Accelerated biodegradation testing of slowly degradable polyesters in soil

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ABSTRACT

According to the impending EU regulations, all polymer products used in agriculture and applied into soil must be biodegradable to prevent their accumulation in the environment. However, some of these products must serve their purpose for months up to years before their degradation and mineralization is desirable. Current international standards describing the biodegradability in soil are more directed towards materials that undergo relatively fast biodegradation, which hampers the development and certification of slowly biodegrading polymeric materials. Here, an accelerated soil biodegradation test is proposed, where by increasing the incubation temperature from 25 °C to 37 °C, up to about 4-fold increase in the biodegradation rate is achieved. The polymers tested include TUV AUSTRIA Belgium certified polyhydroxyalkanoate (PHA), polybutylene succinate (PBS), polybutylene adipate-terephthalate and polylactic acid blend (PBAT/PLA) and an experimental polyester network (ICL-PN). The biodegradation rates of the given polymers at the two temperatures was examined by carbon dioxide evolution. Independent testing was done in two independent laboratories and the results were compared. The biodegradation of the materials was further assessed by electron microscopy and the microbial community present was analyzed by next-generation sequencing. Criteria were proposed for the accelerated test method, which include that the polymers eligible for the accelerated test should not manifest any phase transition, like e.g. a glass transition, in the interval from 25 °C to 37 °C. The proposed accelerated test can be helpful in the development of polymeric materials with a functional life of several years and with ultimately quantitative biodegradability.

Keywords: PHA, PBS**,** PBAT/PLA Polyester network DNA sequencing Accelerated biodegradation

1. Introduction

1.1. General

In soil, plastics are often introduced intentionally to serve a purpose in agriculture, horticulture or environmental engineering. Mulch films, plant pots, tree guards, controlled release fertilizers (CRFs) and geotextiles are only a few examples. Traditionally, these products are made of conventional plastics like polyethylene (PE), polypropylene (PP) or polyurethane (PU). However, due to the rising concern on bioaccumulation of microplastics, the use of conventional plastics in soil is increasingly

under discussion and legislators are looking to (1) ban or reduce certain sources of microplastics or (2) replace them with a biodegradable alternative.

For mulch applications in agriculture and horticulture a new standard EN 17033 "Plastics — Biodegradable mulch films for use in agriculture and horticulture — Requirements and test methods" was launched in 2018, specifying the requirements and test methods for biodegradable films **[1].** The new Fertiliser Regulation, which will come into effect in 2022, will support this new standard **[2].** The European Commission proposed that the polymer coating of controlled release fertilizers shall comply with specific biodegradability requirements (i.e. 90% biodegradation in natural soil conditions and aquatic environments across the EU within 48 months after the end of the claimed longevity period) five years after the entry into force date of the new Regulation **[3]**. The European Chemicals Agency (EChA), on the other hand, proposed that the biodegradation test should be done on the intact CRF shells and that the temperature should be 12 °C in water or soil **[4]**. While there is a general consensus between the industry and the legislators that plastics should be biodegradable and non-persistent, when formation of microplastics cannot be avoided, there is a need for a uniform realistic approach to address this issue. Several methodologies for soil biodegradability testing already exist, but most methodologies focus on relatively fast biodegradation. Yet, many soil applications need to remain intact for months up to years, before biodegradation can start and so a distinction should be made between slowly and fast degrading polymers based on their functional life. The goal of this study was to develop a new fastscreening methodology for slowly degrading polymers to support product development and for fast assessment of the ultimate biodegradation potential under temperature conditions that are representative for real life conditions. A microscopic analysis of the partially degraded test materials and a comparative microbial study of the soil communities were performed to support the biodegradation data.

1.2. Overview of the currently available norms and standards

Over the past few decades several methodologies for biodegradability testing in soil have been developed. In what follows, an overview of the most important methodologies is given.

ISO 11266 (1994) "Soil quality — Guidance on laboratory testing for biodegradation of organic chemicals in soil under aerobic conditions" is designed for testing of pure compounds (chemical purity > 98%) **[5]**. Ultimate biodegradation of unlabeled substances is determined by means of oxygen consumption and/or carbon dioxide production. In addition, a radio-labelled compound can be used to determine the rate of disappearance of the test compound and the formation of metabolites, carbon dioxide, other volatiles and non-extractable residue. Incubation temperature can vary between 25 °C and 35 °C (±2 °C), which is considered the range of maximum microbial activity in soil. For soils from temperate climates a temperature between 10 °C and 25 °C (±2 ° C) is also allowed. No recommendation towards the minimum length of the test is made, but the test should not be run for longer than 120 days, as microbial activity in soils decreases during long incubation periods. Natural soil should be sampled from a site where chemical contact is anticipated. The water content of the soil should be appropriate for the specific goal of the study. In general, maximum microbial activity is found between -0.01 MPa and -0.031 MPa pore-water pressure, or between 40 and 60% of the maximum water holding capacity.

ISO 17556 (2019) "Plastics — Determination of the ultimate aerobic biodegradability of plastic materials in soil by measuring the oxygen demand in a respirometer or the amount of carbon dioxide evolved" **[6]** determines the optimum degree of biodegradation of plastics by measuring the oxygen consumption or the carbon dioxide production. The incubation temperature is maintained constant to

within ±2 °C in the range between 20 °C and 28 °C, preferably 25 °C. The test period should not exceed 6 months. If significant biodegradation is still observed and the plateau phase has not yet been reached, the test may be extended up to 24 months. Natural soil collected from the surface layer of fields and/or forests is used as the inoculum. The soil is sieved over 5 mm, or preferably 2 mm. Alternatively, standard soil composed of industrial quartz sand, clay, natural soil and mature compost can be used as the inoculum. The water content is adjusted to 40—60% of the total water holding capacity of the soil and pH is set between 6.0 and 8.0. C/N ratio should be at least 40:1. The test material should preferably be used in powder form. If necessary, test samples may be reduced in size by means of cryogenic milling. The test is considered valid if the degree of biodegradation of the reference material is more than 60% at the plateau phase or at the end of the test and if the BOD (Biological oxygen demand) values of, or the amount of CO_2 evolved from, the controls are within 20% of the mean at the plateau phase or at the end of the test.

