

Estimating the biodegradation of PHB/PBAT films – An experimental design approach

Miguel Fernandes^{a,b,*}, Andreia F. Salvador^{a,b}, Daniel A. Madalena^{a,b}, António A. Vicente^{a,b}

^a Centre of Biological Engineering, University of Minho, Campus de Gualtar, Braga 4710-057, Portugal

^b LABBELS – Associate Laboratory, Braga, Guimarães, Portugal

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ABSTRACT

The massive use of plastics in various applications, particularly packaging, generates enormous amounts of plastic waste that can be found almost everywhere, including in soil. This represents a serious environmental pollution issue since the biodegradability in soils can take several years, depending on the microbial composition, the physical and chemical characteristics of plastics, and other relevant environmental factors such as the soil temperature, the soil moisture holding capacity (MHC), and the carbon:nitrogen (C/N) ratio. In this work, we evaluated the importance of these soil physical-chemical parameters on the biodegradation of a plastic film composed of PHB/PBAT. A design-of-experiments methodology, namely the Central Composite Rotational Design (CCRD) was used to determine the effects of these parameters in the biodegradation of the films in soil. The carbon dioxide evolution was followed for 6 months following the guidelines of the ASTM D5988 (2018). The results showed that the most important factor was the temperature. Higher temperatures ($\geq 37^\circ\text{C}$) accelerated biodegradation while the adding of nitrogen (aqueous solution of ammonium chloride) had no impact on this process, probably because the C/N ratio pre-existing in the soil was suitable to guarantee microbial activity. Although a MHC between 80% and 100% is recommended by international standards, 60% MHC produced similar results. According to the methodology, the best combination possible includes a temperature of 37°C and a MHC ranging from 60 to 100%.

1. Introduction

Almost 50 % of plastic is used in disposable applications, mainly packaging such as food packaging, bottles, plates, cups, and clothing [1]. Concerning packaging, only a small portion of the waste generated, about 29.5 million tons (collected plastic post-consumer waste: 34.6 % recycling, 42 % energy recovery, 23.4 % landfilling), is recovered, and recycled in Europe [2]. Recycling is a very laborious process, that involves the collection from consumers and manual sorting. The plasticizers, additives, and coloring elements, that are part of plastics significantly complicate the recovery [3]. In the case of food packaging, the recycling rate is much smaller due to contamination with organic substances including oil and leftover foods [4]. Without an economic incentive, most plastics collected are sent to under-regulated countries and destined to the landfill [5]. In the last decades, soil has become one of the environments most heavily polluted due to the disposal of several types of waste, including hazardous waste, organic pollutants, and non-degradable plastics [6].

Beyond reducing the application of single-use plastics, the replacement of conventional plastics by biodegradable alternatives, that maintain the ideal characteristics for packaging, is believed to be a valuable solution to reduce plastic pollution in the oceans and soils. This way, soil pollution can be reduced with the use and development of bio-based biodegradable plastics. Biodegradable plastics have the advantage of being more susceptible to microorganisms, and can be more easily recycled (e.g., through composting), which should be their main destiny (Gómez and Michel, 2013). Nonetheless, biodegradable plastic may also offer resistance to biodegradation in natural environments, which is highly dependent on the environmental conditions. It is of utmost importance to understand the impact of several parameters in the biodegradation process in order to evaluate the impact of plastic waste disposal in soil. The effect of temperature is normally considered to be significant during biodegradation of polymers in soil, even though its impact is more significant to some polymers than others. Nishide et al. [7] found that 52°C was an optimal temperature for the degradation of poly(butylene-succinate-co-adipate) (PBSA) in soil, but the same

* Corresponding author.

E-mail address: d13825@ceb.uminho.pt (M. Fernandes).

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temperature did not affect Polycaprolactone (PCL) and Polybutylene succinate (PBS) biodegradation when compared to mesophilic temperatures (30 °C). At lower temperatures, below 10 °C, it is challenging to have significant degradation [8]. Since the mesophilic microorganisms growth range of temperature is 10 to 45 °C, values outside this range may in fact limit biodegradation due to the low microbial activity [9].

Another relevant parameter is water, which is considered a crucial factor for the development of microorganisms. Very low water content, i.e., 10 % results in no disintegration for more than two months [10]. Another important factor to be considered is the nutrient content of the soil. the most important nutrient for biodegradation is nitrogen since it is required for the growth of microorganisms, due to its expressive presence in nucleic acids and amino acids [10]. Hoshino et al. [11] showed through a study conducted in several soil sites that the degree of degradation of several polymers was more correlated with the total nitrogen content in several sampling sites than with the soil carbon content. According to the authors, this may be related to the plastic composition (carbon, hydrogen, oxygen), which lacks nitrogen.

