Toxstat 3.5 (Rissato *et al*., 2005).

For comparative purposes, ecotoxicological tests were performed with *Ceriodaphia dubia* and methamidophos using natural water as a matrix for determining the LOEC.

Determination by LC-MS/MS

An analysis method was developed to evaluate the level of adsorption of methamidophos in sandy and clayey soils. This developed method was responsible for measuring the parameters of analytical validation, selectivity and specificity, linearity, precision, accuracy, limit of detection, and limit of quantification. The equipment used a Shimadzu high-efficiency liquid chromatograph. This instrument comprised a system controller (CBM20A), a quaternary pump (LC20AB), an auto-sampler (SIL20AC), and column oven (CTO20AC). It had, a detector coupled to the chromatograph with a mass spectrometer Triple TOF 5600, brand AB SCIEX, hybrid quadruple – TOF, ion source DuoSpray; a data acquisition system via software Analyst TF 1.5.1; and a chromatographic column Phenomenex Hydro C-18, which was 50mm long, had 2mm internal diameter and particle size of 4 µm, with a pre-column pf equivalent size. The column temperature was maintained at 35ºC. The injection volume was 5 µL. The mobile phase used contained 25% water with formic acid and 75% methanol.

Table 2. Methamidophos Concentration in µg L^{-1} versus time of contact with sandy and clayey soil

Contact time (h)	Concentration in µg L^{-1} (initial)	Concentration in µg L^{-1} sandy soil (LC-MS/MS)	Concentration in µg L^{-1} clayey soil (LC-MS/MS)
6	0	0	0
12	0	0	0
18	0	0	0
24	0	0	0
36	0	0	0
48	0	0	0
72	0	0	0
6	0.2	0.18319	0.10659
12	0.2	0.17104	0.04412
18	0.2	0.18492	0.02330
24	0.2	0.13807	0.10746
36	0.2	0.12940	0.06408
48	0.2	0.11118	0.09271
72	0.2	0.13373	0.05280
6	1.5	0.78250	0.46078
12	1.5	0.61774	0.51132
18	1.5	0.65312	0.36374
24	1.5	0.61471	0.45168
36	1.5	0.55710	0.32634
48	1.5	0.54193	0.43349
72	1.5	0.36100	0.24346
6	5.0	4.72946	3.39078
12	5.0	3.78859	3.43473
18	5.0	4.23505	3.29690
24	5.0	4.03999	3.17205
36	5.0	4.22207	2.41795
48	5.0	3.95439	2.45291
72	5.0	3.61979	2.22718
6	15.0	13.78276	8.14704
12	15.0	13.41980	8.41102
18	15.0	12.65187	9.00296
24	15.0	12.67987	10.73279
36	15.0	12.81186	7.58210
48	15.0	11.92795	8.95996
72	15.0	9.92915	7.60510

Table 3. Isotherms parameters of Langmuir and Freundlich of sandy and clayey soils in the methamidophos adsorption

Soil type	Langmuir			Freundlich		
	K	qm	R^2	1/n	K	R^2
Sandy	0.2167	0.0534	0.9994	0.6323	0.00446	0.8767
Clayey	0.2610	0.0443	0.8776	0.7784	0.00757	0.9273

The formic acid was prepared with 0.3g of ammonium formate 99% (sigma) in deionized water, stored in a volumetric flask. One pipetted 5 mL of ammonium formate and 1mL of formic acid, HPLC grade. Then, a 1 L volumetric flask was filled with Milli-Q water, obtaining the formic acid solution.