The American standard ASTM D5988 (2018) "Standard Test Method for Determining Aerobic Biodegradation of Plastic Materials in Soil" is equivalent to ISO 17556 (2019), with some exceptions **[7]**. The test method was designed to measure the biodegradability of plastic materials relative to a reference material (e.g. cellulose or starch) in an aerobic environment by measuring the amount of carbon dioxide evolved. The soil used should be a mixture of natural and fertile soils collected from the surface layers of at least 3 diverse locations. Alternatively, a mixture of natural soil and mature compost (ratio 1 g compost to 25 g soil) can be used. Moisture content of the soil is adjusted to 80—100% of the maximum water holding capacity. The test is considered valid if after 6 months more than 70% biodegradation is achieved for the reference material and if the amount of CO_2 evolved from the control reactors is within 20% of the mean at the plateau phase or at the end of the test.

OECD 304A (1981) "Inherent Biodegradability in Soil" is used for the evaluation of the mineralization rate of a14C-labelled compound in soil **[8]**. The method is applicable to volatile or nonvolatile, soluble or insoluble compounds, which are not inhibitory to micro-organisms. The incubation temperature is set at 22 °C ± 2 °C and the water content of the soil is adjusted to 40% of the total water holding capacity. No validity criteria or biodegradation requirements are defined.

OECD 307 (2002) "Aerobic and Anaerobic Transformation in Soil" describes a method designed for evaluating aerobic and anaerobic transformation of chemicals in soil **[9]**. The experiments are performed by using 14C-labelled materials. The test is run at 20 °C ± 2 °C for exposure in temperate climate, or 10 °C ± 2 °C in case of colder climates. A sandy-loamy soil with a pH of 5.5—8.0 and a soil suction of 2.5—5.0 pF is used as the inoculum. The test duration should normally not exceed 120 days. Where necessary test duration can be extended for longer periods (6 or 12 months). Recoveries should range from 90% to 110% for labelled chemicals and from 70% to 110% for non-labelled chemicals.

Next to the abovementioned test methods two standard specifications and two certification schemes exist. These documents contain methodologies and requirements for degradation (biodegradation and disintegration) and environmental safety (heavy metal content and ecotoxicity).

The French norm NF U 52—001 (2005) "Biodegradable materials for use in agriculture and horticulture — Mulching products — Requirements and test methods" determines the biodegradability of agricultural films in soil. It includes a description of the testing method, specifications and labelling of the tested film **[10]**. The biodegradation method in soil is equivalent to ASTM D5988 (2018), with some exceptions. Microcrystalline cellulose is given as the only reference material. The test material is added in the form of fragments (film pieces of 1 —2 cm) or powder. A biodegradation percentage of 60%, relative to reference item cellulose, needs to be reached within 1 year of testing. Moreover, the relative difference between two test results performed simultaneously on the product shall not exceed

20%. Alternatively to the soil test, biodegradation can be evaluated by a freshwater test at 37 °C or a composting test at 58 °C. For both tests 90% absolute or relative biodegradation is required within 6 months. A test material shall be evaluated using at least two of the three abovementioned test methods to pass the biodegradation requirement of NF U 52—001 (2005).

The European norm EN 17033 (2018) "Plastics — Biodegradable mulch films for use in agriculture and horticulture — Requirements and test methods" is the most recent addition to the soil biodegradability standards. It specifies the test methods, as well as requirements for packaging, identification and marking of biodegradable films. The biodegradation test should be performed according to ISO 17556 (2019). Microcrystalline cellulose powder, ashless cellulose filters or poly(3-hydroxybutyrate) can be used as a reference item. If possible, the physical form and size of the reference material should be comparable to that of the test material. A minimum biodegradation percentage of 90% absolute or relative to a reference material needs to be reached within 24 months of testing. Both the reference material and the test item shall be tested for the same length of time and the results compared at the same point in time after the activity of both has reached a plateau. Organic constituents which are present at concentrations of less than 1% do not need to demonstrate biodegradability. However, the sum of such constituents shall not exceed 5%.

The OK biodegradable SOIL certification scheme of TUV AUSTRIA Belgium is inspired by EN 13432 (2001) "Packaging - Requirements for packaging recoverable through composting and biodegradation — Test scheme and evaluation criteria for the final acceptance of packaging" for the requirements for heavy metals and ecotoxicity **[11,12]**. The preferred type of biodegradation test is a soil biodegradation test according to ISO 17556 (2019), ISO 11266 (1994) or ASTM D5988 (2018). Alternatively, an aquatic aerobic biodegradation test can be used with the prerequisite that the test is executed at ambient temperature (between 20 °C and 25 °C). The aquatic biodegradation test is only accepted if positive disintegration results in compost are available. The maximum allowed test duration is two years and 90% absolute or relative biodegradation is required.

The Din-Gepruft Biodegradable in Soil certification scheme of DIN CERTCO is based on EN 17033 (2018) [13]. Testing of ultimate biodegradability is conducted using method ISO 17556 (2019) in accordance with the criteria of EN 17033 (2018) in a temperature range of 20—28 °C (preferably at 25 °C). A biodegradation of 90% absolute or relative to a suitable reference material is required and the maximum testing duration is two years. A fixed biodegradable polymer (microcrystalline cellulose powder, ashless cellulose filter or poly(3-hydroxybutyrate)) is used as the reference material. If possible, the physical shape and size of the reference material should be comparable to that of the test material. A supplementary test (e.g. a composting test at 58 °C) is not accepted.

In order to study the biodegradation behavior of four polyester-based polymers with different biodegradation rates, soil biodegradation tests were set up at two independent laboratories. ISO 17556 (2019) was selected as the basic method for our study, as it is one of the more recent and more elaborate test methodologies and the method is accepted by both certification agencies. Biodegradation is calculated from the amount of carbon dioxide evolved. The use of 14C-labelled compounds, as described in ISO 11266 (1994), OECD 304A (1981) and OECD 307 (2002), is less favorable for a fast screening methodology, because of the high cost to produce the radio-labelled compounds and the inability to label all carbon atoms in a polymer. The incubation temperature was set at the preferred temperature of 25 °C ± 2 ° C for the standard test. For the accelerated test the temperature was increased to 37 °C ± 2 °C, which should be within the range of maximum microbial activity in soil as described by ISO 11266 (1994). No alternative inoculums (freshwater or compost, as described in NF U 52—001 (2005)) were investigated to stay as closely as possible to real-life conditions and to avoid false positive results. The biodegradation tests were performed simultaneously by two

laboratories as a first screening of the reproducibility of the methodology and to examine the acceleration factor.