Most biodegradation studies usually evaluate the effects of a single factor at a time by changing the value of one variable and keeping the other variables constant [9]. However, in many areas, experimental design techniques have been employed in product and process optimization aiming at minimizing costs and saving time, as well as maximizing productivity and product quality. Beyond reducing the number of trials, experimental design analyses factors simultaneously and allows the optimization of more than one response at a time [12]. Furthermore, the application of experimental design strategies (e.g., central composite rotational design - CCRD) results in the mathematical modulation of the assessed scenario so that estimations can be made. This approach can be used in research, development and production, for example to optimize culture media [13].

In this work we evaluated simultaneously the effect of several physical-chemical parameters of the soil on the biodegradation of PHB/PBAT bilayer films, by applying the central composite rotational design (CCRD). The parameters tested were the temperature, soil moisture-holding capacity (MHC), and nitrogen. The interactions between the parameters were investigated. A prediction model was developed and tested, to estimate the PHB/PBAT film biodegradability in soil.

2. Material and methods

2.1. Soil sampling

The soil was sampled from the University of Minho (Campus of Gualtar, Braga, Portugal), according to ISO 18,400 (2018) and as described in other work [14]. Sampling was performed from the surface to the plowing depth, and vegetation, roots, moss-covered litter layer, or woody plants were removed to diminish the addition of fresh organic carbon which could mask or alter the CO₂ produced from the tested material incubations.

In total 19 trials were conducted using combinations of the 3 factors tested temperature (9 - 45 °C), soil moisture-holding capacity (60 - 100 %), and the C/N ratio (nitrogen added to the soil in relation to the carbon existing in the plastic) (0 - 40), further detail can be found in Table 1 and S1 (supplementary methods). In the end, a validation trial was conducted using the best combination possible. The two samplings occurred in October 2020 and July of 2021. The sampling of the validation trial occurred in July 2022. No long periods of drought, freezing,

or flooding were registered before sampling. The soil was transported in a loosely tied black polyethylene bag as recommended by ISO 18,400 (2018) with free access to air, to avoid exposure to light that could promote the growth of algae on the surface of the soil, physical compaction, and reduce variations in the soil water content. The soil was sieved in the laboratory to 1 mm particle diameter and then stored in trays with 2 cm of height to maintain the aerobic condition and in dark at 4 ± 1 °C.

2.2. Physicochemical characterization of soil samples

The physical-chemical characteristics of the soil were determined by using the recommended guidelines in the ASTM D5988 [15] namely the ASTM D1293 [16], ASTM D425 [17], and SM 2540 G [18] for pH, moisture-holding capacity and the content of total volatile and fixed solids respectively, the carbon to nitrogen ratio was determined by elemental analysis with a LECO TruSpec CHN, and the values are presented in the Table S1.

2.3. Biodegradation of PHB/PBAT films – an experimental design approach

2.3.1. Biodegradation assays

Biodegradation experiments were performed using 38 reactors (3 L wide-mouth glass jars), inoculated with the soil (250 mg). PHB/PBAT 55/45 wt % bilayer films (11.3 × 9 cm with 35 µm of thickness) were added to the reactors (one film per reactor), or control assays were set up, in which the blend was replaced by cellulose paper, Whatman no 1 (9 × 8.75 cm with 197 µm of thickness) as recommended by the ASTM D5988 [15]. The biodegradation was evaluated by respirometric tests, following the ASTM D5988 [15] test method using the measurement of CO₂ production by titration.

2.3.2. Measurement of CO₂

The evolved CO₂ can be used to follow the aerobic biodegradation since it is an indirect measure of O₂ consumption, reflecting the biological degradation of the polymer. The Titration method to measure the CO₂ was accomplished accordingly to the ASTM D5988 [15] with the following modifications: jars were used instead of desiccators, and 0.5 M potassium hydroxide (KOH) solution (Labkem) was placed in a 150 ml beaker instead of a 100 ml beaker. An automatic titrator Titrando 888, with the tiemo™ 2.5 software, was used to perform the titration with hydrochloric acid (HCL) solution 0.25 M (Labbox AGR ISO). When replacing HCL, distilled water was added to the soil to conserve the initial moisture-holding capacity. Briefly, the evolved CO₂ was captured by the KOH solution, creating potassium bicarbonate. Then the quantity of the CO₂ produced was determined by titrating the KOH solution with HCL. The net CO₂ produced from the test material was calculated by subtracting the average amount of CO₂ produced in the soil control jars from the amount of CO₂ produced in the test material jars. The biodegradation percentages were calculated from the ratio between the net CO₂ production and the theoretical CO₂ production based on the carbon content, of the tested material.

