Modelling aerobic biodegradation of atrazine and 2,4-dichlorophenoxy acetic acid by mixed-cultures

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Abstract

The aim of this work was to study and to model the biodegradation of atrazine and 2,4-dichlorophenoxy acetic acid by aerobic mixed cultures. Slow removal rates were observed when biodegrading atrazine, in spite of the initial concentrations. However, high removal rates were obtained when biodegrading 2,4-D, removing up to 100 mg/L in about 2 months. Regarding the 2,4-D it must be highlighted that a lag phase appears, being its length proportional to the initial 2,4-D concentration. The biodegradation trends were fitted to a Monod based model and the value of the main parameters determined. In the case of atrazine they were $\mu_{\text{max}}$: 0.011 1/d and $Y$: 0.53 g/g and in the case of 2,4-D $\mu_{\text{max}}$: 0.071 1/d and $Y$: 0.44 g/g, indicating the higher persistence of atrazine. Once finished the experiments the microbial population was characterized being the major genus *Pseudomonas* when treating atrazine and *Rhodococcus* when treating 2,4-D.

Keywords: Biodegradation; atrazine; 2,4-dichlorophenoxy acetic acid; mixed-culture; kinetics.
1. Introduction

Pesticides are a group of organic and inorganic compounds widely used to control pests or kill weeds in order to enhance crops’ productivity (Biziuk et al., 2013). During the last years, two of the most used herbicides in agriculture have been 2,4 – dichlorophenoxy acetic acid (2,4-D) and atrazine. 2,4-D is a polar herbicide, whereas the atrazine is non-polar; however, both herbicides have similar uses to control broadleaf and grassy weeds in crops (Debasmita & Rajasimman, 2013; Ralebitso et al., 2002).

Pesticides can reach soil, water or air in their primary form or as sub-products. These chemicals are the most common pollutants in surface and groundwater all around the world (Celis et al., 2008; Macur et al., 2007; Mitchell et al., 2005). Once the pesticide is in the environment, it can suffer various transformations, generating some problems in living organisms, including humans. Contact with pesticides can take place in different ways: inhalation, ingestion or dermal contact (Geed et al., 2017). The contact increases the possibility of suffering some diseases or medical conditions such as cancer, genetic mutation or fertility problems (Nawab et al., 2003). For these reasons, it is important to remove these chemicals from natural environments.

Traditionally, physical and chemical treatments have been used to remove these pollutants from water and soil (Chair et al., 2017a; Chair et al., 2017b). These treatments are effective, but they are expensive and sometimes difficult to execute, especially in extensive agricultural areas. Because of this, new techniques based on the ability of microorganisms to remove pollutants from contaminated sites have been developed (Ellegaard-Jensen et al., 2017; Sam et al., 2017). This process is known as bioremediation (Fernández et al., 2008). This alternative treatment can be effective,
minimally hazardous, economical, versatile and environmentally-friendly (Cao et al., 2015; Castillo Díaz et al., 2016; Ishag et al., 2017). The bioremediation can be implemented by using different process configurations. One of the most common is the batch reactor. Batch reactors are used when the toxicity of the influent is high, so it can be considered as a good option for the removal of pesticides (Celis et al., 2008). In addition, batch reactors can be used to determine the kinetics of the bioprocess, which is very important for the design and scale-up of the process (de los Ángeles Fernandez et al., 2016; Imamoglu & Sukan, 2013).

The pesticide biodegradation can be carried out under aerobic or anaerobic conditions. Some authors have studied the biodegradability of herbicides in both conditions, evidencing more effective treatment when operating under aerobic conditions (Elefsiniotis & Wareham, 2013). Another important aspect is the inoculum source: the inoculum used to carry out the experiments in biological reactors could be from pure or from mixed cultures (Fernández-Morales et al., 2010). The best performance is always reached when working with pure cultures. However, to reduce the operational cost of the process, sometimes mixed cultures are used (Lobato et al., 2012).

In this context, the aim of this study was to evaluate the biodegradability, by mixed cultures under aerobic conditions, of two herbicides, 2,4-D and atrazine. To do that, activated sludge from a conventional wastewater treatment plant (WWTP) was used as seed. Another objective was to determine the value of the main parameters of the process in order to accurately describe the biodegradation process.
2. Materials and Methods

2.1 Chemicals and inoculum source

High purity 2,4-D (purity, 98%) and atrazine (purity, 99.1%) pesticides were supplied by Alfa Aesar and Sigma-Aldrich respectively.

