Anaerobic biodegradation of atrazine under different redox conditions

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Abstract—The indiscriminate use of atrazine herbicide in Brazil and worldwide has several adverse effects on human health and ecosystems, and can be found in soil, ground and surface water, in the air and also in living beings. The biodegradation of this compound can occur through different redox conditions, with the participation of aerobic and anaerobic microbial consortia, generating different degradation metabolites intermediates. However, due to its leaching potential, it is more commonly found in deep soil layers under anaerobic conditions, which highlights the importance of studies in these conditions. This research was carried out with the enrichment of denitrifying microorganisms, sulfate reducing bacteria and methanogenic arches, in anaerobic reactors under different redox conditions (denitrifying, sulfetogenic and methanogenic). Our experimental procedure consisted of two distinct tests, the first being called pure anaerobic reactors (PAR) and the second called composite anaerobic reactors (CAR). The two assays differ in the inoculum used and the carbon sources available in the reactors. We made 6 reactors for each different redox condition, 3 biotic, 2 abiotic, and 1 blank (control). We observed in this study that the removal of atrazine depends on biotic and abiotic factors, which may occur in both ways, and that physicochemical factors such as adsorption and chemical hydrolysis may have significant effects on this process. The results of the tests indicate that there was no variation in atrazine removal between different redox media, 87% (± 7%) for the denitrifying condition, 88% (± 7%) for the sulfetogenic and 92% (± 7%) for methanogenesis, in biotic reactors with atrazine and soil organic content as the only carbon sources for bacteria in the reactors. However, the variation in the results found in RCA supplemented with complementary sources of acetate carbon for denitrifying reactors, lactate for sulfetogens and acetate and formate for methanogens, and reactors without this supplementation (RPA), indicates that high atrazine removal (100%) within 70 days of analysis for supplemented reactors should be done in shorter time periods.

Keywords—Herbicides, Microbiology, Agriculture, Bioremediation.

I. INTRODUCTION

The use of pesticides is justified in agricultural improvement, being atrazine a herbicide widely used in several countries around the world. In the last 50 years of use of this pesticide, it has stood out for its effective results in controlling weeds in the crop. (CAMPANARI, 2017). However, pesticides can have harmful characteristics to living beings and the environment, such as atrazine, a herbicide widely used in Brazil since mid-1958, which has toxicity and other adverse effects on human health and ecosystems (MA et al. al., 2017; VAIL et al., 2014).

According to the National Bulletin of Commercialization of Pesticides made by Ibama in 2016, from 2014 to 2016 the consumption of atrazine in Brazil doubled, from 13,911.37 tons to 28,615.70 tons of active ingredient. With this large amount of application in crops, atrazine has already been detected in degraded areas corresponding to places of intense agricultural activity, in the central and southern regions of the state of Goiás, Mato Grosso and the Paraíba do Sul River (RJ) near areas of sugarcane crop (DELLAMATRICE; MONTEIRO, 2014). In 2017, atrazine occupied 6th place in the ranking of best selling active ingredients in Brazil, with 24730.90 tons of active ingredient.

In the Bacia do Paraná 3, the presence of atrazine is a matter of social concern and the impacts of this herbicide on human health through the ingestion of contaminated waters was discussed in the Plan of the Paraná Basin 3 made in 2014, when 250 deaths Infants associated with
fetal malformation were raised in the region. Although there is no evidence of a direct link between death and contamination of local water with atrazine, the presence of this compound in accessible kinds of water should be treated carefully.

The presence of this product in groundwater and other water bodies is justified by its application in high concentrations (above those indicated for each type of cultivation), the intense rainfall regime, the types of soils, topography, slope of the land, and its organic properties. Thus, information on the degradation of atrazine herbicide is important to deal with the problem involving this compound (DELLAMATRICE; MONTEIRO, 2014).

Microbial degradation of atrazine is well known in aerobic conditions whose degradation occurs upon the presence of molecular oxygen as an electron receptor using atrazine as an electron donor in sequential biochemical reaction processes (KABRA et al., 2014; KOLEKAR; PHUGARE; JADHAV, 2014). However, depending on the environmental characteristics of the region where this pollutant is present, this compound can be leached into deeper soil and groundwater areas, where the potential for oxireduction is characteristic of anaerobic conditions (TOMASSONI et al., 2014).

