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Article

EFFECTIVENESS OF DIQUAT, BOTH ISOLATED AND ASSOCIATED WITH COPPER SOURCES IN CONTROLLING THE *Hydrilla verticillata* SUBMERGED MACROPHYTES AND ANKISTRODESMUS *Gracilis microphyte*

Eficácia do Diquat Isolado e em Mistura com Fontes de Cobre no Controle da Macrófita Submersa ***Hydrilla verticillata*** *e da Microalga* ***Ankistrodesmus gracilis***

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ABSTRACT - The goal of this study was to evaluate the effectiveness of diquat, both isolated and associated with copper sources (oxychloride and hydroxide) in controling the *H. verticillata* submerged macrophyte and the *A. gracilis* microalgae. For this purpose, 10.0 cm *H. verticillata* young branches and 300 mL of *A. gracilis* culture were used. The experiments were performed in laboratory and the tested diquat concentrations were: 0.1; 0.2; 0.4; 0.8; 1.2; and 1.8 mg L^{-1}, either isolated or added with 1.0% copper oxychlorideand hydroxide, as well as a control sample. On day 3, 7, 11, 21 and 30 after application, phytotoxicity signs were evaluated and on day 60 after application, green and dry biomass production and plant length were measured. To obtain dry mass, plants remained in a greenhouse with forced air circulation at 65.0 ^{o}C, until constant weight. On day 1, 7, 15, 21, 30, 45 and 60 after application, the concentration of chlorophyll *a* in the water was assessed. The herbicide diquat used alone or in combination with sources copper oxychloride and hydroxide was effective in the control of *H. verticillata* and microalgae *A. gracilis*.

Keywords: chemical control, water plant, eutrophication, phytotoxicity, chlorophyll *a*.

RESUMO - O objetivo deste estudo foi avaliar a eficácia do diquat isolado e em mistura com fontes de cobre (hidróxido e oxicloreto) para o controle da macrófita submersa ***Hydrilla verticillata*** *e da microalga* ***A. gracilis****. Para isso, foram utilizados ponteiros com 10,0 cm de* ***H. verticillata*** *e 300 mL de cultura de* ***A. gracilis****. Os experimentos foram conduzidos em laboratório, e as concentrações de diquat testadas foram: 0,1; 0,2; 0,4; 0,8; 1,2; e 1,8 mg L^{-1} isoladas ou acrescidas de 1,0% de oxicloreto de cobre e de hidróxido de cobre, além de um controle. Aos 3, 7, 11, 21 e 30 dias após aplicação foram avaliados os sinais visuais de fitotoxicidade, e aos 60 dias após aplicação foi mensurada a produção de biomassa fresca e seca e o comprimento das plantas. Aos 1, 7, 15, 21, 30, 45 e 60 dias após aplicação foi avaliada a concentração de clorofila a presente na água. O herbicida diquat utilizado de forma isolada ou em mistura com as fontes de cobre oxicloreto e hidróxido foi efetivo no controle de* ***H. verticillata*** *e da microalga* ***A. gracilis****.*

Palavras-chave: controle químico, planta aquática, eutrofização, fitotoxicidade, clorofila *a*.

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INTRODUCTION

Macrophytes work as a habitat for many organisms and as a shelter for fishes, and they help the sedimentation of particulate matter and the release of energy and carbon at the basis of food chains (Wong et al., 2010). However, anthropogenic activities have decisively contributed to the eutrophication of hydric bodies and to the uncontrolled growth of these plants; this damages their multiple uses (Silva et al., 2012).

The reflexes of these infestations are the accumulation of waste and sediments, the difficulty in navigation and cargo transportation, the damages to tourism and fishing and the generation of energy in hydroelectric power stations. Submerged macrophytes can also decrease water oxygenation, especially at night (Carvalho et al., 2005), and even unbalance the carbon/nitrogen relation due to degradation and decomposition processes with oxygen consumption (Yuan et al., 2016).

Among the submerged species, *Hydrilla verticillata* Royle comes from Asia and has been described as one of the most problematic plants in various regions, especially tropical and sub-tropical, and as an exotic invasive species(Sousa et al., 2010). This plant has great colonization capacity, since it vegetatively reproduces by tubers, stem fragmentation and seeds, turning the colonized environment into one with single-species or poorly diversified vegetation (Pitelli et al., 2012).

