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MACROPHYTE BIOASSAY APPLICATIONS FOR MONITORING PESTICIDES IN THE AQUATIC ENVIRONMENT[1]

Utilização de Bionsaios com Macrófitas no Biomonitoramento de Agrotóxicos em Ambiente Aquático

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ABSTRACT - The objective of this study was to evaluate the feasibility of the use of macrophytes Lemna minor and Azolla caroliniana as biomarkers of exposure (acute toxicity) for atrazine, bentazon + imazamox and clomazone, insecticide alpha cypermethrin + teflubenzuron and fungicides pyraclostrobin and mixture pyraclostrobin + epoxiconazole, as well as the risk for environmental intoxication. For this purpose, four plants of L. minor and five of A. caroliniana were selected in a 50 mL Hoagland medium. For both plants, the following concentrations were used: 0.10; 1.07; 3.44; 11.16; 36.40 and 118.0 mg L^{-1} of each test product and a control with three replicates. The results of this study demonstrated higher sensitivity of L. minor to the tested pesticides, when compared to A. caroliniana. L. minor can be used in the monitoring of herbicides bentazon, atrazine and clomazone and pyraclostrobin-based fungicide, and A. caroliniana can be used for pyraclostrobin-based fungicides, due to the sensitivity of these organisms when exposed.

Keywords: bioindication, aquatic plants, acute toxicity, environmental monitoring, pesticide.

*RESUMO - O objetivo deste estudo foi avaliar a viabilidade na utilização das macrófitas **Lemna minor** e **Azolla caroliniana** como bioindicadores de exposição (toxicidade aguda) dos herbicidas atrazina, bentazon + imazamox e clomazone, do inseticida alfacipermetrina + teflubenzuron e dos fungicidas piraclostrobina e mistura piraclostrobina + epoxiconazol, bem como o risco de intoxicação ambiental. Para isso, foram selecionadas quatro plantas de **L. minor** e cinco de **A. caroliniana** em 50 mL de meio de cultivo Hoagland. Em ambas as plantas foram utilizadas as concentrações de 0,10, 1,07, 3,44, 11,16, 36,40 e 118,0 mg L^{-1} de cada produto testado e um controle, com três réplicas. Os resultados obtidos neste estudo demonstraram maior sensibilidade da planta **L. minor**, comparada a **A. caroliniana**, aos agrotóxicos testados. **L. minor** pode ser empregada no monitoramento de herbicidas à base de bentazon, atrazina e clomazone e do fungicida à base de piraclostrobina, e **A. caroliniana**, do fungicida à base de piraclostrobina, devido à sensibilidade desses organismos quando expostos.*

Palavras-chave: bioindicação, plantas aquáticas, toxicidade aguda, monitoramento ambiental, agrotóxico.

INTRODUCTION

The use of pesticides is an important factor to maintain the high agricultural productivity; however, the intensification and inadequate use of these products may contribute for the pollution of the earth (Jennings and Li, 2014) and waters (Santos et al., 2015). Among the herbicides, a atrazine (photosystem II inhibitor) has caused some environmental issues (Santos et al., 2015) due to its diversified use to control pre- and post-emergence weeds; bentazon + imazamox (inhibitor of photosystem II and the acetolactate synthase enzyme) is used to control mono and eudicotyledons during the post-emergence state (Ferhatoglu et al., 2005); and clomazone (carotenoid synthesis inhibitor), which is used

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to promote the loss of virtually all plant pigments (albinism) (Bessegato et al., 2012).

The mixture of the pesticides alpha-cypermethrin + teflubenzuron (pyrethroid and benzoylurea) has been emphasized due to the fact that they are used in several cultures (Pinto Junior et al., 2011), as well as the use of fungicides pyraclostrobin and the mixture pyraclostrobin+epoxiconazole (chemical group of strobilurin and triazole), which have a systemic action and are used for cotton, peanuts, oat, banana, potato, coffee, corn, soy and wheat (Prestes et al., 2013).