The few studies reporting anaerobic degradation of atrazine (CECILIA; MAGGI 2016; DOUGLASS; RADOSEVIC; TUOVINEN 2014; TUOVINEN et al. 2015;) highlight the difficulty of anaerobic microorganisms in degrading this compound, thus remaining a gap in the knowledge needed to the advance in the treatment of anaerobic environments impacted with this herbicide.

Therefore, due to the risks of environmental impacts and potential health hazards, measures aimed at the elimination, mineralization or degradation of atrazine in byproducts with negligible impacts are of utmost importance in environments contaminated with this compound (TOMASSONI et al., 2014). Given this, considering the use of atrazine in agriculture and its potential to contaminate deep soils and groundwater, our objective was to evaluate atrazine biodegradation under different redox conditions (Denitrification, Sulfetogenesis and Methanogenesis).

II. MATERIALS AND METHODS

2.1 Description of the study area

The study area corresponds to the Bacia Hidrográfica do Paraná (BP3), and we chose that due to the intense use of pesticides in the region, especially atrazine due to the extension of corn and soybean planting in the region, which presents a high rate of agricultural occupation from 80% to 90% (PBHP3,2014).

BP3 is located in the western Paraná mesoregion, between latitudes 24° 01 'S and 25° 35' S and longitudes 53° 26 'O and 54° 37' O, with an area of approximately 8000 km² encompassing 28 municipalities, delimited to the north by the Bacia do Rio Piquiri and to the south by the Bacia do Rio Iguaçu (PBHP3,2014).

BP3 has intense agricultural activity, humid subtropical climate, average annual rainfall from 1600 to 2000 mm and average annual evapotranspiration rates are between 1000 to 1200 mm, Semideciduous Seasonal Forest and geology formed by fissural volcanic basaltic rocks defined as general saw formation. Basaltic waters show a strong acid tendency (pH between 5.5 and 6.5) and total mineralization below 300 mg/L, are typically calcium and calcium magnesian, sulfated or chlorinated sodium (PBHP3,2014).

2.2 Sample Collection

We collected the samples under humid atmospheric conditions, in sunny day, with temperatures above 20ºC, in layers of 1.65; 1.75; 1.85 and 1.95m deep soil.

The soil was loamy in the layers just below 20cm, and in the upper layers it was drier in appearance with small aggregates with little cohesion.

2.3 Inoculum

We inoculated from a homogeneous mixture of soils at the different points collected. We made this mixture in a Duran® (1L) flask under nitrogen gas flow applied to the soil in the mixing process in order to maintain anaerobiosis. Then we close the bottle, pack it in foil and store it under refrigeration. All material used to prepare the inoculum was previously sterilized.

2.4 Anaerobic Reactors and Enrichment

For each of the 3 redox conditions (denitrifying, sulfetogenic and methanogenic) we made 7 reactors, 3 biotic, with atrazine and soil organic content being the only carbon sources of the reactors; 2 abiotics, with 1 M azide (NaN₃) and 1 M mercury chloride (HgCl₂) for microbial inactivation, and 1 “white” without atrazine. Figure 1 exhibit the experimental arrangement of anaerobic reactors, which were divided into Pure Anaerobic Reactors (PAR) and Composite Anaerobic Reactors (CAR).

We prepared the biotic reactors to evaluate microbial activity (atrazine growth and degradation), the abiotics to verify atrazine degradation without microbial participation, and the blank to observe if the
microorganisms involved in the atrazine degradation process are developing (or not) from another carbon sources.

We also made 3 biotic enrichment reactors under the different conditions tested, containing larger volume Atrazine (1.0 mg / L) (300mL), with their respective carbon sources, to be used as CAR inoculum.

We prepared the reactors based on the protocol of Deursen (2016), with specific macro and micro nutrient solutions, that are essential for the growth of each microbial group studied (Denitrification, Sulphetogenesis and Methanogenesis).