Among the management procedures to control water macrophytes, physical control with pruning or manual collection, mechanical control with large machines, biological control using live organisms and chemical control with herbicides may be used (Pompêo, 2008).

The National Environment Council approved a regulation that allows the use of chemical products, as long as they are properly registered for water environments through Resolution n. 467, dated July 16th, 2015. Published on the Official Federal Gazette (DOU) on July 17th, 2015, section 1, p. 70 to 71, it "regulates the criteria for the authorization to use physical, chemical or biological products or agents in order to control organisms or pollutants in superficial water bodies. It also contains other provisions" (Conama, 2015).

Diquat is an alternative to control submerged macrophytes, since it is a contact herbicide, non-selective, I photosynthesis inhibitor, belonging to the bipyridyls group; its half-life is lower than 48 hours, leaving water waste-free, since it binds to colloidal soil particles (Rodrigues and Almeida, 2011) with favorable ecotoxicological profile for water organisms like fishes and snails (Garlich et al., 2016a). Studies on its effectiveness were performed with *Hydrilla verticilata*, *Egeria densa* and *Egeria najas* (Henares et al., 2011) and *Ceratophyllum demersum* (Garlich et al., 2016b).

One of the possible problems after the control of macrophyte infestations is the release of nutrients, such as nitrogen and phosphorus, into the water environment, due to the decomposition process; this makes the eutrophication process easier (Mohr et al., 2007), helping the reproduction and proliferation of algae.

The increase in algae density may lead to the decrease of dissolved oxygen in the water, due to night consumption and because of its decomposition; this may lead to fish death, release of toxins secreted by some species of cyanobacteria and water quality degradation; it may also alter water drinkability, increase the price of treatments, cause problems in public supply and prevent recreational use (Vidotti and Rollemberg, 2004; Moura and Firmino, 2014).

Copper may be effective in algae control, because it inhibits cell division and photosynthesis, as demonstrated for *Chlorella* sp. and *Scenedesmus obliquus* (Ma and Liang, 2001), *Pseudokirchneriella subcapitata* (Schamphelaere et al., 2005), *Chlorella pyrenoidosa* (Xia and Tian, 2009) and *Ankistrodesmus gracilis* (Garlich et al., 2016a). Diquat and copper sources mixtures are described as effective to control macrophytes or algae (Martins et al., 2008; Henares et al., 2011; Garlich et al., 2016a,b).

Using the application technology of an herbicide and a algaecide product, blended or in a row, may facilitate the control of macrophytes and algae, and hinder the use of nutrients generated by the decomposition of macrophytes for algae flowering and, thus, minimize the environmental impact of chemical control. Therefore, the goals of this study were determining the effectiveness of the diquat herbicide, both isolated and associated with copper sources (oxychloride and hydroxide), in controlling the submerged macrophyte *H. verticillata* and assessing the effectiveness of secondary control on the *A. gracilis* microphyte, in laboratory conditions.

MATERIAL AND METHODS

In the experiments, 10 cm long young branches of *H. verticillata* were used, according to Henares et al's recommendations (2011). The *A. gracilis* microphyte was obtained from n. 005CH collection, coming from the Broa dam, in Itirapina - São Paulo State (22°15'S and 47°19'W). This microphyte was given by the Limnology and Plancton Production Laboratory from UNESP Aquaculture Center and cultivated in static system at 22.0 ± 2.0 °C, exposed to 30.1 µmol cm^2 s^{-1} light in half NPK (Sipaúba-Tavares and Rocha, 1993).

The tested products were diquat herbicide (CAS 85-00-7), in the Reglone® formulation, with 200.0 g a.i. L^{-1}, isolated and associated with 1.0% copper oxychloride (CAS 1332-65-6), in the Difere® formulation, with 588.0 g a.i. L^{-1} and 1.0% copper hydroxide (CAS 20427-59-2), and in the Supera® formulation, with 537.4 g a.i. L^{-1}.