In order to ensure the availability of trace minerals during the biodegradation stage, a nutrient solution was added at the beginning of the biodegradation experiments. The composition of the nutrient solution was: (NH$_4$)$_2$SO$_4$ 741.5 mg/L, KH$_2$PO$_4$ 445.7 mg/L, NaHCO$_3$ 1152 mg/L, MgSO$_4$·7H$_2$O 502.9 mg/L, CaCl$_2$ 300.4 mg/L and (NH$_4$)$_2$Fe(SO$_4$)$_2$ 31.3 mg/L. All the chemicals were supplied by Panreac. All solutions were prepared with pure Mili-Q water.

The source of the inoculum used in the biodegradability experiments was taken from the conventional sewage WWTP of Ciudad Real (Ciudad Real, Spain). More information about this facility can be found elsewhere (Rodríguez Mayor et al., 2004).

2.2. Experimental set-up

The biodegradation experiments performed in this work were carried out at a pH 7.0±0.2 in discontinuous completely mixed batch reactors. All the biodegradation tests were carried out under aerobic conditions. The batch reactors were open to the atmosphere in order to allow oxygen transfer from the atmosphere to the liquid bulk. The liquid bulk was homogenised by means of a magnetic stirrer rotating at 150 rpm, and during the biodegradation experiments the temperature was controlled at 20ºC by means of a laboratory incubator. Each batch reactor had a total volume of 500 ml, although the working liquid volume was 400 ml. The tests were carried out in the dark in order to prevent any photocatalytic degradation and the growth of algae.
2.3. Operational procedure

The operational procedure was the same in all the experiments. Different concentrations of 2,4-D and atrazine were added to the batch reactors. Besides this, a volume of nutrient solution and a biomass inoculum were added. Samples were taken periodically in order to study the evolution of the pesticides concentration and the biomass growth.

The range of pesticide concentrations studied in this work was from 100 to 10 mg/L in the case of the 2,4-D pesticide and from 40 to 10 mg/l in the case of the atrazine. These ranges were selected according to the literature (Celis et al., 2008; Debasmita & Rajasimman, 2013; Sanchis et al., 2014; Swissa et al., 2014).

Due to the low solubility of atrazine in water, the addition of surfactant was required. In this work, the selected surfactant for the atrazine solubilisation was sodium dodecyl sulphate (SSD). The weight ratio surfactant/atriazine used was 1 in order to ensure the complete dissolution of the atrazine (Sanchez-Camazano et al., 2000).

2.4. Sampling and analytical techniques

The average length of the experiments was about three months for atrazine and two months for 2,4-D. During the experiments, the content of each reactor was analysed about once per week. The parameters analysed included pesticide and biomass concentrations. Both parameters were detected by molecular adsorption spectroscopy.

The evolution of the biomass was analysed by direct optical density (OD) measurements at 600 nm in a Pharma Spec 1700 Shimazu® spectrophotometer. Absorbance offers an accurate indirect measurement of microorganisms’ concentration (Moser et al., 1996; Péquignot et al., 1998). To correlate the absorbance at 600 nm with the real biomass concentration, measured as Volatile Suspended Solids (VSS), an internal calibration was carried out. From the fitting of both pairs of data an accurate
linear trend was observed. The correlation curve relating the OD$_{600}$ to the VSS (g/L) was the following:

$$VSS \ (g/L) = 0.458 \ OD + 0.0028 \quad (r^2 = 0.9889)$$

Before pesticides determination, it was necessary to remove the biomass. To do that, samples were centrifuged by using a Mixtael® centrifuge, rotating at 3700 rpm for 10 minutes. After centrifugation, the supernatant was filtered through a glass fibre filter, pore size 45 µm, to ensure the biomass removal. Once completely removed from the biomass, the pesticides were analysed in a Pharma Spec 1700 Shimazu® spectrophotometer. The spectrophotometric technique has been widely used for the atrazine and 2,4-D determination yielding accurate results. The detection wavelengths were 230 nm for 2,4-D and 229 nm for atrazine (Debasmita & Rajasimman, 2013; Kundu et al., 2005).

The genus and strains identification was performed using MALDI-TOFF Axima Assurance by Shimadzu. The matrix solutions were prepared by saturation of α-cyano-4-hydroxycinnamic acid in a 1:48:2 acetonitrile: water: trifluoroacetic acid matrix solution. Then, the microorganisms were sterilized with ethanol at 75%, the solution centrifuged at 1000 rpm for ten minutes and the supernatant removed. Finally, the microorganisms were then extracted from the precipitate, using 20 µL of acetonitrile/formic acid/water (50:35:15) according to the procedure described in the literature (Mateo et al., 2016).