2.5 Reactor Monitoring

For the monitoring of denitrifying reactors, we evaluated nitrate consumption (NO₃⁻) according to the methodology of Cataldo (1975). We collected 0.2 mL of sample from each reactor and diluted in a 10 mL volumetric flask (1:50 dilution). Reading on a spectrophotometer (410 nm), the results were recorded and calculated according to the calibration curve.

Monitoring of sulphetogenic reactors consists of sulfate and sulfide analyzes. For the sulfate quantifications we followed the 4500-SO₄²⁻ E (turbidimetric) method and for the sulfide (S²⁻) determination we used the colorimetric spectrophotometer method with the hach kit applied for this type of analysis.

We Monitoring the methanogenic reactors by quantifying methane production in the last test using the Shimadzu GC-2014 Gas Chromatograph with Technical Conductivity Detector (TCD) and HP-PLOT / Q column (30 mx 0.53 mm x 40), μm film thickness).

2.6 Hazard detection by high efficiency liquid spectrometry (hplc / uv)

For the detection analysis of atrazine and for the metabolic intermediates desisopropylatrazine (DIA) and adhesetylatrazine (DEA) we used high performance liquid chromatography (HPLC / UV) according to the Thermo Ultimate 3000 HPLC chromatographic method, ACN Mobile Phase: H2O 60:40, Isocratic Elution Mode, 8 Min Run Time, Retention Time: DAY: 3.03 AED: 3.38 ATZ: 5.38, DAD detection, Wavelength: 220 nm

Atrazine detection limits were 0.05 to 1.0 mg / L and for its metabolic intermediates DIA and DEA were 0.025 to 1.0 mg / L.

III. RESULTS

3.1 Pure Anaerobic Reactors (PAR)

3.1.1 Pure denitrifying reactors

We monitored denitrifying reactor activity through nitrate reduction analysis, and are expressed in Figure 2. The presence of atrazine (ATZ) in the reactors throughout the operation is shown in Figure 3.
We can observe that over 130 days of operation, average nitrate consumption (61 ± 2%) in biotic reactors, higher than in abiotic reactors (25 ± 9%), which indicates that there was biological activity.

Nitrate consumption in abiotic reactors may occur due to physicochemical factors such as high pH ammonia stripping, ion exchange, adsorption, reverse osmosis, chlorine oxidation, electrical dialysis, and chemical precipitation (DUARTE, 2018). Further studies on atrazine degradation under abiotic conditions may provide a fuller explanation of the subject.

In the control, which differs from the other biotic reactors only in that atrazine was not added to this reactor, nitrate consumption was higher (81%), which may be an indicator that the absence of atrazine in this biological reactor was favorable to the development of denitrifying microorganisms.

We can also observe that it was not possible to detect atrazine until the thirty-fifth day, however after 71 days it was detected only in the abiotic reactors and in the control.

3.1.2 Pure sulfetogenic reactors

Through analyzes of sulfide production and sulfate consumption we evaluated the microbial activity in sulfetogenic reactors. Figures 4 and 5 show sulfide production and sulfate consumption in these reactors, while the presence of atrazine is shown in Figure 6.
The opposite was observed in the pure denitrifying reactors, higher nitrate consumption in the control reactor. This result suggests that sulphate-reducing bacteria (BRS) are less sensitive to atrazine than denitrifying bacteria.

Denitrifying and sulfetogenic bacteria may be more or less sensitive to certain substances, and there may be a dominance of one population over another, according to the environmental conditions under which they are submitted (BARBOSA, 2017).

Analyzing the atrazine detection plot during 140 days of analysis, it can be observed that herbicide detection only occurred after 72 days in the biotic reactors 1 and 3 (0.103 and 0.123 mg / L, respectively). However, after this period, we detected a subtle increase in atrazine in all biotic reactors, whereas in the control nothing was observed, as expected, as atrazine was not added in this reactor. In abiotic reactors we did not detect atrazine.

3.1.3 Pure methanogenic reactors

Methane production in the pure methanogenic reactors is shown in Figure 7, where we noticed an increase in biotic reactors only. The average production of these reactors was 1 ± 0.16 µmol at the end of the assay, which indicates the methanogenic activity. Figure 8 shows the detection of atrazine in these reactors.