2. Materials and methods

2.1. Materials

Three commercially available polyesters and one experimental polyester network were used for this study. PHA was provided by Danimer Scientific (trade name Danimer Scientific's Nodax PHA), PBS (trade name BioPBS FD92) by MCPP, PBAT/PLA (trade name ecovio® FT2341) by BASF, and ICL-PN polyester network (ICL-PN) by ICL Group (produced according to patent application WO2019086440A1). The test materials were selected on different biodegradation rates. The properties of the materials are shown in Table 1. Differential scanning calorimetry (DSC) thermograms were obtained by Mettler Toledo, DSC 3 with a heating rate of 10 K/min under nitrogen atmosphere (40 ml/min). The samples were scanned from -90 °C — 200 °C. The melting temperatures (Tm) were taken at the onset of the melting endotherm. Glass transition temperatures (Tg) were taken at the inflection point of the curves of the heat capacity changes.

The biodegradation tests were performed on powder after cryogenic milling with liquid nitrogen using a Retsch Ultra Centrifugal Mill ZM 200 with a distance sieve, stainless steel, trapezoid holes 0.75 mm. The cellulose reference was microcrystalline cellulose powder for thin layer chromatography from Merck (laboratory 1) or Sigma-Aldrich (laboratory 2). Before start of the test, the reference and test materials were analyzed for total solids (TS), volatile or organic solids (VS) and total organic carbon content (TOC). The total solids (TS) or dry matter was measured by drying the sample at 105 °C until a constant weight was reached. The volatile solids (VS) or organic matter was determined by heating the dried sample at 550 °C for at least 4 h. The total organic carbon (TOC) content of the reference and test items was determined using a high temperature (950 °C—1200 °C) combustion method. The formed CO2 is measured with IR detection using a Skalar PrimacsSNC-100 analyzer and SNAcces software. The results are summarized in Table 2.

2.2. Biodegradation

During the aerobic biodegradation of organic materials, oxygen is consumed and carbon is converted to gaseous carbon dioxide (CO_2). Part of the organic material is assimilated for cell growth. Biodegradation is calculated as the percentage of solid organic carbon of the test material, which has been converted to CO_2, as described in paragraph 3.1. The reactors are filled with soil and the test material is added as the sole source of carbon and energy. In parallel, a control (without test material) and a reference (with cellulose as a reference item) are run. The biodegradation tests were performed simultaneously in two laboratories. ISO 17556 (2019) was selected as the basic methodology. After the addition of the reference and test substance, the reactors are incubated in the dark at a temperature constant to within ±2 °C. For the standard test an incubation temperature of 25 °C was chosen, for the accelerated test the temperature was set at 37 °C.

2.2.1. Laboratory 1: inoculum, test set-up and CO_2 measurement

The inoculum was a mixture of natural soils, collected from the surface layer of one field and two forests (all located in Belgium). The soils were air-dried for 3 days at room temperature and sieved over 2 mm to remove plant remains, stones and other inert materials.

Table 1 Properties of the test materials.

Material	Morphology	T_m (°C)	T_g (°C)	ΔH ($J.g^{-1}$)
PHA[a]	semicrystalline	100–150	−15 – +5	6.8–56.5
PBS	semicrystalline	78	−45	41.6
PBAT/PLA	semicrystalline	94; 168	−36	3.46; 2.61
ICL-PN	amorphous	–	−9	–

a Data found in literature for Nodax type of polymers [34]).

Table 2 Overview of analyses on the reference and test materials.

Material	Total solid (%)	Volatile solids (% of total solids)	Total Organic Carbon (%)
Cellulose	97.0	100.0	42.7
PHA	99.7	99.9	55.5
PBS	99.7	99.9	55.5
PBAT/ PLA	99.8	80.2	51.4
ICL-PN	97.5	99.4	56.9

The fraction smaller than 2 mm was used as the inoculum and the three soils were mixed in a 1:1:1 ratio. The moisture content was adjusted to 40—60% of the maximum water holding capacity.

A set of 12 equal reactors with a volume of 4 L were used per test. Each reactor was filled with 500 g of soil inoculum (plough layer, anthrosol/cambisol/gleysol mixture; dry weight 81.0%; soil texture, sandy loam, pH(H_2O) 8.17; volatile solids 6.60%; soil organic matter 3.80%). At start-up 2.0 g of reference or test material was mixed with 500 g of soil inoculum, except for the control reactors which contained only 500 g of soil. Two replicates of each test series (control, reference and test items) were prepared. After the reactors were filled, they were closed air-tight and incubated in an acclimatized room at 25 ° C or 37 °C. The total test duration was 270 days.

The amount of carbon dioxide evolved was measured at regular intervals, dependent on the biodegradation kinetics of the test substance. The amount of CO_2 captured in KOH solution was determined by titration with HCl using a Metrohm 888 Titrando and tiamo™ 2.5 software.

2.2.2. Laboratory 2: inoculum, test set-up and CO_2 measurement

The laboratory procedure used was based on ISO 17556 (2019) but was miniaturized and adapted for small laboratory samples of materials. The biodegradation tests **[14]** were realized in 500 mL flasks with septa mounted on the stoppers. The flasks contained polymer samples (50 mg), soil (15 g, plough layer, haplic chernozem; dry weight 88.4%; soil texture, silty loam, pH(H_2O) 7.28; volatile solids 5.55%; soil organic matter 3.05%), perlite (5.0 g) and mineral medium (10.8 mL). The flasks were incubated at 25 °C or 37 °C air-tight as a closed system. Head space gas was sampled at appropriate intervals through the septum with a gas-tight needle and conducted through a capillary into the gas analyzer (UAG, Stanford Instruments, USA) to determine the concentration of CO_2. The percentage of net mineralization with respect to the carbon content of the initial samples was calculated. Three parallel flasks were run for each sample, along with four blank flasks.

2.2.3. Theory and calculations

The theoretical amount of carbon dioxide evolved by the test item ($ThCO_2$) is calculated from the mass (in mg) of test material introduced into the test system, multiplied by the total organic carbon content (in %) of the test material and corrected for the molar mass of carbon dioxide and carbon.

$$ThCO2 = \frac{44}{12} \times m \times TOC$$

where m is the mass of test material (mg), and TOC is total organic carbon content of the test material, expressed as mass fraction.

The percentage of biodegradation is calculated by dividing the cumulative net CO_2 production of the test compound by the theoretical amount of carbon dioxide evolved and multiplying by 100.

$$\%\ biodegradation = \frac{(CO2)T - (CO2)C}{ThCO2} \times 100$$

where $(CO_2)T$ is the cumulative amount of carbon dioxide evolved in each reactor containing test material and $(CO_2)C$ is the mean cumulative amount of carbon dioxide evolved in the control reactors.

