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DCPIP and Respirometry Used in the Understanding of Ametryn Biodegradation

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Abstract

In view of an environmental scenario of degradation of the terrestrial ecosystem due to the indiscriminate use of organic compounds, it is necessary to use complementary techniques to evaluate the remediation processes of contaminated soils. Thus, the objective of this work

was to evaluate the biodegradation of ametryn herbicide, which is applied in sugarcane crops to control broadleaf weeds. The colorimetric assay was carried out by oxidation of 2.6-dichlorophenol-indophenol (DCPIP) in order to quantify the biodegradation of organic compounds present in the soil, in a short period of time. Another method used was the quantification of CO_2 from the microbial respiration responsible for the metabolization of organic compounds in soil. The biodegradation of the ametryn herbicide was evaluated in soil, with the addition of microbial consortium as bioaumentation and addition of surfactant, as a form of microbial stimulation. It was observed that the addition of bioaumentation and biostimulation favored the increased metabolism of ametryn, reducing its residence time in the environment. The colorimetric assay is simple to perform, bringing rapid results. The respirometric assay is simple to perform, however, it has had long-term results. Thus, the use of a colorimetric assay as the primary evaluation of the biodegradation process of organic compounds and the use of the respirometric assay for the long-term evaluation of the metabolization process is suggested. Therefore, the use of complementary methods helps in understanding the process of biodegradation of organic compounds in the environment, as well as in the combination of factors that favor the mitigation of contaminated sites.

Keywords: Oxidation, Herbicide, Colorimetry

1. Introduction

Metabolizing organic compounds in the environment, the microorganisms perform reduction oxide processes, where the path to obtaining energy is respiration, with oxygen as the final acceptor of electrons. Oxygen also acts as an activator of the organic substrate through oxygenation reactions (Díaz, 2004).

Anaerobic processes also use oxide reduction reactions to obtain energy, but with other types of electron acceptors such as nitrates and sulfates. The colorimetric method is based on reactions of oxidation reduction occurring the substitution of a mediator of electrons like oxygen, sulfates, among others, by a synthetic mediator. They are fast and inexpensive processes to detect microbial activity (Cao et al., 2009; Hanson et al., 1993; Hutchins et al., 1991).

A synthetic mediator used to indicate the ability of microorganisms to degrade hydrocarbons is 2.6-dichlorophenol-indophenol (DCPIP). Thus, DCPIP is an indicator that changes its colorless to blue coloration when oxidized in liquid medium. In this way, it can estimate the microbial activity by changing the color of the medium. The color change (Figure 1) is related to the electron exchange, resulting from the biodegradation of organic compounds (Van Hamme et al., 2000).

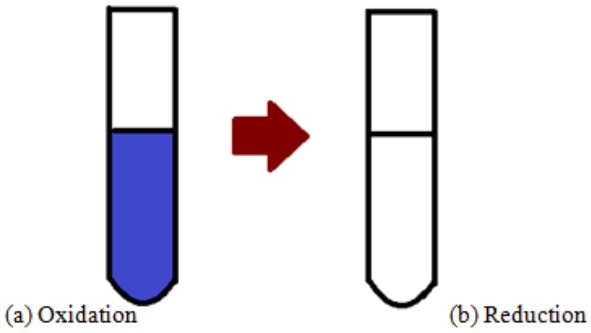

Figure 1. Oxidation-reduction state of 2,6-dichlorophenol-indophenol

The nitrogen present in the DCPIP molecule below functions as an electron acceptor changing the coloration after it is reduced. The change of color is related to the exchange of electrons, resulting from the biodegradation of organic compounds.

Thus, by means of the DCPIP colorimetric assay, the oxidation time of the organic compound can be estimated by microbial routes. Therefore, the objective of this work was to evaluate the oxidation time of the ametryn herbicide in sandy soil, as well as the evaluation of factors such as bioaumentation and microbiol stimulation.