![Fig. 7: Methane production in pure methanogenic reactors](image)

![Fig. 8: Atrazine detection in pure methanogenic reactors](image)

We observed a rapid decay in atrazine concentration under all conditions at the beginning of the assay except for control. As observed in the previous conditions (denitrifying and sulfetogenic), in methanogens we also verified the detection of atrazine after 71 days of operation, and its dynamics in the graph suggests that, once detected, atrazine suffered few variations in its concentration and may be related simultaneous processes of degradation, adsorption and desorption (MAURO; CAMPOS; LANGENBACH, 2007; YUE et al., 2016).

3.1.4 General discussion about pure anaerobic reactors (PAR)

Regarding the initial concentration of atrazine (1 mg / L) added to the PAR, we observed high atrazine removal in the biotic reactors, and total removal in the abiotic reactors. However, in all PAR, we detect atrazine only after 70 days. It is likely that the atrazine applied to the reactors was initially adsorbed to the soil, underwent chemical hydrolysis or other chemical reactions or rapid volatilization.

Correia et al. (2007) observed that atrazine adsorption in clay soil increased as a function of contact time in no-till and native forest soils, and the authors also point out that the quality of soil organic matter can increase herbicide adsorption capacity in the ground. The same authors calculated an atrazine adsorption coefficient in the clayey soil tested, reaching a value of Kf = 11.28, a value considered high by IBAMA, which indicates a higher potential for micro-pollutant adsorption in this soil type. Other authors seeking to evaluate atrazine adsorption in clay soils rich in organic matter have found atrazine adsorption of 22.8% within 24 hours (YUE et al., 2016).

The fact that we have detected atrazine after a long period in the anaerobic reactors tested may signal the permanence of this compound in the soil, taking longer to be charged to the soil solution, where it would in fact be susceptible to biodegradation processes. In this sense, due to soil characteristics, temperature, pH, agitation, and physicochemical properties of atrazine we can believe that the mobility of atrazine in the reactors (adsorption and desorption) influenced the presence of atrazine in the liquid fraction collected for the soil analyzes.

Another important fact that we highlight is about the production of biosurfactants, which are compounds produced by microorganisms extracellularly or as part of the cell membrane. These by-products are capable of improving the solubility and biodegradation of contaminants, such as atrazine (COLLA, 2015).
The fact that atrazine detection was most noticeable in biotic reactors may be related to biosurfactant production. Microorganisms after a long time exposure may have developed the ability to produce these substances to bring atrazine into the soil solution, where the organic micro-pollutant would in fact be susceptible to biodegradation processes.

This reasoning is consistent and has been studied by other authors (ABBASI, 2018; JADEJA; MOHARIR; KAPLEY, 2018; MAURO; CAMPOS; LANGENBACH, 2007). After atrazine has been desorbed from the soil by the action of biosurfactants, it is unlikely to be adsorbed again, a phenomenon not observed in abiotic reactors, where atrazine appears to undergo adsorption and desorption cycles, which strengthens the theory of biosurfactant production in the soil in biological reactors.

Although atrazine has low vapor pressure, which gives it a low volatile potential, this potential is not exclusively evaluated by the intrinsic properties of the compound, as it also depends on the environmental conditions to which the compound is inserted, such as temperature and pressure. In this sense atrazine may undergo volatilization. (LIN, 2017). The atmosphere of the nitrogen gas pressurized reactors subjected to agitation and higher temperatures may have promoted diffusion and liquid-gas mixing phenomena, and considering that only the liquid fraction of the reactors was analyzed, the gas fraction may also contain atrazine.

Under abiotic conditions atrazine may also undergo chemical hydrolysis, which occurs by dechlorination of the molecule and substitution by a hydroxyl, which may give rise to deisopropylatrazine (DIA). Chemical hydrolysis is accelerated at low pH values, by the presence of humic substances, and temperatures above 30ºC (OLIVEIRA, 2015). We conducted the reactors at 30ºC, and the only metabolic intermediate found in the PAR was deisopropylatrazine (DIA). These results may indicate the occurrence of chemical hydrolysis in these reactors, which would justify the formation of this metabolic intermediate in abiotic reactors.