These products used for farming may reach the water bodies due to drifting, surface runoff and leaching (Geoffroy et al., 2004), promoting changes to the several environmental compartments (Prestes et al., 2013). Some organisms may be used as biological indicators for the presence of contaminants on the environment (Costa et al., 2008). In order to create a base for these evaluations, organisms that represent the several levels of the food chain are used on acute and chronic toxicity assays. These studies are easy-to-use tools and they offer results on the possible ecotoxicological effects of pesticides on non-target organisms on the water environments (Florêncio et al., 2014).

Lemna minor is a standardized macrophyte for toxicity assays (OECD, 2002) and it has been used on studies with pesticides 2.4-D (Belgers et al., 2007), diquat and fomesafen (Gorzerino et al., 2009), atrazine, isoproturon and alachlor (Coutris et al., 2011), glyphosate (Kielak et al., 2011), and imazapyr (Cruz et al., 2015). Another macrophyte that may be used as a bioindicator is *Azolla caroliniana*, due to its easy management, short cycle of life, short height and since it is a cosmopolitan species; however, it still requires studies on its sensitivity as a bioindicator. This macrophyte has been used for the evaluation of pesticides glyphosate, clomazone, oxyfluorfen and 2.4-D (Silva et al., 2012), in addition to imazapyr (Cruz et al., 2015).

With the intensification of farming and the large-scale use of pesticides, it is necessary to increase the monitoring of the possible environmental effects. Therefore, the objective of this study was to evaluate the feasibility of using *L. minor* and *A. caroliniana* as bioindicators of exposure (acute toxicity) for the herbicides atrazine, clomazone and bentazon + imazamox, the alpha-cypermetrin + teflubenzuron insecticide and fungicides pyraclostrobin and mixture of pyraclostrobin + epoxiconazole, as well as the risk of environmental intoxication.

MATERIAL AND METHODS

The herbicides tested were atrazine (CAS: 1912-24-9) with active ingredient concentration of 500 g L^{-1} on the Atrazine Nortox®-Nortox S/A formulation; bentazon (25057-89-0) + imazamox (114311-32-9) with 600 + 28 g L^{-1} on the Amplo®-Basf S/A formulation; clomazone (81777-81-1) with 360 g L^{-1} on the Gamit®-FMC Corporation formulation; insecticide alpha-cypermetrin (67375-30-8) + teflubenzuron (83121-18-0) with 75 + 75 g L^{-1} on the Imunit®-Basf S/A formulation; and fungicides pyraclostrobin (175013-18-0) with 250 g L^{-1} on the Comet®-Basf S/A formulation, and the mixture pyraclostrobin +epoxiconazole (106325-08-0) com 260 + 160 g L^{-1} on the Abacus®-Basf S/A formulation.

Macrophytes *L. minor* and *A. caroliniana* were cultivated on a plastic box with volume for 2.5 liters, containing water and substrate constituted by soil, sand and organic matter (2:1:1; vv^{-1}), on a greenhouse. The acclimatization of the macrophytes was conducted on a bioassay room, with temperature at 25.0 ± 2.0 °C, photoperiod of 12 hours and lighting of 1,000 lux, for three days. After the acclimatization, the plants were disinfected with an aqueous sodium hypochlorite 2% solution for *L. minor* and 3% for *A. caroliniana*.

Then, four *L. minor* plants were selected, with three fronds each, as well as five *A. caroliniana* plants, placed on glass containers with capacity for 100 mL, containing 50 mL of Hoagland culture medium, for 24 hours; then, other 50 mL of Hogland with the pesticides were added, at pH 5.86, for 24 hours, for acclimatization.

In order to evaluate the sensitivity of the plant batches, before the acute toxicity assays, assays were conducted with the reference

substance sodium chlorite (NaCl – *P.A*) (OECD, 2002). The CL50;7d (lethal concentration of 50%, for 7 days) of NaCl for *L. minor* was of 0.65 g L^{-1}, with a confidence interval of 95% between 0.69 and 0.62 g L^{-1}; and for *A. caroliniana*, of 2.14 g L^{-1}, with interval between 2.31 and 1.97 g L^{-1}.