2.2.4. Acceleration factor

The experimental data were used to derive basic parameters of the biodegradation curves with the help of a mathematical model adapted from Ref. **[15]**:

$$m(CO_2) = \frac{m(CO_2)_{MAX}}{1 + \exp\left[2 + \frac{4k_{MAX}}{m(CO_2)_{MAX}}(C - t)\right]} - \frac{m(CO_2)_{MAX}}{1 + \exp\left[2 + \frac{4k_{MAX}}{m(CO_2)_{MAX}}C\right]}$$

where, $m(CO_2)$ is the level of evolved CO_2 carbon expressed as the % of the maximal theoretical value; $m(CO_2)_{MAX}$ is the maximal level of evolved CO_2 carbon expressed as the % of the maximal theoretical value; k_{MAX} represents the maximal mineralization rate ($days^{-1}$); C is the length of the lag phase (days); and t is time (days). The modeling was done in open source software Gnuplot (version 5.2. Thomas Williams, Colin Kelley). The acceleration factor (AF) was then calculated as a ratio between the maximal rates at the two temperatures $AF = k_{37°C}/k_{25°C}$.

2.3. Scanning electron microscopy

Film materials were analyzed with the Phenom Pro (Thermo Fisher Scientific, Waltham, MA, USA) SEM. The samples were sputtered with gold and observed at the acceleration voltage of 10 kV in the backscattered electron mode.

2.4. Next generation sequencing

Materials and blank soils were sampled during the middle fast phase of biodegradation. DNA was isolated with DNeasy PowerSoil DNA extraction kit (Qiagen USA) and used for sequencing (SEQme s.r.o., Czech Republic). Specific regions ITS2 (18S) and V3—V5 (16S) of rRNA genes were amplified using primers carrying specific sequences F357 (5'-CCTACGGGAGGCAGCAG-3') and R926 (5'-CCGY-CAATTYMTTTRAGTTT-3'), or ITS3F (5'-GCATCGATGAAGAACGCAGC-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3'), respectively, with a universal overhang. Sequencing adaptors were introduced in 2nd stage PCR. Products were evaluated on agarose electrophoresis and multiplexed into one pool. This pool was quantified using qPCR assay. Sequencing library was sequenced on MiSeq (Illumina) using v2 version of chemistry and 250 nt paired-end reads settings. The data were further processed with phyloseq R package **[16]** and SEED v2.1.05 **[17]** and taxonomy assigned for bacteria using SILVA reference database **[18]**, and for fungi UNITE reference database **[19]**.

3. Results and discussion

3.1. Characterization of the materials

For this study three commercially available polyesters and a non-commercial ICL-PN polyester network were used. Table 1 summarizes the general properties and phase transition temperatures. The three commercial polyesters are linear polyesters which are certified as OK biodegradable SOIL at TUV AUSTRIA Belgium, formerly known as Vingotte **[20]**. Danimer Scientific's Nodax PHA is a medium-chain-length PHA copolymer showing a melting point in the temperature range of 100—150 °C and a glass transition in the temperature range from -15 to +5 °C as reported in literature **[21,22]**.PBS is a poly(butylene succinate) melting at 78 °C which is in line with the specification **[23]**. It shows a glass transition temperature at -45 °C. PBAT/PLA is the blend of the two linear polyesters poly(butylene adipate-co-butylene terephthalate) and polylactic acid. In line with the specification, the material shows two melting points albeit at somewhat different temperatures of 94 °C and 168 °C **[24]**. The PBAT/PLA shows a single glass transition temperature at -36 °C.

In contrast to the commercial linear polyesters the ICL-PN polyester network is an amorphous aliphatic crosslinked polyester with a glass transition (Tg) at -9 °C. All four polyesters thus have glass transition temperatures well below the typical test temperature of 25 °C described in ISO 17556 (2019) (Table 1).

For biodegradation to occur in polyester products, the molecular chains need to have a certain mobility such as in the amorphous phase above the glass transition temperature. For example PLA is degradable under controlled compost conditions at 55 °C **[25,26]**, but reluctant towards biodegradation in soil at ambient temperatures **[27,28]**. In order to avoid false positive results, the biodegradation should be tested at ambient temperature if there is a phase transition between the accelerated test temperature and ambient temperature. As shown increasing of test temperature to 37 °C for soil biodegradation tests enables to accelerate biodegradation of polymers significantly and to shorten test durations. Provided that there is no phase transition between ambient temperature and 37 °C, the soil biodegradation test at 37 °C can be used to evaluate the ultimate biodegradation potential under conditions that are representative for ambient temperature conditions. It can be assumed, that an extrapolation to even lower temperatures (12 °C) will be possible in case the test items have no phase transitions in between. It is expected that testing at this low temperature will lead to impractically long test duration.

3.2. Biodegradation in soil at 25 °C and 37 °C

The acceleration of the microbial processes in soil with temperature can be demonstrated by monitoring the endogenous CO2 production without the addition of an external carbon substrate (Fig. 1). For the two soil utilized in the subsequent experiments with polymeric materials the figure showed about the same specific carbon mineralization and about the same acceleration at the higher temperature throughout the experimental period.

The absolute and relative biodegradation percentages of the reference and test materials are summarized in Table 3. Fig. 2 shows the evolution of the average absolute biodegradation percentages at 25 °C and 37 ° C. According to the international standard ISO 17556 (2019) a test is considered valid when the degree of biodegradation of the reference material is more than 60% at the plateau phase or at the end of the test. This requirement was easily fulfilled in the four tests, indicating the good quality of the soil inoculums. In laboratory 1 a plateau in biodegradation was reached for cellulose after 270 days at a level of 91.4% (25 °C) and 83.0% (37 °C). In laboratory 2 the reference material was stopped after 170 days, when the rate of biodegradation slowed down considerably, at an absolute biodegradation level of 90.0% (25 °C) and 84.0% (37 °C). The somewhat lower final level of biodegradation at the higher temperature was also observed for the other fast degrading materials (PHA and PBS) and may be due to a higher biomass buildup and consequent carbon retention.