Table 4. Correlation coefficients of kinetics of pseudo-second order (ps-2ª) depending on the initial concentration of methamidophos in liquid phase (C) during the adsorption in sandy and clayey soils

Methamidophos	Sandy	Clayey
C (µg/L)	ps-2ª R^2	ps-2ª R^2
0.2	0.8476	0.9181
1.5	0.9811	0.9862
5.0	0.7843	0.9804
15.0	0.6377	0.9274

The best mobile phase results, based on peak methamidophos, derived from the mixture of 25% formic acid with methanol at 75% HPLC grade. A of 2.998-minute post-run time was set after each analysis. The scan used was the TOF MS positive mode. The experiment applied the following operating parameters: the volume of the pump flow was 0,2ml/min, with pressures ranging from 0 to 4000 psi and the number of cycles was 327.

Adsorption of methamidophos in soil

The samples of sandy and clayey soils were classified into sieves. The passing fraction of the sieve of 8 Mesh (d = 2.36 mm) was collected for each sample, followed by the methamidophos contamination. The adsorption tests were performed in a Bench Incubator CT-712-CIENTEC in finite bath, in which was added 8 ml of each of the five methamidophos solutions (0; 0.2; 1.5; 5; and 15 µg L^{-1}), in triplicate, to the 2 grams of soil. In each experiment the temperature was kept constant at 30º C and the agitation at 150 x g. Samples were collected at the following times: 6 h; 12 h; 24 h; 36 h; 48 h; and 72h. After the sample collection, the tests were filtered with a syringe coupled with 0.22 micron membranes millex millipore, and frozen immediately for later analysis by LC-MS/MS. The isotherms were obtained by examining the points corresponding to the time of 72 hours, which was considered the equilibrium time.

RESULTS AND DISCUSSION

Adsorption

This study observed the adsorption of methamidophos in sandy and clayey soils. The concentrations of methamidophos in aqueous phases are presented in Table 2, as a function of contact time. The purpose of studying the adsorption is to verify the affinity of methamidophos behaviour in relation to clayey, sandy, and to the leached soil. Table 2 shows that there is a reduction in the concentration of methamidophos in aqueous phases after the contact with the soils. This concentration drop is more preponderant in clayey soils. This fact may be related to the characteristic of the clayey soil, which is more porous than the sandy one, because of its granular aspect and low porosity. The data presented on Table 2 reveal that the adsorption capacity of the clayey soil is higher than the sandy one (Rissato, 2004).

Adsorptions isotherms

The isotherms of Langmuir and Freundlich (Clark *et al*., 2010) were evaluated from their linearized equations. See calculations (1) and (2), respectively.

$$Ce/qe = Ce/qm + 1/(K.qm) \quad \text{……………..} (1)$$

$$\ln qe = (1/n).\ln Ce + \ln K \quad \text{.......................} (2)$$

The results obtained by adjusting Langmuir and Freundlich models are presented in Table 3. Table 3 shows that each of the soils studied follows a different isotherm. In the case of the sandy soil, the adsorption of methamidophos showed a higher correlation coefficient for the Langmuir isotherm, while in the case of the clayey soil the best correlation coefficient is for Freundlich. These results show that the structure of the sandy soil, being slightly porous and crystalline, favors the mono-layer adsorption. Differently, because the clayey soil has a porous structure and contains alumina, it favors the multilayer adsorption (Ismail *et. al.,* 2002).

Kinetics adsorption

There are several models of kinetics that allow one to observe the behaviour of the adsorbent. These representations also reveal the underlying mechanism which controls the adsorption process and validates the experimental data. The two mostly used kinetic models are the pseudo-first order and pseudo-second order (Clark, 2010). See Equations (3) and (4), respectively.

$$\frac{dq_t}{dt} = k_1(q_e - q_t) \quad \text{…………………} (3)$$

$$\frac{dq_t}{dt} = k_2(q_e - q_t)^2 \quad \text{…………………} (4)$$

The kinetic evaluation, as in the study of isotherms, is based on the correlation coefficient of the model that best fits the experimental data. Table 4 shows the correlation coefficients of the pseudo-second order models for the adsorption kinetic of methamidophos in sandy and clayey soils. The study performed an evaluation of the correlation coefficients of the kinetic of pseudo-first order, but the model did not fit the experimental data, indicating that this kinetic does not represent the data obtained in this study. In the case of kinetic of pseudo-second order, the data showed correlation coefficients close to the unit, especially in the case of the clayey soil, which presented all coefficients above 0.9, indicating that the kinetic adsorption process of methamidophos in studied soils is of pseudo-second order. The results of the kinetic study show that, having a behaviour of pseudo-second order, the more elevated the concentration of methamidophos in aqueous phase is, the higher the speed at which it adsorbs in the soil will be.