Effectiveness experiments for *H. verticillata* submerged macrophyte and *A. gracilis* unicellular algae

The experiments were performed in a bioassay laboratory at a 25.0 ± 1.0 °C temperature, with a 12 hour photoperiod and 1,000 lux lighting. For this purpose, three *H. verticillata* young branches, tied at the bottom with a lead weight, were added to plastic containers with 1.5 L capacity and continuous ventilation. In each experimental unit, 300 mL of microphyte culture in exponential growth phase (10 days) were added, with 5 x 10^3 cells mL^{-1} concentration.

After a 24 hour acclimatization, herbicide application was performed, both isolated and associated with copper sources. The tested concentrations of diquat and of the mixtures with 1.0% copper oxychloride and 1.0% copper hydroxide were: 0.1; 0.2; 0.4; 0.8; 1.2; and 1.8 mg L^{-1} and a control sample, with ten fully randomized design repetitions (DIC).

On day 3, 7, 11, 21 and 30 after application (DAA), phytotoxicity signs were evaluated, being characterized in the following way: 0, no sign; 1, young branches chlorosis; 2, leaf edge chlorosis; 3, leaf edge necrosis; 4, total leaf necrosis; 5, shrinkage; 6, growth inhibition; 7, loss of sustainability capacity of the branches; and 8, total necrosis of the young branches (death). On day 60 after application (DAA), production of green biomass, plant length (cm), and plant dry biomass were measured, as recommended by Arts et al. (2008). To obtain dry mass (g), the plants were kept in a greenhouse with forced air circulation at 65.0 °C until getting constant weight.

Evaluation of chlorophyll *a* and statistic analysis

Chlorophyll *a* was extracted with 90% acetone and quantified at 664 and 750 nm in a spectrophotometer, adapted from the methodology proposed by Cetesb (1990). Concentrations of chlorophyll *a* in the water were evaluated on the first day and on day 7, 15, 21, 30, 45 and 60 after application (DAA).

The obtained results on green biomass, young branches length, dry biomass and chlorophyll *a* were submitted to analysis of variance (ANOVA) and their averages were compared by Turkey test at 5% probability in the ASSISTAT program, version 7.6 Beta (Silva and Azevedo, 2012).

RESULTS AND DISCUSSION

In the experiment with diquat, either isolated or added with 1.0% copper oxychloride and hydroxide, there was 100% control in all tested concentrations, without final biomass production. General signs of phytotoxicity were: young branches chlorosis, leaf edge chlorosis, growth inhibition, loss of sustainability capacity of the young branches and total necrosis of the young branches (Table 1). Total biomass reduction also occurred with the use of diquat, both isolated and associated with 0.1% copper oxychloride and hydroxide. It controlled 100% of *Ceratophyllum demersum* (Garlich et al., 2016b).

On day 3 DAA there was no sign of phytotoxicity caused by the herbicide, except for the paralysis of plant growth compared to the control sample (Table 1). On day 7 and 11 DAA, there was branch chlorosis in all the tested concentrations; on day 21 DAA it was possible to observe the loss of sustainability capacity of the young branches; and on day 30 DAA there was total necrosis of the young branches in all concentrations (Table 1). These phyto-intoxication signs also occurred with *Elodea canadensis*, *Elodea nuttallii* and *Potamogeton crispus* when exposed to asulam herbicide (Arts et al., 2008) and with *C. demersum* when exposed to diquat (Garlich et al., 2016b).

Table 1 - General phyto-toxicity signs of *Hydrilla verticillata* macrophyte after application of diquat, diquat + 1.0% copper hydroxide and diquat + 1.0% copper oxychloride during the experimental period

Concentration (mg L^{-1})	Days after application (DAA)				
	3	7	11	21	30
0.1	0	1, 2, 6	1, 6	6, 7	8
0.2	0	1, 2, 6	1, 6	6, 7	8
0.4	0	1, 2, 6	1, 6, 7	6, 7, 8	8
1.2	0	1, 2, 6	1, 6, 7	6, 7, 8	8
1.8	0	1, 2, 6	1, 6, 7	6, 7, 8	8

0, no sign; 1, young branches chlorosis; 2, leaf edge chlorosis; 3, leaf edge necrosis; 4, total leaf necrosis; 5, shrinkage; 6, growth inhibition; 7, loss of sustainability capacity of the softwood cutting; and 8, total necrosis of the young branches (death).