After determining the sensitivity, the definite assays for acute toxicity on a static system were conducted. For such, 50 mL of Hoagland were added to each test container, with the herbicides, insecticide and fungicides. For both plants, the following concentrations were used: 0.10, 1.07, 3.44, 11.16, 36.40 and 118.0 mg L^{-1} of each product and one control, with three repetitions per concentration. The evaluation of the mortality percentage of the plants was conducted three, five and seven days after exposure.

For *L. minor*, the mortality and percentage of chlorosis and necrosis of the fronds was evaluated, as recommended by OECD (2002). For *A. caroliniana*, the mortality used was according to the grade scale (E and A) (SILVA et al., 2012). The mortality results were subjected to linear regression, and the lethal concentration of 50% (CL50;7d) was estimated by the Trimmed Spermam Karber software (Hamilton et al., 1977).

With the lethal concentration values of 50%, pesticides were classified according to the environmental hazard potential suggested by IBAMA (2016). The environmental intoxication risk of the pesticides was determined and classified using the risk quotient (RQ) method, according to Goktepe et al. (2004). On this method, the risk is calculated by dividing CAE (estimated environmental concentration) by CL50;7d obtained on the acute toxicity assays. In order to determine CAE, the highest recommended doses for application in the field for each pesticide were used, and the uniform distribution of the chemical products on the body of water was considered, over an area of 1.0 ha (10,000 m^2).

RESULTS AND DISCUSSION

Ecotoxicity for *L. minor*

For *L. minor*, the lethal concentration of 50% (CL50;7d) of atrazine was 5.27 mg L^{-1}, with lower limit (LI) of 4.39 mg L^{-1} and upper limit (LS) of 6.33 mg L^{-1}. The CL50;7d of bentazon + imazamox was 31.58 mg L^{-1}, with LI of 23.99 mg L^{-1} and LS of 41.56 mg L^{-1}, and for clomazone, 10.23 mg L^{-1}, with LI of 7.91 mg L^{-1} and LS of 13.23 mg L^{-1}.

L. minor was less sensitive to these herbicides than to flumioxazin, with CL50;48h of 3.6 µg L^{-1} (Geoffroy et al., 2004); to metsulfuron (CL50;7d of 12.0 µg L^{-1}), to triasulfuron (0.24 µg L^{-1}); to MCPA (5765 µg L^{-1}); to terbuthylazine (200 µg L^{-1}); to diquat (60 µg L^{-1}); and to aciflourfen (506 µg L^{-1}) (Cedergreen et al., 2007); to the atrazine, isoproturon and alachlor mixture (50+35+15%), with CL50;96h of 70.0 µg L^{-1} (Coutris et al., 2011); and than imazapyr, with CL50;7d of 1.06 mg L^{-1} (Cruz et al., 2015); however, it was more sensitive to glyphosate (CL50 of 19.6 mg L^{-1}) and to mecoprop, with 12.2 mg L^{-1} (Cedergreen et al., 2007).

For atrazine on control, at 0.10 and 1.07 mg L^{-1}, there was no mortality. At 3.44 mg L^{-1} mortality occurred on 61.6% of fronds; at 11.16 mg L^{-1}, on 66%; at 36.40 mg L^{-1}, on 85.0%; and at 118.0 mg L^{-1}, on 100%, with the linear equation and concentration-mortality correlation shown on Table 1. For clomazone on control and at 0.10 mg L^{-1}, there was no mortality; at 3,44 mg L^{-1}, it corresponded to 36%; at 11.16 mg L^{-1}, to 30%; at 36.40 mg L^{-1}, to 30%; at 118.0 mg L^{-1}, to 100%, with linear equation (Table 1).