2.3.3. PHB/PBAT films

The plastic was a bilayer film of PHB/PBAT 45/55 h wt %. The PHB was an experimental PHB grade (Biomer P309) supplied by Biomer (Krailling, Germany). The PBAT was a commercial aliphatic-aromatic copolyester-based polymer (EcoflexVR F blend C1200, a film-blowing grade). Details about the co-extrusion of the bilayer film can be found in the work of Teixeira et al., [19]. Different amounts of the blend and cellulose were added to the bioreactors to obtain an equivalent mass of organic carbon, according to Table S2.

Table 1
Variables and levels used for the CCRD.

Independent Variables	−1.68	−1	0	1	1.68
C/N ratio	0	6.1	15.0	23.9	30.0
Temperature (°C)	9.0	16.3	27.0	37.7	45.0
Soil Moisture capacity (%)	60.0	68.0	80.0	92.0	100.0

2.3.4. Design of experiments

2.3.4.1. Experiments. A design of experiments (DoE) methodology was used to identify the effects of different parameters and their interactions on the biodegradation process: soil temperature (°C), soil moisture-holding capacity (%), and the C/N ratio (nitrogen added to the soil in relation to the carbon existing in the plastic). The biodegradation was determined through the analysis of the accumulated CO₂ (using the same method described before and based on the ASTM D5988 [15]). A three-variable CCRD (2³) was used, with five repetitions of the central point (0) and six axial points. The addition of axial points (−1.68 and +1.68) enables the adjustment of the data to a second-order model and, consequently, to confirm the existence of curvature [12]. Variables and levels are demonstrated in table 1.

In total, 19 assays were performed, as indicated in Table S3. Since the assays were conducted in two parts, with two soils, 5 assays for the central point were included instead of 3 (i.e., 3 for the first soil and 2 for the second soil). The soils were collected from the same site, and presented similar properties, although slight differences may be possible, for example at the microbial community's composition level. The DoE data were analysed using Protimiza Experimental Design Software (Brazil. <https://experimental-design.protimiza.com.br/>).

A validation trial was further performed to confirm and assess the quality of the DoE. The values of the validation trial, where the most significant for each parameter. In cases where the parameters were not relevant, the central point were selected.

2.3.4.2. PHB/PBAT films biodegradation prediction. The biodegradation of PHB/PBAT films was assessed for 6 months, and a mathematical modulation of the PHB/PBAT films biodegradation for each month of the assay was made. With this approach, it is possible to correlate the physical-chemical characteristics of the tested soil with the films' biodegradation. It is important to correlate and estimate the bioplastics' biodegradability through time by correlating the different physical-chemical characteristics of the soil with the biodegradation time of the biopolymers. For this purpose, a regression model (Eq. (1)) was adjusted using the experimental setup of the previous CCRD (Table S3)

$$y = \alpha * \ln(t) + \beta \quad (1)$$

Where y corresponds to the films' biodegradability and t corresponds to time (in months). It was also possible to observe that the two coefficients of the regression model described in Eq. (1) are related to the physical-chemical characteristics of the soil. As such, a similar CCRD was used to determine the equations to calculate the coefficients α (responsible for the vertical stretch of the curve) and β (responsible for the horizontal displacement of the curve).

These equations can be used, to predict the biodegradation of the material tested in this work in similar soils, with a previous characterization of the soil (temperature and moisture holding capacity). and the sample (elemental analysis for carbon determination).

2.4. Analytical methods

To evaluate the effect of the biodegradation on film properties. in the validation assay, the PHB/PBAT films were analyzed before and after the incubations to verify the effects on the functional groups using Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) and Differential Scanning Calorimetry (DSC) to evaluate the changes in the thermal properties.

2.4.1. Attenuated total reflectance-fourier transform infrared spectroscopy (ATR-FTIR)

To assess the effects of the PHB/PBAT biodegradation on the chemical structure of the films, an ATR-FTIR analyses an ALPHA II-Bruker spectrometer (Ettlingen, Germany) was used according to the

procedure described by Silva et al., [20].

2.4.2. Differential scanning calorimetry (DSC)

A Perkin Elmer DSC 4000 Differential Scanning Calorimeter (Perkin Elmer, Waltham, MA, USA). An assay based on the work of Silva et al. [21] was used for the assessment of the films' thermal properties. However, only portions of the film (8 mg each) were used, and the protocol was set to: heating at 10 °C/min from 5 °C to 190 °C to erase the thermal history; cooled down to −25 °C at −10 °C/min; heating to 190 °C at 10 °C/min. From the final heating run the melting (T_m) and crystallization temperatures (T_c) were determined. The onset temperatures (T_m Onset) and melting enthalpies (ΔH) were calculated using Pyris software version 12.1 (Perkin Elmer, Waltham, MA, USA). The crystallinity (X_c) of the samples was determined accordingly with Beber et al., [22], by the ratio of the melting enthalpy (ΔH_0) for 100 % crystalline PHB or PBAT, which is 146 J/g [23] and 114 J/g respectively [24]. The absolute crystallinity can be calculated according to Eq. (2):

$$X_c = \frac{\Delta H}{\Delta H_0} \times 100 \quad (2)$$

2.5. Statistical analysis

All the DSC values were expressed as mean $\pm$ standard deviation (SD) for the initial and final PHB/PBAT films sheets before and after degradation. The data was assessed using one-way analysis of variance (ANOVA) followed by Tukey's test using OriginPRO 2019b statistical program and the statistical significance was accepted as $p < 0.05$ (95 % significance). The principal component analysis (PCA) conducted on the experimental trials of the CCRD was also performed using the same software.

