Evaluation of the biodegradation of polymeric materials in the freshwater environment—An attempt to prolong and accelerate the biodegradation experiment

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ABSTRACT

Available international standards describing the biodegradability testing in freshwater were designed primarily for chemicals that undergo relatively fast biodegradation (in the order of days). The current study was undertaken to verify their applicability for testing of slowly degradable polymeric materials, and also describes an attempt to accelerate the biodegradation testing by the increase of the incubation temperature from 25 0C to 37 0C. The polymers tested include TÜV 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 were examined by carbon dioxide evolution and additional information was collected from microscopy observations of the materials and the investigation of the microbial community composition and dynamics. Testing was done at two independent laboratories and the results were compared. It was found that at 25 0C the testing environment was relatively stable, and the incubation can be prolonged up to 12 months. At 37 0C however some undesirable phenomena like higher variability between replicates and laboratories, fungi development, and biomass autolysis were observed. Moreover, the increase in temperature did not accelerate the biodegradation process. Based on the comparison of results from the two laboratories, recommendations for the experimental procedure are given.

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

1. Introduction

1.1. General introductio

Biodegradable polymers have recently received a lot of interest due to their potential to reduce the quantity of plastic trash entering the environment and to replace traditional polymers in specialized applications, such as agricultural**[1,2].**

The investigation of polymer material degradation caused by biological agents began in the 60s of the last century with the aim to prolong the life span of the materials. Later, in the 70s and the 80s the

interest started to shift towards eventual negative effects of plastics to the environment, which resulted among others in the development of biodegradable polymer materials in 90s.

There are three main reasons for the booming interest in biodegradable polymers and/or biorenewable plastics which are commonly referred to as bioplastics. Firstly, it is the need to alleviate the negative consequences of the massive leakage of conventional persistent plastics to the environment, which is recognized by the more responsible producers and consumers **[3]**. Secondly, there is a growing awareness and concern about the use of 'intentionally added microplastics' in various consumer, professional, agricultural and industrial products**[4]**. Thirdly, biorenewable and biodegradable plastics are a part of the transition towards a renewable resources and carbon neutral economy. The notion that polymer materials or their residues can be washed from soil to water bodies, where they can accumulate in freshwater and marine ecosystems **[5]**, has led policy makers to pursue a multi-environmem approach. The new EU fertilizer regulation 2019/1009 **[6]** which lay; down rules on the application of polymer products in agriculture, pre scribes that these materials must be tested not only in soil but also in water. The European Chemicals Agency (EChA) proposed an even more extensive, tiered approach for evaluation of 'intentionally addec microplastics', requiring testing in soil, freshwater and marine envi ronment (ECHA 10162, 2019). All this is tightly connected to the need for reliable and representative testing methods proving the biodegrad ability of the materials in different natural environments.

Biodegradation in water environment typically takes many month; or even years depending both on the material investigated and the environment that is simulated. However, during the development pro cess of new biodegradable materials such time span is impractical and there is a growing need to obtain information on the biodegradability faster. Enzymatic tests were recently employed as an alternative. Th basis of such tests is the treatment of the material in aqueous medium with selected enzymes, for example amylase **[7]** for the materials based on carbohydrates, and different type of lipases, proteases or depoly merases **[8-12]**. The results are typically available within days. Such tests can in some cases provide valuable information about the mecha nism of the biological process and can be used to compare differen variants of the material. On the other hand, they do not reflect rea conditions in the environment at all.

Modification of a standard biodegradation test seems to be a good strategy for the development of an accelerated procedure **[13,14]** Generally, it is known that temperature is the key factor that determines the rate of chemical and, to some extent, also biological processes**[15 16]**. E.g., biodegradation of polyhydroxybutyrate-valerate (PHBV tested in seawater under laboratory static incubation at 21 °C reached 99% in just 49 days, but the same sample under real seawater condition; (dynamic flow aquarium) and at temperature varying between 12 °C and 22 °C, depending on the actual weather, attained only 30% in 9C days **[17]**. Based on previous studies **[18,19]** and our own experience [20] it was suggested that the freshwater biodegradation test can be substantially accelerated by shifting the temperature from 25 °C to 37 °C, under the condition that the material tested does not exhibit any kind of inner structural change, e.g. a phase transition in this tempera ture interval.

1.2. Overview of the currently available norms and standards

Over the past few decades several methodologies for biodegrad ability testing in freshwater have been developed **[21,22]**. In what fol lows, an overview of the most important methodologies is given.

The first guidelines were developed by the Organization for Eco nomic Cooperation and Development (OECD) and are designed foi testing of chemicals (i.e., a form of matter that has a constant chemica composition and which cannot be separated into components by phys ical separation methods without breaking chemical bonds). These methods are not always suitable for determining the biodegradation o complex materials, but they are often referred to in legislation and labelling of biodegradable plastics and therefore they are included in this overview.