2.5. Biodegradation model

A mathematical model based on the Monod equation was developed and used for the study of the biodegradation kinetics. Previous researches have shown that pesticides
biodegradation can be described by Monod rather than first order kinetics (Cheyns et al., 2010). Because of that, a model based on the following equations was proposed:

\[
\frac{dx}{dt} = \mu_{\text{max}} \cdot \frac{S}{K_s + S} \cdot X - K_d \cdot X \quad (1)
\]

\[
\frac{dS}{dt} = -\frac{1}{Y} \cdot \mu_{\text{max}} \cdot \frac{S}{K_s + S} \cdot X \quad (1)
\]

where \( Y \) is the yield coefficient (g/g), \( k_d \) is the decay coefficient (1/d), \( X \) is the concentration of microorganisms (g/L), \( \mu_{\text{max}} \) is the maximum growth rate from pesticide biodegradation (mg/d·gVSS), \( S \) is the pesticide concentration (mg/L) and \( k_s \) is the half-saturation coefficient (mg/L).

In order to fit the equations to the data set, Equations 1 and 2 were solved simultaneously, using the Simplex algorithm and the protocol defined in the literature (Fernández et al., 2008; Fernández et al., 2011). As the objective was to achieve the minimum value of the objective function, this function is as follows:

\[
\chi = \sum_{i=1}^{n} \left( (X_i - X_{\text{meas},i})^2 + (S_i - S_{\text{meas},i})^2 \right) \quad (3)
\]

where \( n \) is the number of data points, \( X_i \) and \( S_i \) are the predicted values of the variable at the \( i^{\text{th}} \) measurement, and \( X_{\text{meas},i} \) and \( S_{\text{meas},i} \) are the actual values at the \( i^{\text{th}} \) measurement.
3. Results and discussion

3.1. Pesticide biodegradation

3.1.1. Atrazine

Aerobic mixed culture atrazine biodegradation tests were performed in batch reactors operating for about 12 weeks. As can be seen in Figure 1, atrazine concentration slowly decreased during the study.

![FIGURE 1 NEAR HERE]

The atrazine biodegradation rate was very similar, in spite of the different initial concentrations, in all the cases. This behaviour indicates that the atrazine was slowly biodegradable by the mixed microbial culture used in this work. This behaviour also indicates that, at the concentrations studied in this work, the atrazine does not present toxic effects over the mixed microbial culture used as inoculum. This result is in accordance with toxicity results presented in the literature (Chen et al., 2015).

The slight biodegradability of the atrazine could be explained because it is a very stable molecule, presenting a relative resistance to the microbial attack. The stability of this pesticide could be related to the existence of a heterocycle in its structure. Heterocycle compounds are cycled structures, in which at least one of the ring members is not a carbon atom; this atom is known as heteroatom. These kinds of heterocycles are stable and non-polar (Fischer et al., 2016). In the case of the atrazine, the heteroatom is nitrogen. Due to its chemical structure, the persistence of atrazine is very high, presenting therefore a very low biodegradation rate. This could be explained by the lack of specific enzymes to carry out the biological degradation (Chanika et al., 2011).
Regarding the mixed microbial culture, scarce biomass growth was observed. This could be explained because of the combination of the low biodegradation rate and the influence of the decay rate over the net growth rate of the microorganisms. In order to ratify the biological removal process, trace mineral removal was determined, being the total phosphorous and nitrogen removal about 4 and 10 mg/L respectively. The phosphorous to carbon and ammonium to carbon ratios obtained ratified the biological aerobic degradation.

Once the tests were finished, the mixed microbial culture was characterised by using the MALDI-TOFF technique, identifying *Pseudomonas aeruginosa* as one of the main groups. In the literature, several publications have shown that the *Pseudomonas* genus is able to degrade the chlorinated pesticides (Nawab et al., 2003).

In the literature, mixed culture atrazine biodegradation studies have been reported. On one hand, Sanchis et al. (2014) obtained low percentages of atrazine removal using an inoculum from a conventional activated sludge WWTP. The reactor operated at 30 °C under aerobic conditions. The initial pesticide concentration was 30 mg/l, and after 35 days this concentration started to decrease. The maximum removal percentage was 20% after 180 days (Sanchis et al., 2014). On the other hand, Debasmita & Rajasimman (2013) used a specific inoculum from a pharmaceutical wastewater sludge for the treatment of the atrazine at 29.3°C. In these tests, the effluent atrazine concentrations ranged from 2 mg/l to 14 mg/l, presenting high removal percentages (Debasmita & Rajasimman, 2013). These results indicates that the pharmaceutical sludge contains specific microorganisms able to degrade the atrazine.
Regarding the pure cultures, Swissa et al. (2014) achieved high removal percentages, 94.4% and 100% respectively, by using a monoculture of *Raoultella planticola*, isolated from a herbicides wastewater treatment facility.