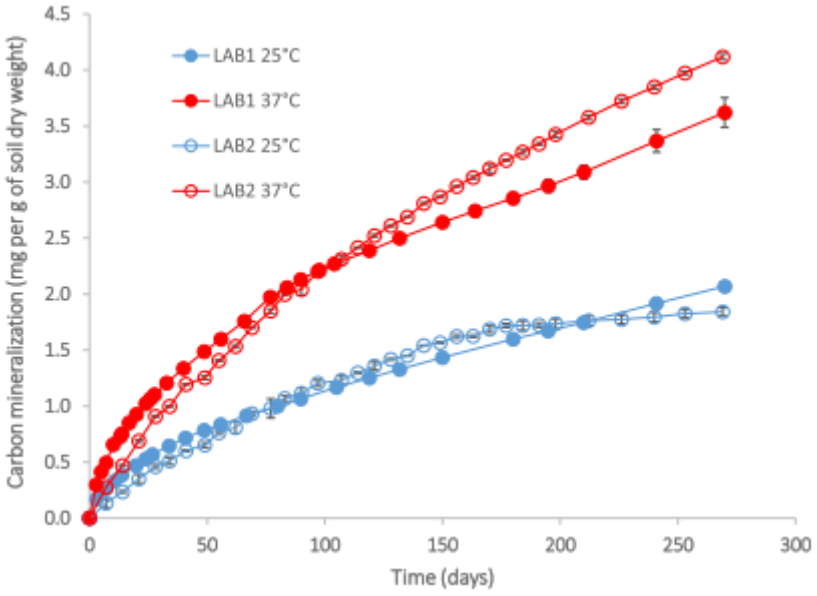

Fig. 1. Endogenous carbon mineralization by blank soils at 25 °C and 37 °C.

Table 3 Absolute (abs) and relative (rel) biodegradation in % of different polyesters at 25 °C and 37 °C.

Material	T (°C)	% of mineralization					
		Laboratory 1			Laboratory 2		
		abs (%)	rel (%)	time (days)	abs (%)	rel (%)	time (days)
Cellulose	25	91.4	100.0	270	90.0	100.0	170
PHA	25	85.8	100.2	150	96.4	107.1	170
PBS	25	85.1	99.4	150	90.0	100.0	212
PBAT/PLA	25	64.4	70.5	270	67.7	75.2	270
ICL-PN	25	23.0	25.2	270	44.8	49.8	270
Cellulose	37	83.0	100.0	270	84.0	100.0	170
PHA	37	71.1	101.6	90	93.0	110.7	170
PBS	37	76.9	109.9	90	78.0	92.9	170
PBAT/PLA	37	70.6	85.1	270	77.2	91.9	270
ICL-PN	37	85.3	102.8	270	82.8	98.6	270

The biodegradation rate and behavior of PHA and PBS was similar to reference item cellulose, with the exception of PBS at 25 °C in laboratory 2. In this test PBS degraded somewhat slower at the beginning but still a similar level of biodegradation was finally reached. At the end of the experiment for PHA, a high level of biodegradation was achieved and the final percentages varied between 85.8% and 96.4% at 25 °C and 71.1% and 93.1% at 37 °C. Test item PBS also degraded well with values between 85.1% and 90.0% at 25 °C and 76.9% and 78.1% at 37 °C reached at the end of the incubation.

A good reproducibility between the laboratories was observed for test items PBAT/PLA and ICL-PN. At ambient temperature (25 °C) PBAT/PLA degraded at a moderate, steady rate throughout the test, resulting in a biodegradation of 64.4% and 67.8% after 9 months in laboratory 1 and 2, respectively, and biodegradation was still progressing. At mesophilic temperature (37 °C) PBAT/PLA degraded slightly faster and a final level of biodegradation of 70.6% and 77.3% was obtained after 270 days.

A significantly higher biodegradation was achieved for test item ICL-PN at 37 °C compared to 25 °C. The biodegradation started at a similar rate during the initial two months at both temperatures, but a steep linear increase in biodegradation was observed between 60 and 180 days of testing at 37 °C. After 9 months an average mineralization of 23.0% and 44.8% was measured at 25 °C in laboratory 1 and 2, respectively, and biodegradation was still progressing.

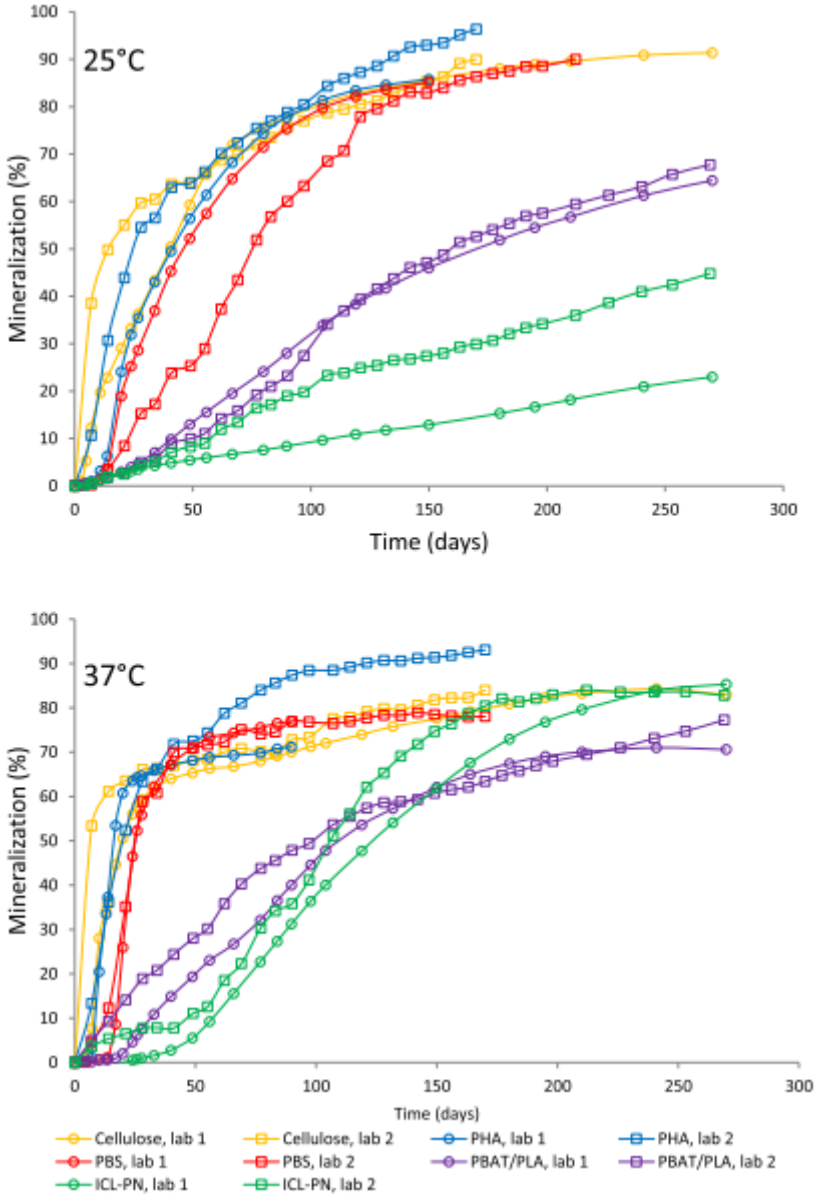

Fig. 2. Carbon mineralization curves of the investigated polyesters in soil.