OECD 301 (1992) "Ready Biodegradability" **[23]** and OECD 31C (2014) "Ready Biodegradability - CO_2 in sealed vessels (Headspace Test)" **[24]** are designed to determine ready biodegradability [25,26] The term "ready biodegradability" refers to an arbitrary classification o chemicals, which have passed certain specified screening tests for ulti mate biodegradability. It is assumed that such substances will rapidly and completely biodegrade in aquatic environments under aerobi conditions. An overview of the guidelines of OECD with regard to aer obic aqueous biodegradation is given in **Table 1.** Degradation is followed by dissolved organic carbon (DOC) analysis (OECD 301A-E) or by determining the amount of CO_2 produced (OECD 301B), O_2 consumed (OECD 301C-D-F) or inorganic carbon (IC) produced (OECD 310).

Table 1 Overview of the OECD guidelines with regard to ready biodegradation in aerobic aqueous environment.

Guideline	Method	Description	Measurement
OECD 301	A	DOC Die-Away	DOC
Ready	B	CO_2 Evolution	CO_2
Biodegradability		(Modified Sturm Test)	
	C	Modified MITI Test (I)	O_2
	D	Closed Bottle	O_2
	E	Modified OECD Screening	DOC
	F	Manometric Respirometry	O_2
OECD 310		CO_2 in sealed vessels	IC
Ready		(Headspace Test)	
Biodegradability			

The duration is always set at 28 days, pre-exposure of the inoculum to the chemical is not allowed, the test substance is provided in a rather high concentration (2 to 100 mg L^{-1}) and the amount of DOC from the inoculum should be kept as low as possible compared to the amount of DOC from the test substance. The pass levels for ready biodegradability are 70% removal of DOC or 60% of theoretical oxygen demand (ThOD), theoretical production of carbon dioxide ($ThCO_2$) or theoretical amount of inorganic carbon (ThIC). The pass level needs to be reached within a 10-d window, which starts when the degree of biodegradation has reached 10% DOC, ThOD, $ThCO_2$ or ThIC and must end before day 28 of the test. The incubation temperature depends on the chosen method, but varies between 20 °C and 25 °C, with a deviation of max 2 °C. The inoculum can be activated sludge, sewage effluent, surface water, soil extract or a mixture of sources, again depending on the selected method. The number of replicates per series is mostly 2 for blank and test material and 1 for reference material, with the exception of OECD 301C (10 replicates for all series) and OECD 310 (min. 17 replicates per series). In order to check the validity of the test procedure, OECD 301 prescribes that a reference compound, which meets the criteria for ready biodegradability needs to be tested in parallel. OECD 301 proposes aniline, sodium acetate or sodium benzoate as reference materials. OECD 310 refers to aniline, sodium benzoate or ethylene glycol for water-soluble test substances and to 1-octanol for poorly soluble test substances. The validity requirement (70% removal of DOC or 60% of ThOD, $ThCO_2$ or ThIC) must be reached within 14 days. The only exception to this rule is OECD 301C in which > 40% ThOD (after 7 days) and > 65% ThOD (after 14 days) is required for aniline. In addition, OECD 301 prescribes that the

difference of extremes of replicate values of removal of test chemical at the plateau, at the end of the test or at the end of the 10-d window, as appropriate, is < 20%. Requirements for background activity are also added for some methodologies (OECD 301B-C-D-F and OECD 310).

For testing of "plastic materials" four standards were developed: two international standards ISO 14851 (2019) and ISO 14852 (2021) **[27]** and two American standards (ASTM D5271-02 (2017) **[28]** and ASTM D6340 98 (2007) **[29]**), but the latter have been withdrawn without replacement. ISO 14851 **[30]** "Determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium — Method by measuring the oxygen demand in a closed respirometer" and ISO 14852 (2021) "Determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium — Method by analysis of evolved carbon dioxide" are designed to determine the optimum degree of biodegradation of plastics by measuring respectively the oxygen demand in a closed respirometer or the amount of evolved carbon dioxide. The incubation temperature is maintained constant to within ± 1 °C in the range between 20 °C and 25 °C. The test period should typically not exceed 2 months. However, if significant biodegradation is still observed and the plateau phase has not yet been reached, the test may be extended up to 6 months. Activated sludge from a sewage-treatment plant treating predominantly domestic sewage is used as the inoculum to simulate natural environment. Alternatively, a pre-exposed inoculum can be used, provided this is clearly stated in the test report and the method of pre-exposure is clarified. Before use, the concentration oi suspended solids of the sludge is determined and, if necessary, concentrated by settling. A suitable volume of sludge is added to the minera medium to obtain suspended solids in the range 30 mg L^{-1} to 1000 mg L^{-1} in the final mixture. The test material should preferably be used in powder form and is added in a concentration of at least 100 mg L^{-1} (ISC 14851) or 30-100 mg total organic carbon (TCC) per liter (ISC 14852). Higher test item concentrations (up to 2000 mg L^{-1} of organic carbon) can also be tested in both methods on condition that the optimized test medium is used. The optimized medium is highly buffered and contains more inorganic nutrients. The number of replicates per series is 3 for blank and test material and 2 for reference material. The test is considered valid if the degree of biodegradation of the reference material (aniline, microcrystalline cellulose powder, ashless cellulose filters or poly-p-hydroxybutyrate) is more than 60% at the end of the test and if the background activity of the blank does not exceed 60 mg C2 L^{-1} (ISC 14851) or 90 mg CO2 L^{-1} (ISC 14852) at the end of the test, in case of an inoculum dosage of 30 mg L^{-1} dry matter.