3. Results

3.1. Design of experiments for the biodegradation assay

The physicochemical properties of the soil were within the recommended ranges by the ASTM D5988 [15], namely the pH was in the range 6 - 8 in all trials as indicated in Table S1. More importantly, the differences among the pH of the different soils were quite small, which contributed to the maximum homogeneity possible between the trials. The values of biodegradation obtained, varied between 28.65 % and 60.73 %, which corresponded to the conditions of trial 11 (lowest temperature) and 8, respectively (Table 2). The 19 trials performed for the DoE demonstrated that the increased temperature resulted in an increased biodegradation. The PCA analysis (Fig. 1) also supported this conclusion. In the trials where 37.7 °C or higher temperatures were used (trials 3, 4, 7, 8 and 12) the biodegradation reached > 51 % after 6 months, while with the lower temperatures tested the maximum biodegradation obtained was of 48. 2 % (trial 15). With the two lowest temperatures tested (9 and 16.3 °C), the biodegradation never passed the 41 % barrier (trial 5). The most significant carbon mineralization curves are represented in Figure S1.

All the parameters which were not significant were eliminated from the CCRDs equations. The results of the CCRDs demonstrated that the temperature was the only parameter tested that was significant in all the CCRDs performed each month (Table 3). The humidity was just significant in the sixth month, and in interaction with temperature in the third and fourth months. The C:N ratio was only significant in interaction with the temperature in all months, except for the first one (Table 3).

3.2. A multivariate perspective

In the PCA analysis (Fig. 1) the principal component 1 (PC1) and principal component 2 (PC2) explain 71.52 % and 11.55 % of the data variability, respectively. Consequently, it is important to mention that

Table 2
Percentage of biodegradation of the PHB/PBAT films during 6 months for each DoE trial. The validation was performed in triplicate. SD - Standard deviation.

Trials	C/N ratio	T (°C)	MHC (%)	Biodegradation (%)					
				Months					
				1	2	3	4	5	6
1	8.1 (−1)	16.3 (−1)	68.1 (−1)	5.31	14.68	18.35	27.31	33.23	38.44
2	31.9 (1)	16.3 (−1)	68.1 (−1)	1.89	6.36	14.67	22.77	27.19	32.93
3	8.1 (−1)	37.7 (1)	68.1 (−1)	34.36	41.31	42.12	46.12	48.89	51.53
4	31.9 (1)	37.7 (1)	68.1 (−1)	32.18	42.55	46.51	49.01	54.36	59.30
5	8.1 (−1)	16.3 (−1)	91.9 (1)	4.50	19.71	26.35	31.93	35.45	40.58
6	31.9 (1)	16.3 (−1)	91.9 (1)	2.79	9.69	18.47	31.22	34.02	39.53
7	8.1 (−1)	37.7 (1)	91.9 (1)	31.05	36.51	38.22	43.85	52.52	57.63
8	31.9 (1)	37.7 (1)	91.9 (1)	27.32	43.65	45.32	47.68	53.50	60.73
9	0.0 (−1.68)	27.0 (0)	80.0 (0)	6.49	23.13	27.33	32.42	40.70	42.64
10	40.0 (1.68)	27.0 (0)	80.0 (0)	9.01	26.97	31.83	36.73	42.42	47.99
11	20.0 (0)	9.0 (−1.68)	80.0 (0)	5.32	14.76	18.06	21.45	25.12	28.65
12	20.0 (0)	45.0 (1.68)	80.0 (0)	12.41	38.36	51.70	55.45	57.06	58.53
13	20.0 (0)	27.0 (0)	60.0 (−1.68)	16.79	25.06	30.84	37.79	43.30	45.06
14	20.0 (0)	27.0 (0)	100.0 (1.68)	14.52	27.12	35.31	37.82	40.22	44.87
15	20.0 (0)	27.0 (0)	80.0 (0)	11.80	23.24	30.70	36.40	45.28	48.23
16	20.0 (0)	27.0 (0)	80.0 (0)	12.45	25.24	34.58	33.70	39.96	43.67
17	20.0 (0)	27.0 (0)	80.0 (0)	14.57	26.30	29.64	33.24	40.97	46.80
18	20.0 (0)	27.0 (0)	80.0 (0)	14.73	25.33	32.74	37.38	43.34	47.64
19	20.0 (0)	27.0 (0)	80.0 (0)	15.32	24.23	28.91	34.03	40.43	45.97
Validation (Average ± SD)	20	37.7	80	22.79 ± 1.58	36.54 ± 1.14	42.0 ± 2.31	45.97 ± 1.85	51.09 ± 2.28	55.28 ± 1.72