At 37 °C a plateau was reached for ICL-PN at a level of 85.3% and 82.8% in laboratory 1 and 2, respectively. This result indicated that the increase in the incubation temperature accelerated the degradation and increased the percentage of biodegradation at the end of the incubation period.

In order to quantify the rate of the different biodegradation curves, the data were fitted **[15]**, and the maximal mineralization rate (k_{MAX}) was determined (Table 4). The k_{MAX} values of the faster degrading Cellulose, PHA and PBS appeared to differ between the laboratories 1 and 2, due to differences in the soils and in the protocols used. On the other hand, the k_{MAX} values compared relatively well for the slower degrading PBAT/PLA and ICF-PN Apparently, the subtle differences in biodegradation test conditions have a relatively larger effect on the k_{MAX} of fast degrading materials than on slow degrading materials.

Table 4 Maximal mineralization rates and acceleration factors.

Materials	Laboratory 1			Laboratory 2		
	k_{MAX}, $days^{-1}$ 25 °C	k_{MAX}, $days^{-1}$ 37 °C	AF	k_{MAX}, $days^{-1}$ 25 °C	k_{MAX}, $days^{-1}$ 37 °C	AF
Cellulose	1.60	2.87	1.79	6.56	9.88	1.51
PHA	1.53	5.73	3.75	2.61	3.11	1.19
PBS	1.53	5.73	3.75	0.82	2.85	3.48
PBAT/PLA	0.40	0.58	1.45	0.42	0.61	1.45
ICL-PN	0.14	0.68	4.86	0.23	0.78	3.36

k_{MAX}, maximal mineralization rate; AF, acceleration factor.

The AF was defined as the ratio of the k_{MAX} values at 37 °C and 25 °C (Table 4). The values obtained were relatively consistent between the laboratories except for PHA where a significantly higher acceleration was found in laboratory 1. The AF varied between 1.5 and 1.8 for the PBAT/PLA blend and cellulose to a value between 3.5 and 4 for PBS and ICL-PN. Hence a significant reduction of the biodegradation time can be obtained by increasing the test temperature from 25 to 37 °C. In line with the general principles of chemistry the value of AF is expected to be proportional to a pseudo activation energy of the overall biodegradation process which depends on the physico-chemical properties of the given biodegradable material.

3.3. Scanning electron microscopic analysis of biodegradation process

The degradation of the test materials and the development of the biofilm on material surfaces was observed by scanning electron microscopy (Fig. 3). On all samples the material degradation of their surfaces was clearly discernable, seen as general roughening of the surface and appearance of various cracks and surface erosion. It was also evident that at the higher temperature these signs were more profound. The two slowly degradable polymers (PBAT/PLA and ICL-PN) were not covered with microorganisms. Sparsely, objects that could be bacterial cells were observed on the surface. Much more dense growth of bacterial cells could be seen on the surface of PBAT/PLA at 37 °C, some of them organized in chains. The situation was different for both fast degrading materials (PHA and PBS), where a dense network of fungal filaments and fungal spores covered the entire surface. Intensive erosion of the material was seen along the filaments, in the case of PBS the filaments were observed to bore through the material. These observations are well in agreement with the CO_2 measurements and especially with the microbiological assessment of the biodegradation process.

3.4. Sequencing analysis of the microbial communities present during the biodegradation

The microbial community is one of the key elements of the biodegradation process. In general, soils contain a great diversity of microorganisms that should in most cases fulfill the requirements for the biodegradation of polyesters. Hydrolytic enzymes, necessary for their biodegradation are probably the most abundant group of extracellular enzymes, thus it could be expected that suitable enzymes can be produced by many species that can eventually substitute their roles in the biodegradation process. In the case of the accelerated biodegradation test, the question should be answered, whether the increase of the temperature does not create an important limitation for the effective function of the microbial community. To answer such objection, DNA was isolated from the soil of laboratory 2 alone and from the materials incubated in this soil and sampled during the intensive phase of their biodegradation. Fragments of rRNA genes were sequenced and the compositions of the bacterial and fungal communities were analyzed.