Next to the abovementioned test methods one standard specification and one certification scheme exist. These documents contain methodologies and requirements for degradation (biodegradation and solubil-ity/dispersibility) and environmental safety (heavy metals).

The European norm EN 14987 (2006) **[31]** "Plastics - Evaluation of disposability in wastewater treatment plants - Test scheme for fina acceptance and specifications" specifies the test methods and criteria tc verify if a solid plastic material can be considered as disposable in wastewater treatment systems. A distinction is made between cold and hot "water soluble/dispersible plastics". The hot water soluble/disper-sible plastics can only be disposed of through the sewage after exposure to hot water. Moreover, also a distinction is made between soluble and dispersible plastics. The dispersible plastics are not suitable for applications where the final solution shall pass through small diameter pipes or orifices (e.g., laundry bags for washing machines). The biodegradation test should be performed according to ISC 14851 (2019) or ISC 14852 (2021). The test temperature shall be room temperature (from 20 °C to 25 °C). It is excluded to run the tests under thermophilic temperature conditions. The inoculum shall be obtained only from municipal or industrial sewage sludge. Soil or compost inocula are not allowed. Soluble starch or microcrystalline cellulose powder can be used as a reference material. A minimum biodegradation percentage of 90% absolute or relative to a reference

material needs to be reached within 56 days of testing. Both the reference material and the test material shall be tested for the same length of time and the results compared at the same point in time.

The OK biodegradable WATER certification scheme of TUV AUSTRIA Belgium is inspired by EN 14987 (2006) for the requirements for biodegradation and by EN 13432 (2000) "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. Biodegradation should be tested according to ISC 14851 (2019) or ISC 14852 (2021). The maximum allowed test duration is 56 days and 90% absolute or relative biodegradation needs to be reached. No disintegration and ecotoxicity requirements have to be met, but constituents that appear on the (candidate) list of Substances of Very High Concern (Annex XIV or the REACH) are not accepted.

The goal of this study was to develop a 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. In order to study the biodegradation behavior of four polyester-based materials with different biodegradation rates, freshwater biodegradation tests were set up at two independent laboratories. ISC 14851 (2019) and ISC 14852 (2021) were selected as the basic methods for our study, as they are the most recent and most elaborate test methodologies, specifically designed for plastics and accepted for certification. There were several questions that the study aimed to answer. Mainly, to show whether the biodegradation experiments in freshwater environment can safely be prolonged and/or accelerated. Also, it was interesting to find whether the results were reproducible between the two laboratories and to what extent they were influenced by the different inocula used.

To achieve this not only the mineralization of materials was followed by CO_2 measurement but simultaneously the microbiology of the process was monitored.

2. Materials and Methods

2.1. Materials

Three commercially available polyesters and one experimental polyester network were used for this study. The identical materials were also used in the previous study aimed at the biodegradation testing in soil **[20]**. 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 covalently crosslinked polyester by ICL Group (produced according to patent application WC2019086440A1) labeled here as ICL polyester network (ICL-PN). The test materials were already characterized [20] hence only selected properties are now presented **(Table 2).**

The respirometric biodegradation tests were performed on powdered samples obtained 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 (Lab1) or Sigma-Aldrich (Lab2).

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 (or mineralization more precisely) is calculated as the percentage of solid organic carbon of the test material, which has been converted to CO_2. The reactors are filled with a mixture of mineral medium and sludge 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. ISC 14851 (2019) and ISC 14852 (2021) were selected as the basic methodologies. In Lab1 the test was performed according to Annex G of ISC 14851 (2019). In Lab2 the test was based on ISC 14852 (2021) but was modified and optimized for laboratory closed respirometer MicroCxymax CO_2/C_2 with IR detector for CO_2 detection and paramagnetic sensor for oxygen detection in gas phase (Columbus Instruments, Chio, USA) *[33]*. After the addition of the reference and test substance, the reactors are incubated in the dark at a temperature constant to within ±1 °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. Labi: inoculum, test set-up and CO_2 measurement

The inoculum was a mixture of activated sludge, collected from 3 sewage-treatment plants (all located in Belgium) treating domestic and/ or industrial wastewater. After filtration over an 80 µm sieve, mixing in equal parts, decantation of the supernatant and replacement with mineral medium, the final sludge inoculum was obtained.