This herbicide did not show an adequate correlation as to the concentration-mortality relationship, with $R^2 = 0.54$ (Table 1), indicating that some type of degradation of the herbicide may have occurred; however, the degradation of clomazone due to the sunlight on aqueous solutions has not been reported in the literature (Tenbrook and Tjeerdema, 2006).

For bentazon + imazamox, on control, at 0.10 mg L^{-1} and at 1.07 mg L^{-1}, there was no mortality. At 3.44 mg L^{-1}, the mortality corresponded to 10% of the fronds; at 11.16 mg L^{-1}, it corresponded to 25%; at 36.40 mg L^{-1}, to 56.25%; and at 118.0 mg L^{-1}, to 72%, with the linear equation and concentration-mortality correlation shown on Table 1.

Table 1 **-** Representation of linear equations and correlation (R^2) of the concentration-mortality relationship for the tested products

Lemna minor	Linear equation	R^2
Atrazine	y = 21.711x - 23.973	0.90
Clomazone	y = 13x - 5.666	0.54
Bentazon + imazamox	y = 15.964x - 28.167	0.90
Alpha-cypermethrin + Teflubenzuron	y = 1.576x + 12.125	0.42
Pyraclostrobin + Epoxiconazole	y = 5.103x - 5.995	0.65
Pyraclostrobin	y = 14.57x + 10.667	0.96
Azolla caroliniana	Linear equation	R^2
Atrazine	y = 10.511x - 2.673	0.89
Clomazone	y = 6.857x - 1.800	0.54
Bentazon + imazamox	y = 6.631x - 0.126	0.61
Alpha-cypermethrin + Teflubenzuron	y = 0.158x - 0.370	0.42
Pyraclostrobin + Epoxiconazole	y = 6.4294x - 12.960	0.65
Pyraclostrobin	y = 18.549x - 8.653	0.96

For the alpha-cypermetrin + teflubenzuron mixture at CL50;7d > 100.0 mg L^{-1} with the concentration-mortality correlation of only 0.42 for *L. minor* (Table 1), similar to teflubenzuron also for this bioindicator, with CL50;7d > 1,000.0 mg L^{-1} (Medeiros et al., 2013). The mortality of *L. minor* exposed to the associated insecticides alpha-cypermetrin + teflubenzuron varied from 17.76% at 0.10 mg L^{-1} to 23.87% at 36.40 mg L^{-1}. For the pyraclostrobin fungicide at CL50;7d, it reached 1.82 mg L^{-1}, with LI of 1.18 mg L^{-1} and LS of 2.82 mg L^{-1}; and for the pyraclostrobin + epoxiconazole mixture at CL50;7d > 100.0 mg L^{-1}, with the concentration-mortality relationship shown on Table 1.

According to Prestes et al. (2011), the pyraclostrobin + epoxiconazole mixture showed CL50;72h of 0.20 mg L^{-1} for the *Pseudokirchneriella. Subcapitata* alga, while pyraclostrobin alone showed CL50;72 of 5.57 mg L^{-1}, and epoxiconazole, of 1.14 mg L^{-1}; therefore, these authors mention that the magnification factor of the toxicity corresponded to 13.6 times in relation to the individual toxicity of each compound; however, this effect was not characterized for toxicity on *L. minor.*

OECD (2009) determines the maximal concentration (limit for acute toxicity assay) of 100.0 mg L^{-1}, since the lack of mortality at this concentration indicates that the organism does not belong to the most sensitive group to the substance at short-term exposures. Based on this rule, it is not possible to use *L. minor* for the biomonitoring of alpha-cypermetrin + teflubenzuron and pyraclostrobin + epoxiconazole, since no sensitive response occurs (correlation between concentration and mortality) for these products (Table 1).