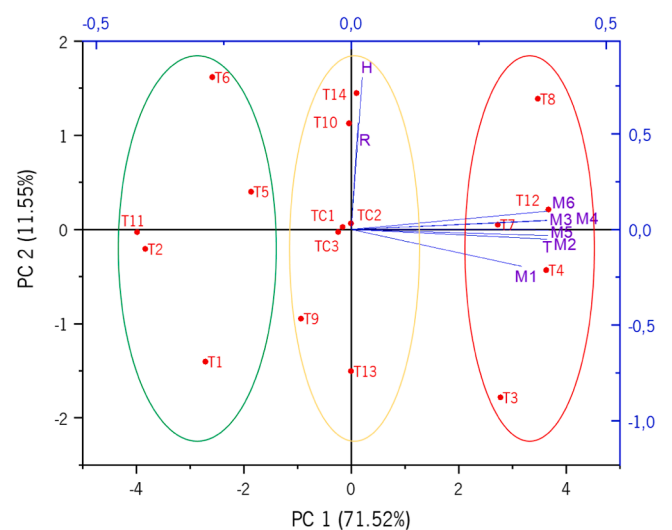


Fig. 1. Principal component analysis of the biodegradation experimental trials (represented by T1-T14, and TC for the central points) where H represents the soil's humidity, R corresponds to the C/N ratio, T corresponds to the temperature and M(1–6) to the month. 919.

Table 3
DoE equations for each month. x_1 – C/N ratio, x_2 – temperature, x_3 – moisture holding capacity.

Months	DoE Equations	
1	$Y_1 = 14.38 + 8.98 x_2$	$R^2 = 52.86 \%$
2	$Y_2 = 26.18 + 11.07 x_2 + 2.93 x_1 x_2$	$R^2 = 89.24 \%$
3	$Y_3 = 31.66 + 11.07 x_2 + 2.88 x_1 x_2 - 2.09 x_2 x_3$	$R^2 = 94.67 \%$
4	$Y_4 = 36.15 + 9.56 x_2 + 0.99 x_2^2 + 1.50 x_1 x_2 - 2.08 x_2 x_3$	$R^2 = 95.89 \%$
5	$Y_5 = 41.99 + 9.75 x_2 + 1.74 x_1 x_2$	$R^2 = 95.45 \%$
6	$Y_6 = 46.51 + 9.37 x_2 + 1.17 x_3 + 2.19 x_1 x_2$	$R^2 = 92.90 \%$

the horizontal distance between the different points is significantly more important than their vertical displacement. As such, it is possible to see in Fig. 1 the categorization of the experimental trials according to the experimental temperature, with the conditions with the higher temperatures on the right (45 and 37 °C), the middle temperature (27 °C) on

the center and the lower temperatures on the left (9 and 16 °C). The PCA clearly shows that the temperature was the most important factor since it is mainly represented by the PC1. Moreover, it is also possible to confirm that in fact, higher temperatures promote a higher degradation of the PHB/PBAT films since the loadings indicate a correlation between the experimental temperature and the biodegradation on each month. It was also possible to confirm that both humidity and C/N did not significantly impact the biodegradation of the polymers due to their orthogonal placement when compared to the temperature and biodegradation on each month.

3.3. Analytical analysis of the validation trial

The analytical techniques revealed that biodegradation during the validation trial (C/N ratio of 20, 37.7 °C temperature and 80 % of soil holding capacity), was mainly attributed to the PHB layer. In Figure S2 it is possible to see the difference between the initial film and the final residues for the validation trial. The ATR-FTIR demonstrated that the PHB layer was the main contributor to the biodegradation since no PHB spectra were present on the residues at the end of the assay (Figure S3). The PBAT layer presented clear signs of serious biodegradation with a marked reduction in all peaks, such as the aromatic (1267 cm^{-1}) and the carbonyl absorbance peak (1709 cm^{-1}) (Figure S3) [25]. Several small new peaks developed (e.g., 3937 3872, 3745, 3694, 3617, 3289 cm^{-1}) some of them related to the formation of free O—H and hydroxyl and carboxylic, linked to the scission in the main chain resulting from the hydrolysis or the action of enzymes [26]. The DSC analysis also revealed that the PHB disappeared from the sample and all the parameters were significantly affected ($p < 0.05$), except for the temperature melting onset (Table S4). The increased crystallinity (Table S4) demonstrated the preference for biodegradation of the amorphous areas of the PBAT layer [27]. The PBAT melting temperature increased significantly, indicating the biodegradation and hydrolysis of the aliphatic part which lead to an improved crystalline structure with fewer defects [28]. It is also noteworthy that in the trials with the temperature in the range recommended by the standard method (20–28 °C) the control biodegradation (cellulose), was equal or superior to the 70 % < necessary for the assay to be considered valid. The same was observed at 16.3 °C and 45 °C, however at 9 °C and 37.7 °C the condition was not achieved (Table S5). This may happen, because some temperatures favour the cellulose-degrading microorganisms or there are some differences in the