According to Henares et al. (2011), *Hydrilla verticillata* is more sensitive to diquat than other Brazilian submerged macrophytes, such as *Egeria densa* and *Egeria najas*, with a three times bigger growth reduction of the young branches when compared to the one of these macrophytes after the application of 1,6 mg L^{-1}. Comparing these studies, it was verified that this plant has greater sensibility to diquat, both isolated and associated with copper sources, since, according to Henares et al. (2011), 14 days after diquat application (1.6 mg L^{-1}) there still was *E. densa* and *E. najas* fresh biomass. According to Langeland et al. (2002) and Glomski et al. (2005), there was *H. verticillata* dry biomass on day 21 with the application of 0.25 mg L^{-1} and 0.37 mg L^{-1}, respectively.

As shown by this experiment, to obtain total control over the biomass of *H. verticillata* macrophyte, a more extended evaluation is needed, up to 60 days after application.

Van et al. (1987) waited for the minimum time of a two day exposure of this macrophyte, in laboratory condition, to 0.25 mg L^{-1} to obtain control, whereas for the 2.0 mg L^{-1} concentration, the necessary time was 6 to 12 hours.

According to Glomski et al. (2005), diquat in 0.09, 0.185, and 0.37 mg L^{-1} concentrations, with a 12 hour exposure, showed excellent control of *E. canadensis,* whereas the control of *H. verticillata* was not satisfactory. Diquat (0.37 mg L^{-1}) presented excellent control of this macrophyte and of *E. densa*, in static condition or with an exposure time of three hours minimum, but in a water flow simulation scenario it was not effective in the control of *H. verticillata* (Skogerboe et al., 2006).

The increase of 1.0% copper in the diquat commercial formulation did not interfere in the herbicide effectiveness (Table 1), indicating that there is no need for antagonistic effect between the active ingredient (6.7-dihydrodipyride [1.2-*a*:2',1'-c] á pyrazinediium ion) and copper oxychloride and hydroxide. This effect was also described by Sutton et al. (1970), with the addition of 0.1 to 2.0 mg L^{-1} copper sulphate to diquat herbicide, in order to control *E. densa, Najas guadalupensis* and *H. verticillata*; according to Pennington et al. (2001), with a mixture of 1.0 to 3.0 mg L^{-1} endothall + 0.5 mg L^{-1} copper, with similar control results (> 99%) over *H. verticillata*; according to Garlich et al. (2016b) with diquat, either isolated or associated with 0.1% copper oxychloride and hydroxide, for *C. demersum.*

In the experiments performed with diquat, either isolated or increased with copper sources, the experimental period lasted 60 days in static system, since one of the goals was to verify the possible control effect over *A. gracilis* microphyte; however, the absence of final macrophyte biomass in all tested concentrations indicates that the main problem in experiments with diquat are exposure time and effect evaluation time.

In the evaluation of chlorophyll *a* on day 1 after diquat application, the pigment concentration was around 12,0 µg L^{-1} in all tested concentrations (Table 2). On day 7 and 15 DAA, in the sample treatment, chlorophyll *a* concentration was 13.77 and 16.39 µg L^{-1} respectively, whereas in all concentrations there was a drastic decrease in chlorophyll *a*, significantly differing from the

Table 2 - Averages of chlorophyll *a* values (mg L^{-1}) in *Ankistrodesmus gracilis* microphyte crop water with application of diquat herbicide during the experimental period

Conc. (mg L^{-1})	Days after application						
	1	7	15	21	30	45	60
0.0	12.67	13.77 a	16.39 a	17.16 a	17.73 a	18.71 a	20.69 a
0.1	12.45	1.92 b	1.30 b	0.61 b	1.81 b	4.63 b	5.45 b
0.2	12.37	1.65 b	1.07 b	0.50 bc	1.65 b	4.38 b	5.22 b
0.4	12.61	1.52 b	0.74 b	0.46 bc	1.57 b	4.10 b	4.51 b
0.8	12.62	0.74 c	0.52 b	0.35 bc	1.05 bc	3.33 b	4.39 b
1.2	12.69	0.61 c	0.44 b	0.26 bc	0.72 c	1.56 c	2.84 c
1.8	12.53	0.36 c	0.37 b	0.23 c	0.68 c	0.92 c	2.64 c
VC	1.21	7.86	9.46	4.67	8.03	9.04	8.36
DMS	0.42	0.64	1.61	0.36	0.80	1.50	1.52