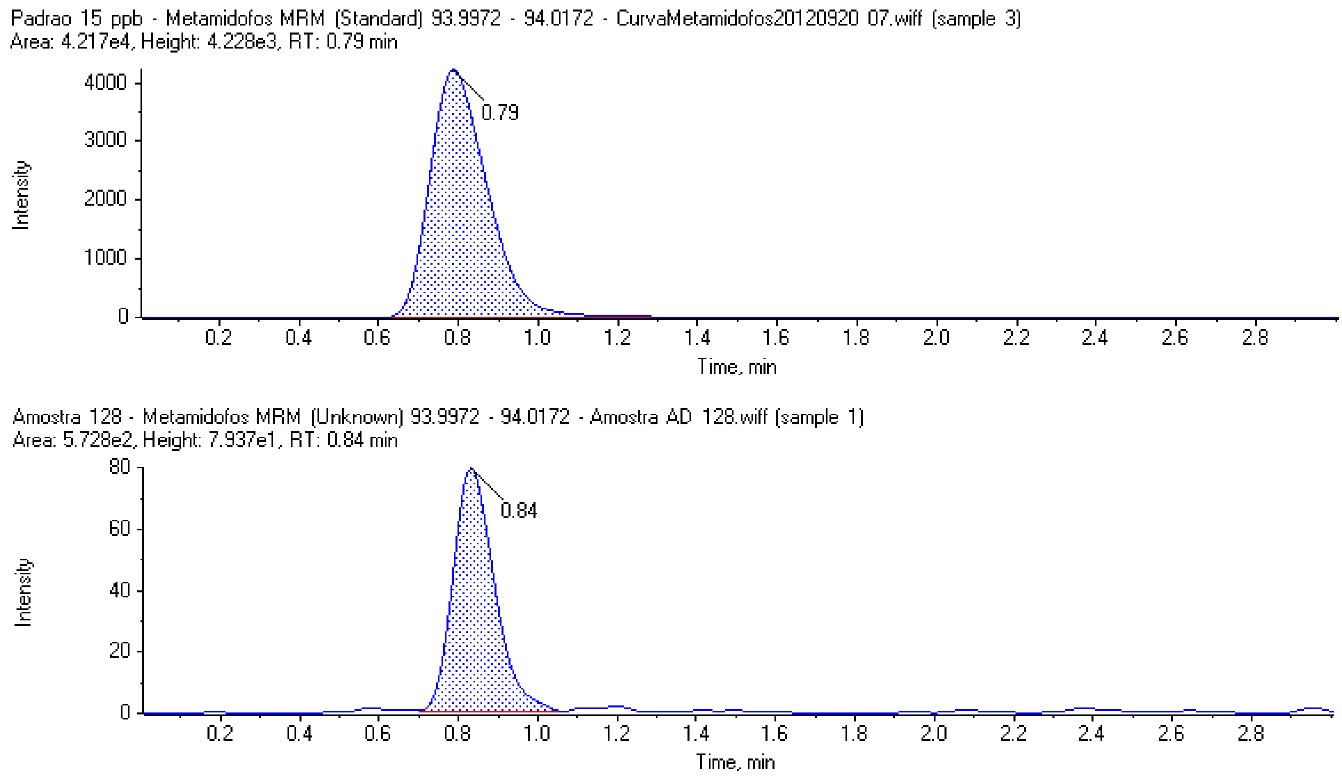

Figure 2. LC/MS/MS analysis chromatograms of standard (a) and sample (b)