communities in each reactor, and thus different biodegradation potential. Furthermore, the higher biomass build-up and consequent carbon retention, may happen at superior temperatures in this case 37.7 °C [29]. This could explain the inferior biodegradation observed in some trials using this temperature.

3.4. Validation and estimation the biodegradation of PHB/PBAT films

Estimating the biodegradation of PHB/PBAT films could be a very useful strategy to determine the biodegradation of PHB/PBAT films in soils. For this purpose, a validation assay was used, within the tested parameters' range, with a different combination of experimental conditions, i.e., a C/N ration of 20, at 37.7 °C and a soil moisture capacity of 80 %. The results of the validation trial are depicted in Fig. 2.

Fig. 2 shows that the estimated and experimentally obtained biodegradation values in the validation trial have a 0.98 correlation coefficient. A logarithmic regression model was further used to estimate the biodegradation of PHB/PBAT films by correlating the physico-chemical characteristics of the soil with the biodegradation at a specific time. Thus, the equations to calculate the model's coefficients were obtained using a CCRD and the results are expressed in Eq. (3) and Eq. (4).

$$\alpha = 17.34 + x_2 + 0.13x_2^2 + 0.37x_3 + 0.07x_1x_2 + 0.42x_2x_3 \quad (3)$$

$$\beta = 13.80 + x_1 + 9.97x_2 + 0.02x_2^2 - 0.21x_3 + 1.43x_1x_2 + 0.79x_2x_3 \quad (4)$$

Where x_1 corresponds to C/N ration, x_2 corresponds to the soil temperature and x_3 corresponds to the moisture-holding capacity. This way, the logarithmic model (Eq. (1)) can be further used to estimate the biodegradation of PHB/PBAT films through time and the results are depicted in Fig. 3.

It is possible to observe in Fig. 3 that the prediction model (Eq. (1)) can predict with high accuracy the biodegradation of PHB/PBAT films since a correlation between the estimated and experimental results is ca. 0.99 which can be considered a high correlation, considering that this process is mainly mediated by the presence of microorganisms which can present a high variability. The correlation is 0.93 between all the experimental values indicated in table 2 and the estimations of the model under the same conditions.

4. Discussion

Considering the values recommended by the standard method [15] for the C:N ratio (nitrogen added to the soil in relation to the carbon in the test specimen, in this case in the form of ammonium chloride), although 0 and 40:1 were used, this parameter had no clear impact on the final biodegradation, not being significant in any equation (Table 3).

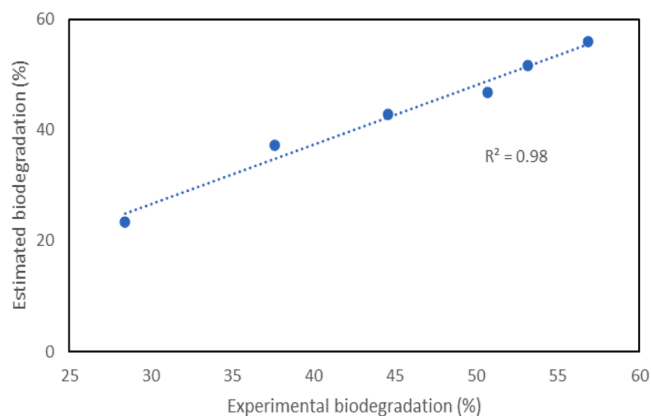


Fig. 2. Comparison between the experimental validation trial and the prediction model using monthly equations.

Kijchavengkul et al. [27] found that compost with a higher C:N ratio than the recommended, resulted in less polymer biodegradation. It has been observed in other work that the carbon input presented by the PHBV degradation may cause the microbial immobilization of nitrogen sources to sustain microbial growth, since the PHBV degradation increased by 45 % the microbial biomass nitrogen and reduced the dissolved organic nitrogen by 66 %. [30]. In this work, the addition of nitrogen (ammonium chloride) did not have a significant impact on the biodegradation of the PHB/PBAT films, probably because the carbon of the polymer and nitrogen added were not sufficient to significantly shift the C:N ratio existing in the soil, which explains the similar biodegradation between all the C:N ratio conditions tested. In these biodegradation tests, nitrogen was not limiting as shown by the close biodegradation values between the intermediate (20) C:N ratio (43.7 % - 48.2 %, trials 14 to 19) and the lowest (0) and highest (40) C:N ratio tested, 42.6 % (trial 9) and 48 % (trial 10) respectively.