Averages followed by the same letter on the column do not statistically differ among themselves by the Turkey test ($p<0.05$).

control sample, with values between 1.92 and 0.36 μg L^{-1}; 0.8, 1.2 and 1.8 mg L^{-1} treatments differed even more from the other concentrations (Table 2). The high chlorophyll *a* reduction on day 15 DAA for *A. gracilis* algae is similar to what was described for diquat; copper oxychloride and hydroxide reduced photosynthetic activity, with values between 0.09 and 0.14 μg L^{-1} (Garlich et al., 2016a).

From day 21 to 45 DAA this tendency remained constant with 1.2 and 1.8 mg L^{-1} concentrations, differing from the control sample and from the other concentrations (Table 2). On day 60 DAA, a process of chlorophyll *a* increase in the water from the experimental containers was started, although still significantly lower than the control sample (Table 2), indicating the beginning of algae growth recovery. During this period, values varied between 5.45 and 2.64 μg L^{-1} (Table 2).

Diquat has been vastly evaluated for macrophyte control, especially to promote fast control over many species (Martins et al., 2008; Garlich et al., 2016b). This is a photosystem-I inhibitor herbicide, that reduces the effectiveness in electron transfer; this system can be found both in macrophytes and algae. This secondary effect over *A. gracilis* microphyte is fundamental in management decision, since one of the main problems in chemical control use is the quick release of nutrients into water, which may cause eutrophication and help new microphytes colonization (Rattray et al., 1991) or algae flowering (Hessen et al., 2002).

Diquat was effective in controlling *A. gracilis* microphyte, similar to *Scenedesmus vacuolatus*, exposed to 13 to 30,000 μg L^{-1} S-metalachor for 48 hours (Copin et al., 2016) and to *Chlamydomonas reinhardtii* exposed to paraquat herbicide with 26 μM effective concentration (EC50) (Jamers and De Coen, 2010).

In the test with diquat + 1.0% copper hydroxide, on day 1 DAA the detected chlorophyll *a* concentration was about 12.0 μg L^{-1} (Table 3). On day 7 DAA there was a significant decrease in all tested concentrations, compared with the control sample. The most effective concentrations were 1.2 and 1.8, which presented the lowest chlorophyll *a* values in water. (Table 3). From day 15 to 60 DAA a similar control behavior was observed in all concentrations (Table 3). Compared with the isolated diquat test, the final concentration of chlorophyll *a* was lower in this test, indicating that the presence of 1.0% copper hydroxide in the herbicide formulation increases the algaecide effect in the experimental unit, similar to copper sulphate, in 55.8, 117.5, and 187.5 μg L^{-1} concentrations ($CuSO_4$), to control *Raphidocelis subcapitata* (Murray-Gulde et al., 2002) and similar to diquat + 0.1% copper hydroxide, which was effective from 0.8 mg L^{-1} in controlling *A. gracilis* as well, with 0.32 μg L^{-1} chlorophyll *a* values (Garlich et al., 2016a).

In the test with diquat + 1.0% copper oxychloride, the concentrations of chlorophyll *a* demonstrated control over *A. gracilis*, similar to what was described, with the addition of copper hydroxide in the diquat formulation (Table 4); however, the concentration of chlorophyll *a* on day 60 after application was lower in this experiment, with values between 0.39 e 0.05 μg L^{-1} (Table 4).