### 3.1.2 2,4-D removal

Figure 2 illustrates the obtained results in the batch experiments where the biodegradation of 2,4-D was studied.

![FIGURE 2 NEAR HERE]

The curves represent the pesticide evolution along the experiment. The evolution is due to the biological consumption of 2,4-D as a carbon source. The results revealed a lag phase in all cases. Once the lag phase was over, the mixed culture was able to completely degrade the pesticide 2,4-D, indicating that it is an easily biodegradable pesticide. As can be seen in Figure 2, the length of the lag phase increases as 2,4-D concentration increases. The relation between the lag phase and initial concentration is presented in Figure 3, where a linear relationship between both parameters is shown.

From the results presented in Fig 3, it can be concluded that every 2.5 mg/L of pesticide prolonged the lag phase by about 1 day. In Fig 3, the grey area represents 95% confidence intervals.

![FIGURE 3 NEAR HERE]

From the characterization of the microbial culture the following microbial groups were identified: *Rhodococcus ruber* and *Ochrobactrum anthropi*. In the literature, *Rhodococcus ruber* has been identified as a herbicide-degrading strain (Bell et al., 1998; Cycoon et al., 2017; Hongming et al., 2015). Regarding *Ochrobactrum anthropi*, in the
literature this has been described as a xenobiotic (Chudasama & Thaker, 2017) and pesticide degradation culture (Talwar et al., 2014).

In the literature, 2,4-D biodegradation studies have been reported. Celis et al. (2008) used a mixed culture inoculum taken from a WWTP. In these tests, a 100% 2,4-D degradation was achieved under aerobic conditions. The tests were performed in a batch reactor, the highest concentration removed being 500 ppm. In these tests, the lag phase took 30 days, and the length required to remove the pesticide 185 days. Sanchis et al. (2013) also worked with continuous aeration, obtaining 95% 2,4-D degradation in 135 days. The initial pesticide concentration was 50 ppm and the length of the lag phase was 21 days (Sanchis et al., 2014).

3.2. Mathematical modelling

For the design and scale-up it is necessary to accurately determine the model parameters of the biodegradation process (Imamoglu & Sukan, 2013; Makinia et al., 2006). In this work a Monod based model was fitted to the experimental results and the values of the main parameters estimated. These parameters provide interesting information about biomass growth and pesticide removal due to the organism’s intrinsic characteristics and the existing environmental conditions. Regarding the importance of the parameters, biomass yield coefficient (Y), maximum specific growth rate (μ<sub>max</sub>), biomass decay coefficient (k<sub>d</sub>) and half-saturation coefficient (K<sub>s</sub>) provide crucial information for the evaluation of biomass concentration during the treatment. All these parameters are very important to appropriate design treatment processes (Imamoglu & Sukan, 2013).

In order to determine the biodegradation parameters, the mathematical model describing the pesticide removal and the biomass growth was fitted to the experimental data set obtained in the batch experiments. An accurate estimation of the model’s parameter
requires an initial set of values. These values should guarantee the convergence of the
mathematical functions to obtain a minimal squared error. These initial values can be
taken from the literature or directly evaluated from the data sets used. In this work the
initial \( k_d \) was experimentally determined from blank tests where only biomass was
present. The value of the \( k_d \) was 0.005 l/h. In order to obtain accurate initial
approximations for \( \mu_{\text{max}} \), \( K_s \) and \( Y \), linear regression analysis of the pesticide and
biomass data series were carried out according to the literature (Robinson & Tiedje,
1983). General least squares regression, for linear or nonlinear models, is derived from
the maximum likelihood method under the assumption that the variance is constant and
the errors are normal for all observations.

Nonlinear regression analysis require accurate initial estimates of its parameters. This
can be obtained by fitting the transformed data to a linear version of the model but the
Monod model is intrinsically non-linear and cannot be transformed into a linear
equation. Anyway, provisional estimates can be obtained by replacing the infinitesimal
times (\( dt \)) with finite time increments (\( \Delta t \)). This supposition is correct when operating at
very low \( \Delta t \) values. The finite-differential equations obtained in this way were also non-
linear, but they can be linearized as follows:

\[
-\frac{\Delta t}{\Delta S} \cdot X = \frac{K_s \cdot Y}{\mu_{\text{max}}} \cdot \frac{1}{S} + \frac{Y}{\mu_{\text{max}}} \quad (4)
\]

\[
\frac{\Delta t}{\Delta X} \cdot X = \frac{K_s}{\mu_{\text{max}}} \cdot \frac{1}{S} + \frac{1}{\mu_{\text{max}}} \quad (5)
\]

These yield straight lines when \( -\Delta t \cdot X/\Delta S \) and \( \Delta t \cdot X/\Delta X \) are represented versus \( 1/S \).