Ecotoxicity for *A. caroliniana*

For *A. caroliniana*, the lethal concentration (CL50;7d) of atrazine, clomazone and bentazon+imazamox and of the alpha-cypermetrin + teflubenzuron insecticide was higher than 100.0 mg L^{-1}, similarly to 2.4-D (708.35 mg L^{-1}) and to clomazone (129.63 mg L^{-1}) (Silva et al., 2012), which shows the low sensitivity of this bioindicator; the correlation between concentration and mortality is shown on Table 1. *Azolla filiculoide* exposed to the mixture of herbicides atrazine, isoproturon and alachlor (50+35+15%) showed CE50;21d of 60.0 µg L^{-1} (Coutris et al., 2011), and for *Azolla pinnata* exposed at 0.001 and 0.002 mg L^{-1} of carbofuran (Furadan®), there was no inhibition of the NPK fixation by the plant (El-Shahate et al., 2011), considering that they are much more sensitive than *A. caroliniana.*

For atrazine, bentazon+imazamox and clomazone on control and at 0.10 mg L^{-1}, there was no mortality of plants during the

experimental period. Between 1.07 and 118.0 mg L^{-1}, mortality reached 23.3 and 66.6%, respectively. For the alpha-cypermetrin + teflubenzuron insecticide and for the pyraclostrobin + epoxiconazole fungicide, the mortality of *A. caroliniana* occurred only at 36.4 and 118.0 mg L^{-1}. For pyraclostrobin, on control at 0.10 mg L^{-1}, no mortality occurred. At 1.07 mg L^{-1}, 36.6% of mortality occurred; at 3.44 mg L^{-1}, 51.0% occurred; at 11.16 mg L^{-1}, 70%; at 36.40 mg L^{-1}, 80%; and at 118.0 mg L^{-1}, 100% of mortality occurred with the concentration-mortality correlation shown on Table 1.

For Silva et al. (2012), glyphosate (Scout® and Trop®) showed CL50;7d of 23.66 mg L^{-1} and 38.91 mg L^{-1}, respectively; oxyfluorfen, of 80.50 mg L^{-1}; and imazapyr, of 18.98 mg L^{-1} (Cruz et al., 2015), which indicates a higher sensitivity of *A. caroliniana* to these herbicides than to the ones tested on this study using the same plant (*A. caroliniana*).

The CL50;7d of pyraclostrobin for *A. caroliniana* was of 3.22 mg L^{-1}, with LI of 2.36 mg L^{-1} and LS of 4.40 mg L^{-1}; for the pyraclostrobin + epoxiconazole mixture, CL50;7d > 100.0 mg L^{-1}. These values were similar to the evaluation made by Probst et al. (2005) with epoxiconazole (CL50;72h of 1.66 mg L^{-1}) and by Ochoa-Acuña et al. (2009) with pyraclostrobin (1.4 mg L^{-1}); however, they differed from the ones obtained by Coors and Frische (2011) with pyraclostrobin exposed to the alga (CE50 of 0.15 mg L^{-1}).

The mixture of the pyraclostrobin + epoxiconazole fungicides evaluated by Prestes et al. (2011) with the alga *P. subcapitata* promoted different effects than the ones found by this study, with growth promotion at the lowest concentrations (0.01 ad 0.10 mg L^{-1}) up to 48 hours of exposure, followed by growth inhibition at the other doses (0.01, 0.10, 1.0, 10.0 and 100.0 mg L^{-1}), and for the products alone (pyraclostrobin and epoxiconazole), there was a promotion on the growth of algae.

Comparative analysis across bioindicators, ecotoxicological classification and environmental intoxication risk

According to the CL50;7d data obtained, *L. minor* was sensitive to the herbicides atrazine, bentazon+imazamox and clomazone, and to the fungicide pyraclostrobin, while *A. caroliniana* was sensitive to the fungicide pyraclostrobin. The order of CL50;7d for *L. minor* was pyraclostrobin > atrazine > clomazone > bentazon+imazamox > alpha-cypermetrin + teflubenzuron = pyraclostrobin + epoxiconazole and, for *A. caroliniana*, pyraclostrobin > atrazine = clomazone = bentazon+imazamox = alpha-cypermetrin + teflubenzuron = pyraclostrobin + epoxiconazole.