Table 3 - Averages of chlorophyll *a* values (mg L^{-1}) in *Ankistrodesmus gracilis* microphyte crop water with application of diquat herbicide + 1.0% copper hydroxide during the experimental period

Conc. (mg L^{-1})	Days after application						
	1	7	15	21	30	45	60
0.0	12.68	13.77 a	13.39 a	17.16 a	17.73 a	18.71 a	20.69 a
0.1	12.62	3.91 b	1.06 b	0.98 b	1.24 b	1.25 b	0.79 b
0.2	12.71	3.27 bc	0.77 b	0.66 bc	1.06 b	0.52 bc	0.54 b
0.4	12.47	3.25 bc	0.60 b	0.57 bc	1.02 b	0.37 bc	0.46 b
0.8	12.50	2.14 cd	0.26 b	0.54 bc	0.88 bc	0.32 c	0.31 b
1.2	12.69	1.76 d	0.23 b	0.53 bc	0.32 c	0.25 c	0.09 b
1.8	12.79	1.54 d	0.15 b	0.45 c	0.29 c	0.24 c	0.08 b
VC	1.46	8.12	9.72	5.52	7.73	8.43	9.98
DMS	0.51	1.19	1.60	0.45	0.69	0.90	1.00

Averages followed by the same letter on the column do not statistically differ among themselves by the Turkey test ($p<0.05$).

Table 4 - Averages of chlorophyll *a* values (mg L^{-1}) in *Ankistrodesmus gracilis* microphyte crop water with application of diquat herbicide + 1.0% copper oxychloride during the experimental period

Conc. (mg L^{-1})	Days after application						
	1	7	15	21	30	45	60
0.0	12.66	13.77 a	16.39 a	17.16 a	17.73 a	18.71 a	20.69 a
0.1	12.60	3.88 b	0.47 b	0.92 b	2.07 b	0.48 b	0.39 b
0.2	12.74	2.26 bc	0.44 b	0.44 c	0.73 c	0.27 b	0.27 b
0.4	12.63	1.85 bc	0.40 b	0.40 c	0.27 c	0.18 b	0.18 b
0.8	12.65	1.61 c	0.31 b	0.28 c	0.21 c	0.12 b	0.07 b
1.2	12.77	1.47 c	0.30 b	0.27 c	0.17 c	0.08 b	0.06 b
1.8	12.96	0.89 c	0.22 B	0.19 c	0.14 c	0.04 b	0.05 b
VC	1.50	9.77	8.51	5.13	8.69	7.97	9.48
DMS	0.53	2.02	1.59	0.40	1.16	0.63	0.99

Averages followed by the same letter on the column do not statistically differ among themselves by the Turkey test ($p<0.05$).

For *A. gracilis*, the mixture diquat + 0.1% copper oxychloride, on day 15 DAA, in 1.2 mg L^{-1} concentration, reduced chlorophyll *a* values to 0.26 µg L^{-1} (Garlich et al., 2016a).

According to Franklin et al. (2002), the addition of only 1.4 mM ion, copper or combined with zinc or cadmium, is enough to control the growth activity of the tropical fresh water alga *Chlorella* sp.; this value is far lower than the one used in this study. In some cases, depending on active form, formulation, chemical availability or environmental factors, copper may just temporarily paralyze the growth activity of the alga, since, according to Zhang et al. (2014) for *Chlorella vulgaris* and according to Hook et al. (2014) for *Ceratoneis closterium*, the luminous intensity and highconcentrations of copper damage the metabolic routes of photosynthesis, causing deleterious effects on structural, biochemical and physiological levels in the algae and, consequently, they affect nitrogen fixation, cause disorders in the plasma membrane and reduce the absorption of mineral elements, harming cellular mobility and causing instability in organelles.

The decrease in chlorophyll *a* concentration in the three experiments was similar to the one described for *Scenedesmus obliquus* exposed to 1,0 µg L^{-1} diuron (Eullaffroy and Vernet, 1990). The same alga, exposed for 24 and 48 hours to 1,000.0; 100.0; 10.0; 1.0 and 0.1 mg L^{-1} of flazasulfuron herbicide resulted in final values around 0.1 µg L^{-1} of photosynthetic pigment (Couderchet and Vernet, 2003).

The use of diquat herbicide, either isolated or associated with copper sources (oxychloride or hydroxide) showed excellent control effectiveness over *H. verticillata* submerged macrophyte and *A. gracilis* microphyte in the conditions of this experiment, creating a new possibility for macrophyte and algae management.

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