The fact that we detected DIA in abiotic reactors was not expected since microbial activity in these reactors was nonexistent. However, it is known that physicochemical phenomena such as chemical hydrolysis can occur, promoting the degradation of the compound (GOMES; SANTOS; SILVA, 2004). In addition, atrazine half-life is variable and depends on factors such as agitation, temperature, pH, soil organic matter content and quality, soil type, and even more specific characteristics such as the type of organic colloids and inorganic substances present in the soil.

This set of factors may cause changes in the atrazine degradation process, which opens a gap for further studies to analyze the behavior of atrazine in different soil types and different abiotic conditions.

Thus, it was not possible to observe atrazine biodegradation in the PAR. It is possible that atrazine was adsorbed by the soil, since the soil used as inoculum is clayey and has a high cation exchange potential, which are typical characteristics of the red latosols and nitosols found in the sample collection region. However, the formation of DIA suggests the occurrence of atrazine degradation processes, since this metabolic intermediate stands out among the most well-known organochlorine by-products of atrazine, together with desethylatrazine (DEA) (COELHO; BARNARDO, 2017).

Another important factor to be emphasized is that the time for atrazine degradation under the conditions studied was short, thus requiring more time to assess the occurrence of degradation. The results observed in biotic reactors indicate that in conditions without supplementation with other carbon sources and enriched inoculum, more time is required before atrazine biodegradation can occur.

### 3.2 Composite Anaerobic Reactors (CAR)

#### 3.2.1 Composite denitrifying reactors

The results we found in denitrifying compound reactors are shown in figures 9 and 10.

![Fig. 9: Nitrate consumption in denitrifying composite reactors](image-url)
We observed that after three days of operation there was an average increase of 12 ± 1% (161 mg / L) in nitrate present in all denitrifying composite reactors (Figure 9). This increase occurred due to the inoculum source used, which was from the compound reactor (300 mL) operated for 45 days, which still had 48% (620 mg / L) of the total nitrate added in the reactors.

Considering this increase in nitrate, we detected an average nitrate consumption of 68 ± 2% (923.3 mg / L) for biotic reactors, 24 ± 6% (328 mg / L) for abiotics and 80% (1088 mg / L) for control after 68 days of operation. This discrepancy in the results of different operating conditions indicates the occurrence of biological activity in biotic reactors to the detriment of abiotics.

The control reactor showed higher nitrate removal than biotic reactors (22% more), which may indicate that the microbial community developed better in the absence of atrazine, as it is known that this herbicide can be used by microorganisms after a exposure time, thus requiring microbial adaptation to the compound (SCHLEDER, 2016).

Regarding the detection of atrazine, we observed 100% removal of atrazine in both biotic and abiotic reactors. After 71 days of analysis atrazine was no longer detected, suggesting that under supplemented conditions the biological degradation of atrazine is faster.

3.2.2 Composite sulfetogenic reactors

The accompaniment of sulfetogenic composite reactors is expressed through figures 11, 12 and 13.

We observed an average sulfide production of 0.05 ± 0.01 mg / L in the biotic reactors whereas in the abiotic sulfide production was not detected, being found 0.01 ± 0.00 mg / L of sulfide after 3 days, which remained until the end of the rehearsal. The value found comes from the sulfide residual of the inoculum used. The control reactor produced 0.03 mg / L sulfide, lower than the biotic reactors.
Regarding the average sulfate consumption, we noticed in the biotic reactors higher values, $72 \pm 2\% \text{ (} 1580 \pm 40 \text{ mg / L) compared to abiotic reactors, } 10 \pm 3\% \text{ (} 268 \pm 54 \text{ mg / L) and the control (62 \%) over the 70 days of operation.}$

The results we found, both for sulfide production and sulfate consumption, show microbial activity in biotic reactors. This phenomenon was favored by the previous enrichment and adaptation of the bacteria in the reactors (300 mL) with 1 mg / L atrazine. As expected, the control showed lower values compared to atrazine biotic reactors. Probably because sulfate-reducing bacteria need a longer time to adapt to a new condition, where there is no longer one of the resources they were adapted to in their growth (DURRUTY; GONZALEZ, 2015).