The herbicide ametryn is applied in sugarcane crops to control broad-leaved weeds, and is moderately persistent in the environment. Therefore, it is necessary to add factors of bioaumentation and microbial biostimulation in order to favor the increase of the biodegradation of its molecule as well as to decrease its time of permanence in the

environment (Carmo et al., 2013. Jacomi et al., 2009; Laabs et al., 2001; Mudhoo and Garg, 2011).

In this work, the biodegradation of the ametryn molecule was evaluated by two different methods. The first is through the evaluation of the oxidation of DCPIP, correlating with the metabolism of ametryn in a short time. The second method is through the respirometry of Bartha and Pramer, quantifying the generation of CO_2, referring to metabolization through the microbial activity present in the analyzed soil. In addition, the addition of microbial consortium inoculum and as biostimulation the addition of Tween 80 surfactant was used as a form of bioaumentation, in order to evaluate the acceleration of ametryn metabolism in sandy soil.

2. Material and Method

2.1 Preparation of the DCPIP Solution, Bushnell Haas Culture Broth and Tween 80 Surfactant

The colorimetric test with 2.6-dichlorophenol-indophenol (DCPIP) was performed using the concentration of 1.000 g/L together, the Bushnell Haas (BH) broth, read at 600 nm (Difco, 2009).

The 2.6-dichlorophenol-indophenol (DCPIP-VETEC P.A.) is an indicator that changes its colorless to blue coloration when oxidized in liquid medium. In this way, it can estimate the microbial activity by changing the color of the medium, according to the adaptation of the methodology described by Bidoia, Montagnolli and Lopes (2010).

DCPIP was used at the concentration of 1.000 g / L dissolved in deionized water. Tween 80 Synth surfactant (Lot 82925) was used at 1%. The Table 1 below shows the constituents of the Bushnell Haas broth (BH).

Table 1. Constitutions of the BH

Reagents	Quantity
$MgSO_4.7H_2O$	0.2 g/L
$CaCl_2.2H_2O$	0.02 g/L
K_2HPO_4	1.00 g/L
KH_2PO_4	1.00 g/L
NH_4NO_3	1.00 g/L
$FeCl_3.6H_2O$	0.05 g/L

2.2 Ametryn Herbicide

The ametryn herbicide (N-ethyl-N- (1-methylethyl) -6- (methylthio) -1,3,5-triazine-2,4-diamine) from Fluka (Germany), 98.5% pure, was used in granular form. The concentration used was 3.75 g/L, based on field application in sugarcane cultivation.

2.3 Microbial Consortium

The soil was enriched with the highest concentration of the ametryn (125.00 µg/ml) was taken and added into 50.00 g of soil and 100 ml of mineral medium (Bushnell Hass), contained in 250 ml Erlenmeyer flasks. The flasks were placed for incubation at 30°C and constant shaking at 180 rpm (Shaker SOLAB) for 5 days.

After this period, the plating was done up to the order of 10^{-6}, resulting in $1.2x10^{7}$ CFU/g of soil. Saline solution 0.85% sodium chloride, NaCl, was used for sample dilution. The plating was performed in glass Petri dishes, in mineral medium (Bushnell Hass) solidified with agar. Plates were incubated at 30°C for 48 hours and colony growth was observed.

After growth, the morphologically different colonies were chosen and inoculated into 50 ml of liquid mineral medium (Bushnell Hass) under constant stirring at 150 rpm on a shaker table at 30°C.

2.4 Experimental Design

For the colorimetric assay Hach type tubes (10 mL) containing 1.00 g of sandy soil, BH medium were used. The assay was performed according to Bidoia et al. (2010). The table 2 shows the constituents of each test sample.