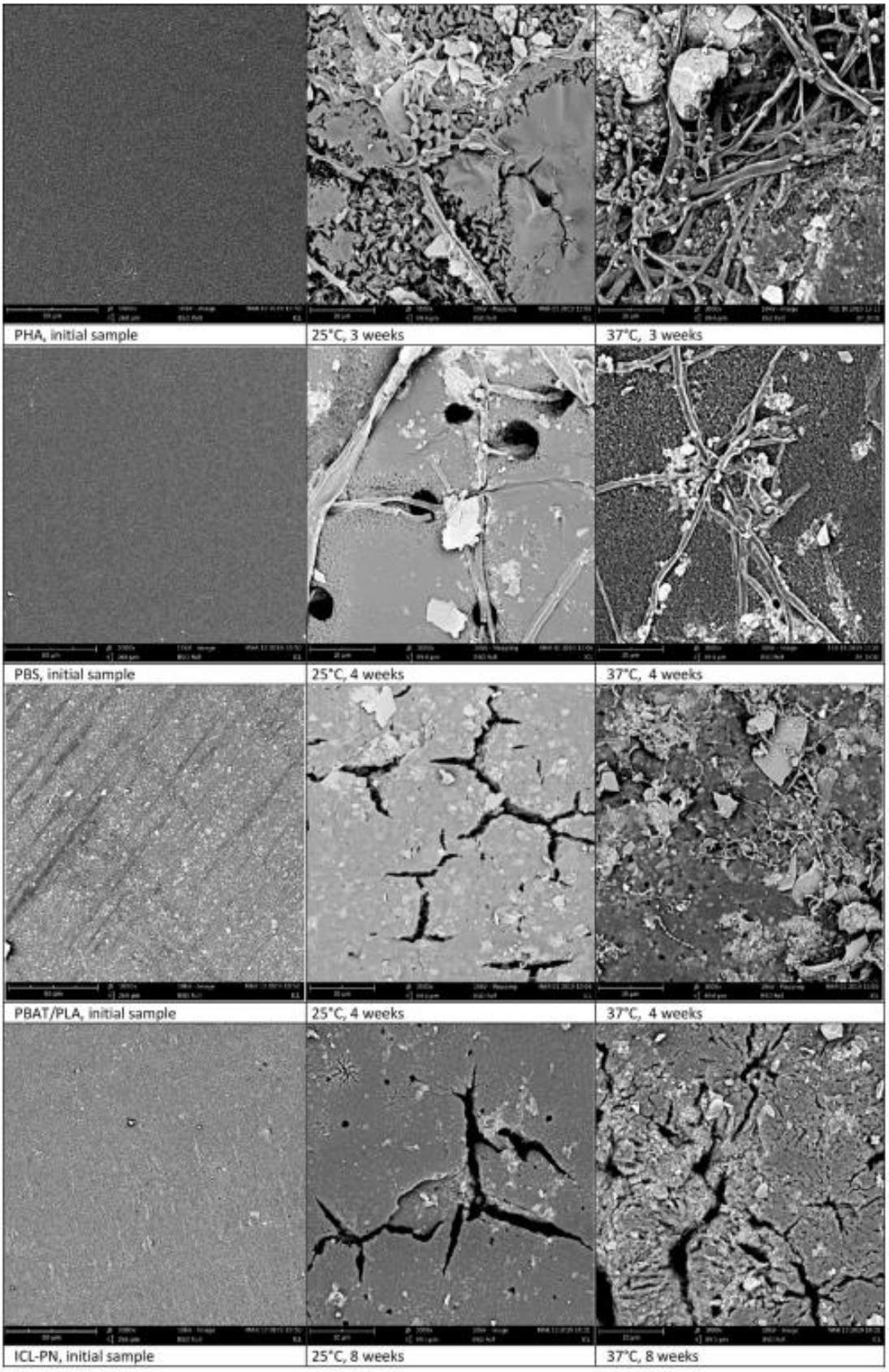

Fig. 3. Scanning electron micrographs of the polyester films investigated.

At the phylum level, the bacterial communities at both temperatures are dominated by Proteobacteria (Fig. 4). There is a notable difference between the fast degrading PHA and PBS, where the proportions of Proteobacteria, and also Actinobacteria, are even higher, and the more slowly degrading PBAT/PLA and ICL-PN, where Chloroflexi seems to be important and even more abundant at 37 °C. This phylum is known to encompass many active xenobiotic degrading strains and also many thermophilic species **[29]**. At the genus level, it is evident that the communities contain an important diversity of species in the soil alone but also in the vicinity of the biodegraded materials, which is apparent from the important part of the species that occupy less than 3% or less than 1% of the community. This proportion is systematically slightly lower at the higher temperature which could indicate slightly lower bacteria diversity. Again, a difference can be seen between the slow biodegrading materials, where the gross of the community, especially at 25 °C, is very similar to the blank soil, and the fast degrading materials, where the genera, which are probably directly connected to biodegradation, occupy a more important part of the community. Judging the differences between the two temperatures, for the slower degrading PBAT/PLA and ICL-PN, there are mostly differences in the relative abundance of the genera while the general composition of the community seems to be similar. For the faster degrading PHA and PBS more marked differences in the community composition were evident. The identified organisms that could be mentioned include Thiohalorhabdaceae **[30]** and Woeseia **[31],** both thermophilic bacteria capable to withstand higher salinity, which were identified in a higher proportion during PBS biodegradation at 37 °C. Thermophilic Chloroflexi member Litorilinea **[32]** seen in connection with PBAT/PLA biodegradation at 37 °C and interestingly Povalibacter **[33]**, a polyvinyl alcohol degrading Proteobacteria, which was identified in the PHA degrading community at 25 °C.

Fungal communities at the phylum level were totally dominated by Ascomycota and this dominance was absolute for the two fast degrading materials PHA and PBS. Apparently, fungi had an important role in the biodegradation of these materials as could also be judged by their high presence on their surface (Fig. 4). On the species level, only a limited number of species comprised the majority of the fungal communities on PHA and PBS at 25 °C with the thermophilic fungi Aspergillus fumigatus being totally dominant at 37 ° C. Fungal filaments were not observed on the surface of the slower degrading materials (PBAT/PLA and ICL-PN) and the fungal communities seemed to be more diverse at both temperatures but their compositions differed at the species level.