The initial estimates of \( \mu_{\text{max}} \) and \( K_s \) can be obtained from the y intercepts and slope of
the transformed biomass data. The \( \mu_{\text{max}} \) value can be obtained from the y intercepts of
the fitting line corresponding to the transformed biomass data. The \( K_s \) value can be
obtained by dividing the slope by the y intercepts of the fitting line corresponding to the transformed biomass data. The Y value can be obtained by dividing the y intercepts of the fitting line for the transformed substrate data by the y intercepts of the fitting line corresponding to the transformed biomass data. The Y value can also be obtained by dividing the slope of the fitting line for the transformed substrate data by the slope of the fitting line corresponding to the transformed biomass data.

Below, the determination of the initial estimates, as well as the modelling of the atrazine and the 2,4-D removal, are presented.

3.2.1. Atrazine modelling

As previously indicated, in order to estimate initial values of $\mu_{\text{max}}$, $K_s$ and $Y$, the equations of the model proposed were linearized and fitted to the atrazine data set. An example of the atrazine linearization fitting is shown in Figure 4A.

From the linearization, the initial estimates obtained were $\mu_{\text{max}}$ 0.015 1/d, $K_s$ 7 mg/L and $Y$ 0.52 g/g. These initial estimates of the variables were used to start the fitting of the model to the experimental data obtained when degrading the atrazine. All the data series were fitted simultaneously, in order to determine universal values of the $Y$, $K_s$ and $\mu_{\text{max}}$ for the concentration interval studied. This could be done because no toxicity events were observed in any of the data series. Once the model fitting was finished, accurate predictions of the biomass and atrazine trends were obtained. Figure 4B illustrates the profile obtained when degrading an initial atrazine concentration of 37 ppm.
From the fitting, the best estimation of $\mu_{\text{max}}$, $K_s$, $K_d$ and $Y$ were those presented in Table 1. As can be seen in Table 1, the kinetic parameters presenting very low values leading to very low atrazine biodegradation rates in all the tests. The biodegradation rate was about 18 mg pesticide/(g VSS·d), despite the initial atrazine concentration.

|TABLE 1 NEAR HERE|

3.2.2. 2,4-D modelling

As in the case of the atrazine, the initial estimates of $\mu_{\text{max}}$, $K_s$ and $Y$ for the 2,4-D biodegradation were determined by means of model linearization. The results obtained in the 2,4-D linearization procedure are presented in Figure 5A.

|FIGURE 5 NEAR HERE|

From the linearization, the initial estimates obtained were $\mu_{\text{max}}$ 0.06 1/d, $K_s$ 1 mg/L and $Y$ 0.43 g/g. These initial estimations were used to fit the mathematical model to the experimental results corresponding to the 2,4-D biodegradation.

All the data series were fitted simultaneously, in order to determine universal values of the $Y$, $K_s$ and $\mu_{\text{max}}$ for the concentration interval studied. After the model fitting, accurate predictions of the biomass and 2,4-D biodegradation trends were obtained. The fitted values obtained when degrading 2,4-D are presented in Table 1.

As an example, the fitting of the data series corresponding to a 2,4-D initial concentration of 63 ppm is shown in Figure 5B.

As can be seen in Figure 5B, once the model parameters were fitted, the profile of experimental data (biomass growth and pesticide biodegradation) was accurately predicted.
Comparing the biodegradation parameters of both, atrazine and 2,4-D, it must be highlighted the very different maximum specific growth rate values obtained. This difference in the microbial growth leads to lower biodegradability rates in the case of atrazine when it is compared with the biodegradability rates of 2,4-D.
4. Conclusions

In conclusion, the atrazine is a persistent pesticide presenting very low degradation rates. Conversely, the 2,4 D presented higher degradation rates but presented a lag phase proportional to its initial concentration. Atrazine and 2,4-D biodegradation processes were fitted to Monod equations, being the main difference the $\mu_{\text{max}}$ values: 0.011 1/d for atrazine and 0.071 1/d for 2,4-D, which indicates that the 2,4-D biodegradation rate is about six-fold that obtained when biodegrading atrazine. Finally, biomass characterization analyses were carried out at the end of the experiments identifying *Pseudomonas* and *Rhodococcus* as the main genus biodegrading atrazine and 2,4-D respectively.

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