Table 2. Samples used in the colorimetry test in sandy soil, with addition of ametryn, surfactant Tween 80 and microbial consortium

Samples	DCPIP	BH broth	Microbial consortium	Ametryn 3.75 g/L	Tween 80
1	250.0 µL	7.75 mL	-	-	-
2	250.0 µL	7.50 mL	-	2.30 µL	v
3	250.0 µL	7.50 mL	250.0 µL	2.30 µL	-
4	250.0 µL	7.50 mL	250.0 µL	2.30 µL	125.0 µL
5	250.0 µL	7.50 mL	-	-	250.0 µL
6	250.0 µL	7.50 mL	250.0 µL	-	-
7	250.0 µL	7.75 mL	-	2.30 µL	-

The shaker tubes were shaken for Phoenix tubes and then packaged in an oven with stirring at 35°C and 0.069 g. Prior to readings, the tubes were withdrawn and allowed to stand for 30 minutes. After this period the absorbance was measured in Hach Odissey DR-2500 spectrophotometer at 600 nm.

The readings were performed at 12 hours intervals until the sample changed from blue to colorless. Measurements were taken until the DCPIP was completely reduced.

2.5 Respirometry Test

The respirometry experiment was according to the technical standard ABNT (1999), with Bartha and Pramer respirators evaluating the generation of CO_2 (mg) as a result of the time (Table 3).

Table 3. Samples used in the respirometry experiment in sandy soil with addition of ametryn, surfactant Tween 80 and microbial consortium

Samples	Constituintes
1	Control soil
2	Soil + ametryn + Tween 80
3	Soil + ametryn + microbial consortium
4	Soil + ametryn + microbial consortium + Tween 80
5	Soil + Tween 80
6	Soil + microbial consortium
7	Soil + ametryn

Quantifications of CO_2 generation were carried out by means of the titration method described by Régo et al. (2014).

2.6 Analises Estatísticas

Statistical analyzes of the results were performed using Kruskal-Wallis non-parametric test, considering a significant difference $p \leq 0.05$, in order to evaluate the different types of treatments, since the data are not distributed normal. The analyzes were performed in Origin 9.0 software.

3. Results and discussion

3.1 Colorimetric Assay with DCPIP

The figure 2 shows the colorimetric assay, analyzed for 96 hours, with soil contaminated by herbicide, but with addition of surfactant (biostimulation) and microbial consortium (bioaumination).

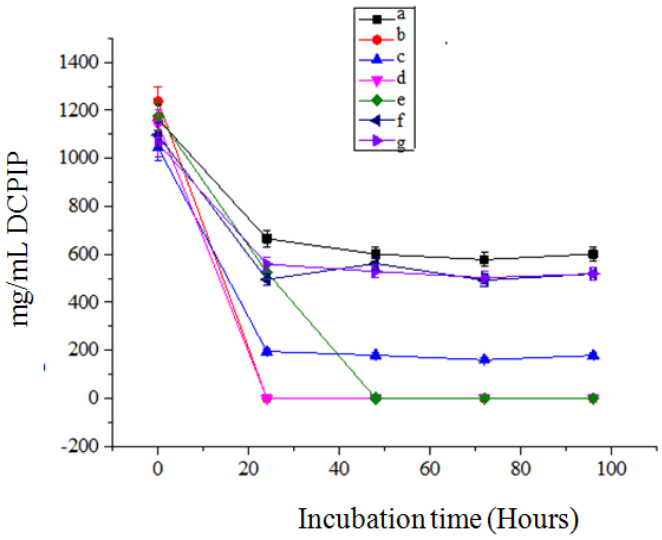

Figure 2. Colorimetric assay with soil, herbicide, microbial consortium and surfactant (600nm). (a) Control soil / b: Soil + ametryn + surfactant / c: Soil + ametryn + microbial consortium / d: Soil + ametryn + microbial consortium + surfactant / e: Soil + surfactant / f: Soil + microbial consortium / g: Soil + ametryn

It can be observed that from 0 to 24 hours there was a great fall in the concentration of oxidized DCPIP in all the samples and that from that time a stabilization occurred in the oxidation.

The control samples containing only soil was the one that presented higher concentration of oxidized DCPIP, since it had little carbon source, because the soil had sandy composition.