Validation of the analytical method for determination of methamidophos in soil by LC-MS/MS

The parameters of analytical validation of LC-MS/MS established in this study were: selectivity and specificity, linearity, precision, accuracy, limit of detection, and limit of quantification. The selectivity of the method was demonstrated by the absence of peaks in injection of whites. Regarding linearity and interval, for the concentration range from 0.19 to 15 µg L^{-1}, the calibration curve, the coefficient of determination (r^2), is 0.9999, the correlation (r) is 0.9999; and the equation of the line representing the mean between the three analytical curves was represented by $y = 11845x - 1662.4$. The repeatability was evaluated for five different concentrations of methamidophos, distributed along the linear working range: 0.19; 0.38; 0.75; 1.5; 5; and 15 µg L^{-1}. These concentrations were injected in triplicate, yielding a minimum coefficient of variation of 1.53 % and a maximum of 16.79 %.

The intermediate precision was tested with an intermediate concentration of 5 µg L^{-1}. The concentration was injected 16 times, presenting a variation coefficient of 4.8%. According to Silva *et al*. (2008), variation coefficients of up to 20% are deemed acceptable in the analysis of pesticide residues. In this study, repeatability and intermediate precision showed satisfactory coefficients of variation. The accuracy of the method was established for six different and known concentrations, considering the linear working range. The resulting recovery values ranged between 88.75 and 115.5%. According to Guedes (2011), the acceptable recovery intervals for the analysis of residues are generally between 70 and 120%. In the evaluation of sensitivity, the lowest concentration of methamidophos determined with acceptable precision and accuracy, in accordance with the established experiments, was 0.19 µg L^{-1}, which is considered the limit of quantification (LOQ). The limit of detection (LOD) of the method was 59 ng L^{-1}, which corresponds to the estimated detection limit. This value was, calculated by multiplying the intercept deviation multiplied by 3, which is the minimum to which corresponds to the signal (noise) ratio of the base, determined by Equation (5) (ANVISA, 2003):

Table 5. Parameters of merit

Parameters	Value (unit)
Linear range	0.19 - 15 µg L^{-1}
Inclination	11,867.69 L μg^{-1}
r^2	0.9999
LOD (theoretical)	59 ng L^{-1}
LOQ (theoretical)	177 ng L^{-1}

Table 6. Results of ecotoxicological tests with *Ceriodaphnia dubia* and methamidophos in elutriate of different soils

Methamidophos concentration in elutriate (ng L^{-1})	Methamidophos concentration in elutriate (ng L^{-1})	Average number of neonates of *Ceriodaphnia dubia* per female	
(sandy soil)	(clayey soil)	Sandy soil	Clayey soil
0.0 (Control)	0.0 (Control)	23.3	23.3
4.93	3.24	19.3	18.4
6.48	4.27	13.7*	14.8*
8.30	5.46	12.3*	16.0*
10.37	6.82	14.5*	16.8*
12.97	8.53	13.2*	-

*Significant difference compared to control (P<0,05)

$$LOD = \frac{DPa \times 3}{IC} \quad \text{.......................... (5)}$$

Where, DPa is the standard deviation of the linear coefficient with the y-intercept of, at least, 3 calibration curves containing methamidophos concentrations close to the established limit of quantification. IC is the slope. Table 4 summarizes the validation criteria involving key parameters. In Figure 1, LC/MS/MS analysis chromatograms of standard (a) and sample (b).