Regarding humidity, it is recommended an 80 to 100 % MHC [15]. However, lower values were used in this study and this parameter had no significant impact on biodegradation in the tested range (from 60 to 100 %) only differing by 0.2 % (trials 13 and 14). Water absorption can interact with the polymer structure causing hydrolytic degradation (abiotic process), resulting in the cleavage of ester bonds [31]. In the case of PBAT, the absorption of water in the amorphous regions of the aromatic polyester can cause swelling and stress resulting in micro-cavitation [32]. The degradation of PHB is also higher in soils with more water content than in soils with less water [33]. The moisture content can thus influence the degradation. Products with several materials, such as PHAs can even present a superior water absorption percentage, for example, PHBHHx/KF presented a higher water absorption percentage and a higher percentage of weight loss during a soil degradation study when compared to the PHBHHx [34]. Briassoulis and Mistrionis [8] used the same standard methods, to test lubricants, and recommended also a range of 60 % to 100 % of the soil water holding capacity and an ideal value of 80 % for the greatest possible disintegration rate. Chinaglia et al., [35] indicated differences in the biodegradation of Mater-bi (a commercial, biodegradable plastic) between soils with 55 % and 88 % MHC using the same standard method, although with both soils, the plastic material has exceeded the biodegradability threshold of 90 % defined by the standard EN 17,033. These results may explain why the soil's moisture in the present biodegradation assay did not present significant differences in the range used, i.e., it was already within the optimal range.

It is expected that environmental parameters have an immediate effect on biodegradable plastic build-up after release (accidental or intentional) since they directly influence the plastic biodegradation rates. For modeling pollutant degradation half-lives in chemical fate assessment and exposure models used for risk assessment purposes, the temperature is used as a predictor variable [36]. The range of temperatures recommended by the standard methods is between 20 °C and 28 °C. The increased temperature tested, namely 37.7 and 45 °C, increased the biodegradation by 10.8 % and 11.5 % respectively. This observation could be due to the abiotic degradation or/and the enhanced activity of some microorganisms in these conditions. Kim et al. [37] demonstrated that the degradation of PHB was higher in different soils at 37 °C than at 28 °C. These differences were caused by the activity of the soil microbial community, which is strongly influenced by the temperature of the environment. PBAT degradation is also faster at composting temperatures (i.e., 55 °C) [38,27]. It was expected that higher temperatures should increase or at least accelerate the biodegradation process. This was confirmed by the PCA analysis (Fig. 1). Temperature can effectively disturb chemical and biochemical reactions and influence the taxonomic structure and metabolic activities of the communities [39]. Usually, the biodegradation process increases with temperature increase until it reaches a plateau (i.e., where it is included in the thermal optimum) and finally, as the temperature increases more, the biodegradation decreases, because enzymes start to denature, and the biodegradation

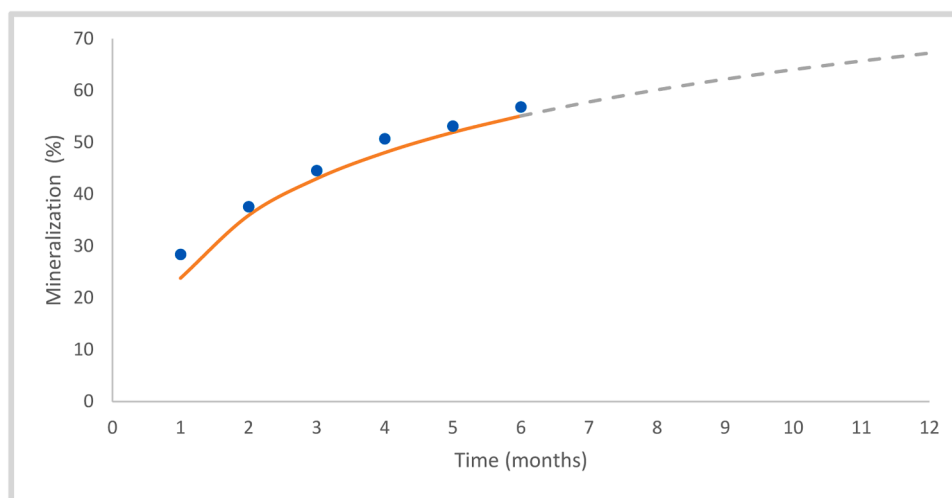


Fig. 3. Comparison between the experimental validation trial (dots) and the prediction model using the final equation (line) for 6 months and prediction using the validation parameters beyond the 6 months tested (dashed line).

outcome becomes ineffective [40]. This effect explains the small difference in the biodegradation between the 37.7 °C and 45 °C conditions, which was only 1.3 % more, for the higher temperature.