Atrazine and pyraclostrobin were classified as very toxic, and clomazone and bentazon + imazamox, as moderately toxic, for *L. minor* (IBAMA, 2016). The other tested products were considered slightly toxic. *L. minor* was more sensitive to the herbicide metsulfuron-methyl in comparison to *Elodea canadensis*, *Callitriche platycarpa*, *Potamogeton crispus*, *Ceratophyllum demersum*, *C. submersum*, *Batrachium trichophyllum*, *Berula erecta*, *Sparganium emersum*, *Spirodela polyrrhiza* and *L. trisulca* (Cedergreen et al., 2004), which indicates its potential to be used for the biomonitoring of pesticides, specially herbicides, on water environments. According to Fairchild et al. (1997), this plant is sensitive to triazines, such as atrazine, sulphonylureas (metsulfuron and clorsulfuron), pyridines (diquat and paraquat), dinitroaniline (trifluralin) and acetanilide (alachlor and metolachlor).

The herbicides atrazine, clomazone and bentazon+imazamox, insecticide alpha-cypermetrin + teflubenzuron, and fungicide pyraclostrobin + epoxiconazole were classified as slightly toxic, and the fungicide pyraclostrobin, as very toxic, for *A. caroliniana* (IBAMA, 2016). *A. caroliniana* was sensitive to pyraclostrobin, which indicates the possibility of using it for the biomonitoring of this fungicide e group.

The insecticide alpha-cypermetrin + teflubenzuron and fungicide pyraclostrobin + epoxiconazole were the molecules that shoed the lowest toxicity for the tested bioindicators, which may be due to the fact that the insecticide is an inhibitor for chitin (benzoylureas) and the central nervous system (pyrethroids) and that the association of fungicides promotes an antagonist effect of

Table 2 - Risk quotient values (RQ) for the tested products

Pesticides	*Lemna minor*	*Azolla caroliniana*
Atrazine	0.0759	0.0040
Clomazone	0.0148	0.0015
Bentazon + imazamox	0.0049	0.0015
Alpha-cypermethrin + Teflubenzuron	0.00007	0.00007
Pyraclostrobin + Epoxiconazole	0.0002	0.0002
Pyraclostrobin	0.0109	0.0062

toxicity (Savoy, 2011). Cypermetrin, according to Wendt-Rasch et al. (2003), promoted a change on the composition of species, increasing the phytoplankton population on concentrations higher than 0.13 $\mu g\ L^{-1}$. Fungicides pyraclostrobin + epoxiconazole promote high toxicity for the microcrustaceans *Daphnia similis* and the fish species tambaqui (*Colossoma macropomum*), both in combination and in isolation (Prestes et al., 2012, 2013). The herbicide atrazine is the one that is most detected on surface and underground waters (Mudhoo and Garg, 2011; Santos et al., 2015), and bentazon has been identified on underground waters on the states of Virginia, Missouri, California and, more recently, in Florida (Bessegato et al., 2012).

On the environmental intoxication risk evaluation, according to Goktepe et al. (2004), atrazine is classified as a moderate environmental risk herbicide (0.05<RQ<0.5), and the other tested products are classified as low risk (RQ<0.05), for *L. minor* (Table 2). For *A. caroliniana*, all tested products were classified as low environmental risk (RQ<0.05) (Table 2), even if 100% of the highest recommended dose for application in the field gets to the water environment.

The results obtained on this study showed a higher sensitivity of the *L. minor* plant, in comparison to *A. caroliniana*, to the tested pesticides. It shows low environmental intoxication risk for macrophytes. Therefore, it is concluded that *L. minor* may be used to monitor bentazon, atrazine and clomazone-based herbicides and the pyraclostrobin-based fungicide, and *A. caroliniana* may be used to monitor the pyraclostrobin-based fungicide.

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