The samples that contained soil, ametryn, surfactant and microbial consortium and the samples that had soil and surfactant were the ones that presented lower concentration of oxidized DCPIP. This is due to the presence of the surfactant, which contains carbon source.

Surfactant is a major source of carbon available for consumption of the microbiota. In this way, it was rapidly metabolized. The herbicide is also a source of carbon to be degraded, however it is not always in the bioavailable form. Thus, when adding herbicide, with surfactant and microbial consortium, all carbon source was rapidly biodegraded (Paria, 2008; Kararundu and Karasuloglu, 2007; Barros et al., 2007).

This was observed in figure 4, in the respirometry assay of Bartha and Pramer. In this trial, the samples that accumulated more CO_2 were those that had soil, herbicide, surfactant and microbial consortium and the one that contained soil with surfactant. Thus, the respirometric assay and the colorimetric assay can be used to evaluate the degradation of ametryn in soil.

The second sample that presented lower concentration of oxidized DCPIP was that it had soil with herbicide and microbial consortium. Thus, the microbial consortium has the ability to degrade the herbicide molecule. However by adding Tween 80 surfactant to the pool the degradation becomes greater.

The surfactant control was the one that suffered the most decay over time, due to its high carbon source, which facilitated the oxidation.

Microbial consortium control and herbicide control did not present significant differences ($p = 0.091$). However, the colorimetric assay is fast and there probably was not enough time to differentiate between the samples.

There was no significant difference between the samples containing soil, herbicide and surfactant, with the samples with soil, herbicide, microbial consortium and surfactant.

The samples with surfactant presented higher decay of the DCPIP concentration, even in the presence of the herbicide. The same can be observed in the respirometric assay of Bartha and Pramer (Figure 4). From Table 4, the percentage decay rate of each sample can be observed over time.

Table 4. Percentage (%) of decay of DCPIP concentration over time

Samples/ Hours	a	b	c	d	e	f	g
24 hours	42.79	99.99	70.63	99.99	21.00	25.61	15.96
48 hours	9.66	99.98	70.06	99.99	99.97	14.86	12.10
72 hours	3.83	99.99	72.24	99.99	99.99	13.63	13.07
96 hours	3.96	99.99	70.19	99.99	99.99	6.50	13.63

a: Control soil/ b: Soil + ametryn + surfactant/ c: Soil + ametryn + microbial consortium/ d: Soil + ametryn + microbial consortium + surfactant/ e: Soil + surfactant/f: Soil + microbial consortium/ g: Soil + ametryn

The samples with soil, herbicide, microbial consortium and surfactant obtained a high percentage of decay after 42 hours. The samples with soil and surfactant already presented 99.99% reduction after 24 hours.

The control soil obtained low oxidations of the DCPIP concentration, since the soil was sandy, with low organic matter content.

Observing the samples with soil and herbicide, there were low percentages of decay. This is probably due to its molecular geometry that is difficult to degrade and persistent in the environment (Farré et al., 2002).

The figure 3 shows the results obtained with the respirometry test of Bartha and Pramer, being quantified the generation of CO2 over time of 315 days of evaluation.