Ecotoxicological studies

As described in Table 6, the highest concentration attained without causing a significant statistic effect in the reproduction of organisms (NOEC) for the sandy soil was 4.93 ng L^{-1}, with an average of 19.3 neonates. For the clayey soil, the NOEC (No observed effect concentration) was 3.24 ng L^{-1}, with an average of 18.4 neonates. Thus, the intensity of the toxic effect of elutriates was similar for both clayey and sandy soils. Although demonstrated that *C. dubia* and *Dapnhia magna* have similar sensitivity to various substances with less than one order of magnitude (Elnabarawy *et al*., 1986), in the present study this did not happen.

A *Daphnia magna* study (Vega *et al*., 2005), reported a NOEC = 4.5 µg L^{-1} to methamidophos in water, whereas in the tests (Table 6) the NOEC found were of 4.93 and 3.24 ng L^{-1} in the elutriate, that is, a value of approximately 1000 times lower was found in this study. The intensity of toxics effects for *Ceriodaphnia dubia* in elutriate can be attributed exclusively to methamidophos, since the soil samples showed no concentrations of pollutants capable of causing any toxic effect on the organism tested. In addition, the result of the chronic survival test with *Ceriodaphnia dubia* in natural water (not shown) demonstrated the same intensity (LOEC = 6.13 ng L^{-1} of methamidophos) of tests with elutriate, allowing one to attribute the toxics effects solely to methamidophos. In light of the exposed, one can assume that the toxic effects, more pronounced to *Ceriodaphnia dubia,* were caused by the chemical characteristic of the product used in this study (99% active ingredient), which is different from the product used in the study with *Daphnia magna* (USEPA, 2008) that utilized 78.5% of methamidophos.

Comparing the ecotoxicological test and analytical methods, the limit of quantification (LOQ) for the ecotoxicological test (3 to 6 ng L^{-1}) was much smaller than the method of LC-MS/MS (0.19 µg L^{-1}). Despite the exposed, the LOD of the LC-MS/MS was 59 ng L^{-1}, indicating that its LOQ can be reduced by adjusting in the parameters of the analytical validation. The LOD and LOQ of LC-MS / MS was used for the determination of the adsorption methamidophos study elutriate in clay and sandy soils in a concentration higher than that applied to the study of the *Ceriodaphnias dubia.* Thus, the first step in the analysis of methamidophos in soils should be the performance of the ecotoxicological test with *C. dubia.* The use of this method will allow the verification of the occurrence of toxics effects in the elutriate sample. If there is a toxic effect in the elutriate sample of soil it will mean that the values are above the NOEC (greater than 3 ng L^{-1}), which is an indication of the presence of methamidophos in the sample. This fact indicates the need for confirmation of quantities of the analyte by LC-MS/MS. Moreover, the ecotoxicological test will serve as a screening tool for the analysis of elutriate of soil to methamidophos, independently of the pesticide formulation, because the methamidophos has an affinity with the leachate (water). One should note that the (The use of biological and chemical methods) proposed in this paper applies only to the active principle methamidophos. The analysis of the results suggests that this same methodological approach could be followed in the study of other active ingredients. Thus, the approach suggested in this paper, provides a direction to the quantification of the analytical method to be used in the determination of the environmental quality of the soils, regarding the contamination by active ingredients with characteristics similar to methamidophos.

Conclusions

Based on the findings of this study, one can conclude that:

- The methamidophos adsorption capacity in clayey soil is greater than in sandy soil.
- In sandy soil, the methamidophos adsorption showed a better correlation coefficient for the Langmuir isotherm, while for clayey soil the best correlation coefficient was for the Freundlich isotherm.
- Regarding the kinetic models that allow one to observe the behaviour of adsorption and the mechanism of the adsorption process, both the clayey and the sandy soil follow the model of pseudo-second order for the methamidophos adsorption.
- The validation of the chemical method LC-MS/MS was satisfactory. The parameters of linearity, interval, precision, accuracy, and appropriate sensitivity were observed in the results.
- The methods described previously can be used as analytical tools of methamidophos in soils. The ecotoxicological analysis should be used as a screening tool and the LC-MS/MS as confirmatory analysis of the analyte molecule.

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