After 70 days of analysis we detected 100% removal of atrazine in all sulfetogenic composite reactors except in the control that at no time did we detect atrazine. We also observed that after 37 days there was an average removal of $89 \pm 7\%$ atrazine in the biotic reactors to $53 \pm 3\%$ atrazine removal in the abiotics. We also highlight that the removal of atrazine in the biotic reactors after 3 days went from $59 \pm 6\%$ to $53 \pm 3\%$ in the abiotic reactors in the same period, which means that from the third day to the thirty-seventh day, there was no atrazine removal in the same period in the abiotic reactors.

As the sulphate-reducing bacteria (BRS) were adapted to the larger (300 mL) biotic reactors with atrazine and sodium lactate as a supplementary carbon source, when inoculated in reactors under the same conditions, varying only by the volume of the reactors (50 mL) and the absence of soil, it was expected that the BRS would have a good growth, as it was verified by the monitoring of these reactors. When inoculated in an environment without atrazine (control reactor), sulfide production and sulfate consumption values were lower than the biotic reactors, which was also expected, since there was a lack of resources that the bacteria were already adapted to metabolize.

The atrazine removal process in these reactors shows that the microbial activity of the biotic reactors was more significant in a shorter time (37 days) than the nonbiological processes that may have occurred in the abiotic reactors.

### 3.2.3 Composite methanogenic reactors

The results of studies on methanogenic composite reactors are shown in Figures 14 and 15. In which we noticed a total average production of $3.9 \pm 0.2 \mu \text{mol of methane only in biotic reactors (Fig. 14).}$ This shows more intense microbial activity than in pure methanogenic reactors.

![Fig.14: Methane production in methanogenic composite reactors](image1)

![Fig.15: Atrazine detection in methanogenic compound reactors](image2)

We observed rapid removal of atrazine after three days of operation (100%). This result suggests that, for methanogenic condition, it is necessary to monitor the removal of this compound at shorter time intervals.

#### 3.2.4 General discussion about composite anaerobic reactors (CAR)

In all composite reactors we noticed rapid removal of atrazine. These results reveal that studies involving the degradation of this compound under conditions with enriched bacteria, adapted and inoculated in reactors supplemented with other carbon sources should be studied in a shorter time interval than the study of reactors without previous enrichment and supplementation.

In addition, the results found in abiotic reactors show the need for more specific studies on the physicochemical processes that can occur within these reactors. Another important fact to note is that there was no detection of atrazine metabolic intermediates in any of the compound
reactors studied, which may be another indicator that the degradation processes were so fast that no detection of metabolic intermediates was possible.

IV. CONCLUSION

With this research we show that atrazine is a compound that can undergo different types of degradation processes, and that these processes depend on specific environmental conditions, such as microbial action and physicochemical conditions of the environment, as well as intrinsic characteristics of this herbicide.

We also showed that for pure anaerobic reactors (PAR) the removal of atrazine was slower, which may be related to adsorption processes and slow biological activity, since there was no previous enrichment of the microbial populations present in these reactors. For the composite anaerobic reactors (CAR), it was observed that the atrazine removal process was faster, which demands shorter time interval for the monitoring of the degradation of the compound in this operational condition.

For both PAR and CAR there was no variation considered relevant in the removal of atrazine, which puts the different oxidation media studied in a very close range.

From the results obtained in this study, it is suggested further research in abiotic reactors, including analyzes of the sedimentary fraction, as well as the PAR gas fraction as well as the CAR, in order to detect atrazine in these matrices, since atrazine it is mobile and can be found in the liquid fraction as well as in the solid and / or gas fraction.

Our results may contribute to a more complete analysis of the behavior of this compound in the environment, as well as the main variables that imply its displacement and degradation. Thus, our work sheds light on the breadth of research that can be developed in this area, and we stress the importance of this information for decision-making by public agencies and for bioremediation projects for atrazine-contaminated areas.

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