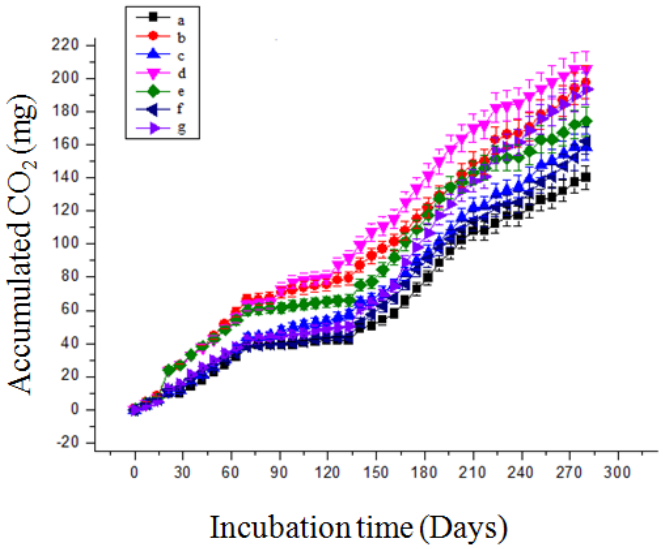

Figure 3. Accumulated CO_2 generation during 315 days at 28°C with addition of ametryn, microbial consortium and surfactant. a: Control soil/ b: Soil + ametryn + surfactant/ c: Soil + ametryn + microbial consortium/ d: Soil + ametryn + microbial consortium + surfactant/ e: Soil + surfactant/f: Soil + microbial consortium/ g: Soil + ametryn.

At the beginning of the test, the samples with soil and Tween 80, was the one that stood out the most, due to the large carbon source present in this compound. However, it is observed that at 60 days samples with ametryn and Tween 80 and sample with ametryn, Tween 80 and microbial consortium began to increase CO_2 production.

It is also observed that from 60 to 130 days of incubation, there was a stabilization period of CO_2 production in all samples. This period is a phase of adaptation of the microorganisms in the presence of ametryn, since from the 130 days the accumulation of CO_2 was resumed.

This was observed by Alexander (1999), in which the community tends to establish the balance of the population, due to the response of the microorganisms to the presence of herbicides in the environment, occurring in four phases: a) drastic reduction of bacterial and fungal populations; b) adaptation of the microbial population, especially adapted bacteria and with increasing population; c) establishing a balance; d) restoring a population balance similar to the original one.

It can be seen that the samples that generated the most CO_2 were the ones that had the addition of ametryn, microbial consortium and Tween 80. This may be due to the presence of Tween 80 surfactant, which has the ability to emulsify the substances making the molecule of ametryn bioavailable to the soil microorganism and the presence of microbial consortium, brings the microbial bioaumentation process facilitating the increase of CO_2 generation in the presence of ametryn herbicide.

The samples containing soil and Tween 80, after 280 days, accumulated less CO_2 in relation to the samples with soil and ametryn, totaling 173.947 mg, because all the carbon present in Tween 80 was consumed for the metabolic activities of the microbiota.

At 280 days, the samples with ametryn and Tween 80 were matching to that having soil and ametryn. This is due to the consumption of the carbon source of Tween 80, with the ametryn carbon source standing out, corroborating with the literature, that amethrin is a persistent compound, even in low quantity. This was observed by Farré et al. (2002), concluding that ametryn is persistent and bioaccumulates in the environment.

The samples with soil and ametryn and inoculum of microbial consortium and the samples with soil and inoculum of microbial consortium total in 158,767 mg and 162,287 mg of CO_2 accumulated, remaining below the samples that contained Tween 80. Thus, when adding the surfactant and the consortium with the application of ametryn, caused it to increase CO_2 production. Probably Tween 80 acted as a disintegrator of the ametryn molecule for soil particles, making ametryn available for biodegradation through the action of microbial consortium.

Probably, the microbial consortium adapted to the presence of ametryn in the soil, using this as a source of carbon and energy. The same was observed by Navaratna et al. (2012), because there was an increase in bacterial growth in the presence of ametryn, which may have used the herbicide as a carbon source. In this way, biodegradation is the main form of ametryn molecule breaking.

From 250 days, the curve of all the samples tends to constancy, that is, there is no more carbon source coming from the herbicide, the surfactant or the microbial consortium. The curve in the graph remains constant due to the presence of microbial biomass.

In the whole incubation period of the samples, the control soil was the one that generated the least CO_2, since this one presents sandy composition, with low amount of carbon source.

4. Conclusion

By adding the combination of surfactant and microbial consortium inoculum to contaminated soil by application of ametryn, increased oxidation of DCPIP decreased, correlating with increased degradation of the organic compound.

The addition of microbial consortium and Tween 80 surfactant favored the detoxification of soil contaminated by ametryn, being observed by the increase of oxidation of DCPIP and increase of CO_2 generation over time.