For this specific bilayer film, an increase in temperature was beneficial under the tested soil characteristics. Although with 45 °C the biodegradation was slightly better, a temperature around 37 °C should be used to maximize soil biodegradation since it requires less energy to be maintained. Furthermore, under this temperature condition (i.e., 37 °C) the maximum temperature of 45 °C for the growth of mesophilic microorganisms is avoided, and the microbiome is probably not significantly shifted. The microbiome is normally efficient in the degradation of neat PHAs in temperatures between 20 - 30 °C. PBAT is compostable and several thermophilic organisms have been identified, with higher temperatures benefiting the degradation of this polymer [41]. The abiotic effect at these temperatures may be also higher [41]. This effect in combination (or not) with the more effective action of the PBAT degrading microorganisms caused the enhanced degradation in this work. The analysis of the communities in different conditions can be very interesting to test in future works.

The results indicate that the PHB fraction was more biodegraded in the validation trial (Table S4 and Figure S3). The PHB polymer was no longer detected after the assays in the film by the ATR-FTIR and DSC analyses. The PBAT was less degraded but also contributed to the CO₂ evolution, which was confirmed by the development of small peaks related to the scission in the main chain, and the PBAT increased crystallinity related to the degradation of the amorphous regions.

The 0.98 correlation coefficient (Fig. 2) obtained for the estimated and experimentally biodegradation values (Validation trial) indicates that the DoE approach can be used, not only to optimize a pre-determined process (the most common application of DoE) but also used to modulate the biodegradation of bioplastics using fewer trials. The quality of the prediction model (Fig. 3) enables the possibility to predict the soil biodegradation of these films at any given temperature, MHC and C:N ratios, within the tested range. For example, in Braga (the area where the soil was sampled) the annual temperature of soils at a plough depth in 2021 was between 1.3 °C and 26.2 °C (IPMA - Instituto Português do Mar e da Atmosfera (personal communication)), being the average soil temperature of 14.4 °C. Considering the MHC of the soil is not inferior to the threshold tested (60 %) and no nitrogen is added to the soil in the case of the release of the films to the environment, and the type of soil is similar to the one used to produce the model, the model estimates that the total degradation will reach in 6 months, 35.74 %. Instead of the temperature annual average, the value of any parameter can be changed over time, during the prediction, using the real values

measured and following the seasonal changes. Using the same model beyond the 6 months tested (Fig. 3), it is possible to consider that the bilayer film would reach almost 70 % biodegradation in 12 months. However, these types of extrapolations beyond 6 months should only be considered indicative since the model was built using tested (experimental) values for 6 months.

This model is a tool that considers several variables, nonetheless, it has several simplifications and represents an approximation of the biodegradation and not an exact prediction. For example, UV light can interact with polymers. The photodegradation can cause crosslinking within the film due to the recombination of the produced free radicals from Norrish I. Kijchavengkul et al. [32] observed the photodegradation of PBAT film in Costa Rica soils, with the formation of free radicals. However, Kijchavengkul et al. [42] discovered that the crosslinking caused a decrease in the biodegradation of PBAT films in composting. This and other factors, including nutrients and type of soil could be considered to enhance the quality of the model. Another aspect that should be addressed is the characteristics of the polymers being tested. Phase transition temperatures (glass transition or melting temperature) of the polymers in this range (20–37 °C) can change the biodegradation process. For example, for PLA, above the glass transition temperature, the biodegradation is improved, because the chains in the amorphous regions become flexible [43]. These types of questions need to be taken into consideration when testing other polymers.

5. Conclusion

The key parameter for the biodegradation efficiency of this bilayer film in soil was the temperature, within the range between 37.7 and 45 °C. In this case, to accelerate the biodegradation in soil, a value of 37 °C was found as the best, since it requires less energy, than the higher temperatures tested. The range of MHC (60 - 100 %) tested, resulted in similar outcomes indicating that any value within this range can be used to properly test biodegradation of plastics. The addition of nitrogen in the form of ammonium chloride didn't influence biodegradation, which indicates that the soil contained the necessary nitrogen and an adequate C:N ratio (within the range recommended by the standards). The results of this work could also help to save time on biodegradation plastic testing, if the best conditions are applied.

CRedit authorship contribution statement

Miguel Fernandes: Writing – review & editing, Writing – original draft, Software, Resources, Conceptualization. **Andreia F. Salvador:**

Writing – review & editing. **Daniel A. Madalena:** Writing – review & editing, Methodology, Conceptualization. **António A. Vicente:** Methodology, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.polymdegradstab.2025.111182](https://doi.org/10.1016/j.polymdegradstab.2025.111182).

Data availability

Data will be made available on request.

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