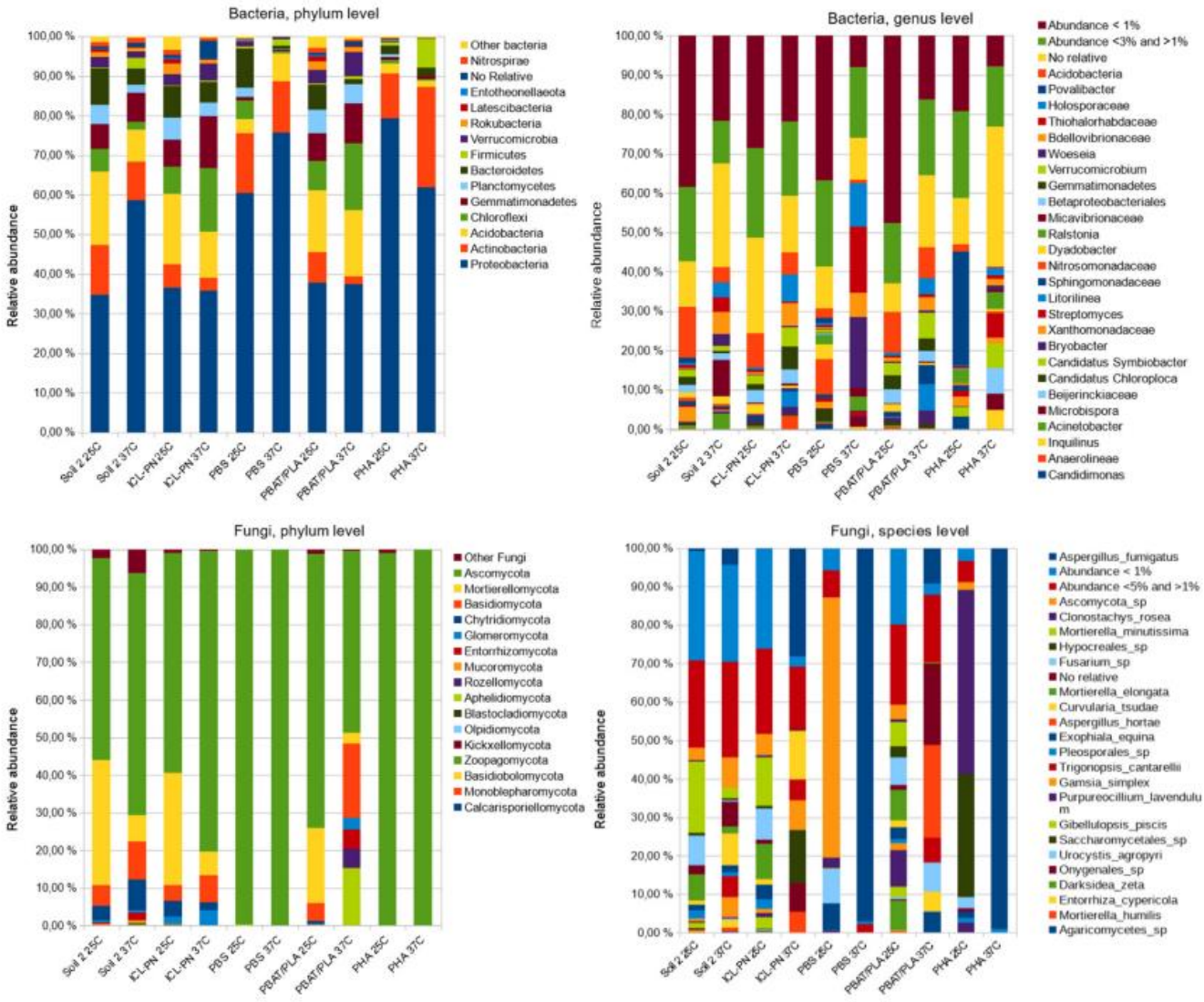

Fig. 4. Composition of fungi and bacterial communities present initially in the soil and during the biodegradation of the investigated polyesters at 25 °C and 37 °C.

In conclusion, it seems that the temperature shift from 25 °C to 37 °C was more reflected for the two fast degrading materials (PHA and PBS), which supported mostly fast-growing microorganisms. For the slow degrading polymers (PBAT/PLA and ICL-PN) the shift of the bacterial communities was not very important. In fungal communities, thermophilic species replaced their mesophilic counterparts but on the phylum level, the Ascomycota dominance was preserved.

3.5. Proposal of the accelerated biodegradation test conditions and validity

The goal of the study was to examine the feasibility of an accelerated soil biodegradation test that would enable to judge biodegradability in a shorter time frame and the approach chosen was to increase the temperature of the test from 25 °C to 37 °C. A moderate increase of testing temperature to 37 °C was chosen to avoid a drastic change of the microbiome towards extremophilic species. As shown, increasing the test temperature to 37 °C for soil biodegradation tests enables to accelerate

biodegradation of polymers significantly and to shorten test durations. Provided that there is no phase transition between ambient temperature and 37 °C, the soil biodegradation test at 37 °C can be used to evaluate the ultimate biodegradation potential under conditions that are representative for ambient temperature conditions. Microbiological assessment showed the microbial community at both temperatures provided substantial capacity and diversity for the biodegradation and that both bacteria and fungi were present and active at both temperatures. It can be assumed, that an extrapolation to even lower temperatures (12 °C) will be possible in case the test items have no phase transitions in between.

As environmental safety under the form of toxicity tests should be determined on residuals and metabolites after biodegradation, an accelerated test would also be beneficial in this context. Soil can be prepared in a much shorter time frame and results become available much faster and at lower cost.

Based on the foregoing observations and considerations, the following test criteria are proposed for the accelerated biodegradation testing:

a. The test temperature is 37 °C.
b. The test matrix is soil.
c. The test is conducted on cryogenically milled samples.
d. The samples should preferentially be homogeneous homo- or copolymers; in case of polymer blends the domain size should be small enough so that the biodegradation behaviour of the blend does not reflect those of the original blend components.
e. There should be no phase transition (Tg, Tm) between 25 ° C and 37 °C. If there is a phase transition, then the biodegradation needs to be tested at 25 °C.
f. Equal or more than 90% biodegradation relative to the reference material should be reached within maximum 2 years at 37 °C.
g. Equal or more than 90%/AF biodegradation relative to the reference should be reached within 2 years at 25 °C whereby biodegradation should be progressing and the plateau phase should not have been reached. AF is the ratio of the maximal mineralization rates at 25 °C and at 37 °C. For AF of 1.5 this means that at least 60% biodegradation should be reached within 2 years at 25 °C whereas for AF of 4 at least 22.5% biodegradation should be reached.

4. Conclusions

1. The study introduces a method that can help in the development of polymeric materials that need to be fully albeit slowly biodegradable in soil. In the current embodiment the standard procedure requires up to two years of incubation which is highly impractical for the development of new materials that should exert a certain function and therefore should only slightly biodegrade during the first year after application.

2. By increasing the temperature from the standard 25 °C—37 °C it is possible to accelerate the processes considerably using the universal rule of the theoretically exponential temperature dependence of the chemical reaction rates and in the same time still retain the relevance of the results in relation to the conditions prescribed by the standard, provided, that there is no phase transition occurring in the material within the temperature interval between 25 °C and 37 °C.

3. The procedure was tested independently in two laboratories on certified biodegradable polyesters and an experimental polyester network sample. It was shown, that the materials could be roughly

divided into two categories. The first group, fast degrading materials (cellulose, Danimer Scientific's Nodax PHA and PBS), which can reach the ultimate mineralization at 25 °C in about 6 months and where the accelerated test is not necessary. The second group was comprised of materials where the biodegradation in soil was relatively slow at 25 °C (PBAT/PLA and ICL-PN). Here the acceleration test at 37 °C could be important and bring benefits. AF observed varied from about 1.5 to about 4.

4. The results showed very good agreement between the two independent laboratories.

5. The method is useful to compare the soil biodegradability of the individual materials in a faster manner during the development phase and to identify prospective candidates for further testing and certification.

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