The colorimetric assay is simple to perform, bringing rapid oxidation results of organic compounds present in the soil. The Bartha and Pramer respirometry test, however, is also simple to perform, but it does provide long-term evaluation results. Thus, it is suggested to perform a colorimetric assay by DCPIP in order to understand the behavior of the metabolization of the organic compound in soil, as well as the most appropriate treatments, as a form of primary evaluation, to later perform the respirometric assay of Bartha and Pramer , obtaining complementary results of evaluation of the biodegradation of organic compounds present in the environment, benefiting processes of mitigation of contaminated soils.

References

Alexander, M. (1999). *Biodegradation and bioremediation*. San Diego: Ed. Academic.

Bidoia, E. D., Montagnolli, R. N., & Lopes, P. R. M. (2010). Microbial biodegradation potential of hydrocarbons evaluated by colorimetric technique: a case study. In: *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology***,** Editora Formatex, *2*, 1277-1288.

Cao, B.; Nagarajan, K., Loh, K. (2009). Biodegradation of aromatic compounds: current status and opportunities for biomolecular approaches. *Applied Microbiology and Biotechnology*, *85*, 207-228.

Carmo, D. A., Carmo, A. P. B., Pires, J. M. B., & Oliveira, J. L. M. (2013). Comportamento ambiental e toxicidade dos herbicidas atrazina e simazina. *Revista Ambiente & Água*, *8*, 133-143.

Díaz, E. (2004). Bacterial degradation of aromatic pollutants: A paradigm of metabolic versatility. *International Microbiology*, *7*, 173-180.

Farré, M., Fernandez, J., Paez, M., Granada, L., Barba, L., Gutierrez, H. M, Pulgarin, C., & Barceló, D. (2002). Analysis and toxicity of methomyl and ametryn after biodegradation. *Analytical and Bioanalytical Chemistry*, *37*, 704-709.

Hanson, K. G., Desai, J. D., Desai, A. J. (1993). A rapid and simples creening technique for potential crude oil degrading microorganisms. *Biotechnology Techniques*, *7*, 745-748.

Hutchins, S. R., Sewell, G., Kovacs, D., smith, G. (1991). Biodegradation of aromatic hydrocarbons by aquifer microorganisms under denitrifying conditions. *Environmental Science Techonology*, *25*, 68-76.

Jacomini, A. E., Camargo, P. B., Avelar, W. E. P., Bonato, P. S. (2009). Determination of ametryn in river water, river sediment and bivalve mussels by liquid chromatography-tandem mass spectrometry. *Journal of the Brazilian Chemical Society, 20,* 107-116.

Laabs, V., Amelung, W., Pinto, A. A., Wantzen, M., Silva, C. J., & Zech, W. (2001). Pesticides in Surface Water, Sediment and Rainfall of the Northeastern Pantanal Basin, Brazil. *Journal of Environmental Quality, 31,* 1636-1648.

Montagnolli, R. N., Lopes, P. R. M., Bidoia, E. D. (2014). Screening the toxicity and biodegradability of petroleum hydrocarbons by a rapid colorimetric method. *Archieves of Environmental Contamination and Toxicity, 67,* 1-12.

Mudhoo, A., & Garg, V. K. (2011). Sorption, Transport and Transformation of Atrazine in Soils, Minerals and Composts: A Review. *Pedosphere, 21,* 11-25.

Régo, A. P. J., Reganhan-Coneglian, C. M., Montagnolli, R. N., & Bidoia, E. D. (2014). CO_2 production of soil microbiota in the presence of ametryne and biofertilizer. *Water Air Soil and Pollution, 225,* 1-6.

Van Hamme, J. D., Odumeru, J. A., & Ward, O. P. (2000). Community dynamics of a mixed-bacterial culture growing on petroleum hydrocarbons in batch culture. *Canadian Journal of Microbiology, 46,* 441-450.

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