Table 2 Selected properties of the test materials.

Material	Morphology	T_m (°C)	T_g (°C)	Organic carbon (%)
PHA [32]	semicrystalline	100–150	−15 – +5	55.5
PBS	semicrystalline	78	−45	55.5
PBAT/PLA	semicrystalline	94; 168	−36	51.4
ICL-PN	amorphous	–	−9	56.9

This inoculum was actively aerated for 2 hours. The mineral medium was prepared by adding 1 mL of the following stock solutions ($CaCl_2 \cdot 2H_2O$ (36.4 g L^{-1}), $FeCl_3 \cdot 6H_2O$ (0.25 g L^{-1}) and $MgSO_4 \cdot 7H_2O$ (22.5 g L^{-1})) and 10 mL of phosphate buffer solution (8.5 g L^{-1} KH_2PO_4, 21.75 g L^{-1} K_2HPO_4, 33.4 g L^{-1} $Na_2HPO_4 \cdot 2H_2O$ and 0.5 g L^{-1} NH_4Cl) to about 500 mL of distilled water into a 1 L bottle. Everything was mixed and made up to 1 liter with distilled water.

A set of 12 equal reactors with a volume of 500 mL were used per test. Each reactor was filled with 250 g of test medium, consisting of 245 g of mineral medium and 5 g of inoculum. A total suspended solids content of 28.0 mg L^{-1} and a total volatile suspended solids content of 20.3 mg L^{-1} were measured in the final test medium. The level of pH at the beginning of the test was 7.2. At start-up 25 mg of reference or test material was added to 250 g of test medium, except for the control reactors which contained only 250 g of test medium. 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 on inductive stirrers in a thermostatic cabinet at 25 °C or 37 °C. A magnetic rod kept the reference item, test items and the growing biomass into suspension throughout the test (450 RPM). The total test duration was 294 days. The amount of carbon dioxide evolved was measured at regular intervals (every 2 to 4 weeks). 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. Lab2: inoculum, test set-up and CO_2 and O_2 measurements

A mixed microbial culture in the form of activated sludge from a municipal sewage treatment plant was used as the inoculum. Prior to testing, the activated sludge was decanted three times with tap water and aerated for 24 hours, after which it was centrifuged (3000 g, 10 minutes, 25 °C). After removing the supernatant, the biomass was suspended in a mineral medium prepared for this type of respirometric test realized in two temperatures (25 °C and 37 °C). The initial sludge dry matter was 250 mg L^{-1}. The mineral medium was prepared by adding 800 mL of aerated distilled water into a 1 L bottle, as well as 1-mL quantities of the following stock solutions: $CaCl_2$ (27.5 g L^{-1}), $FeCl_3 \cdot 6H_2O$ (0.25 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (22.5 g L^{-1}); and solutions of trace elements: H_3BO_3 (0.75 g L^{-1}), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.05 g L^{-1}), $CoSO_4 \cdot 7H_2O$ (0.18 g L^{-1}), $CuSO_4 \cdot 5H_2O$ (0.5 g L^{-1}), $ZnSO_4 \cdot 7H_2O$ (0.1 g L^{-1}), $FeSO_4 \cdot 7H_2O$ (3 g L^{-1}). 20 mL of phosphate buffer solution was also added (8.2 g L^{-1} KH_2PO_4, 21.75 g L^{-1} $K_2HPO_4 \cdot 12\ H_2O$, 44.7 g L^{-1} $Na_2HPO_4 \cdot 12\ H_2O$) and 5 mL $(NH_4)_2SO_4$ (10 g L^{-1}). Everything was mixed and filled up to 1 liter with aerated distilled water. The tested polymer materials were added in the form of powder at the initial concentration of 200 mg L^{-1}. The pH at the beginning of the test was 7.0 ± 0.5 and the mixture was stirred with magnetic rod at 300 RPM. Samples of the suspension for determining the dissolved organic carbon, pH and dry matter of the biomass were always withdrawn at the start and the end of the test. Endogenous respiration (blank) was investigated concurrently. The oxygen consumption and amount of carbon dioxide evolved was measured by the respirometer MicroOxymax CO_2/O_2 at every 8 hours. In order to assess the biodeterioration of polymer materials in the water environment by scanning electron microscopy (SEM), fluorescent microscopy and microbial community analysis a third replicate of each polymer material was prepared, in which the polymer material was added in the form of film (5 x 5 mm).

2.2.3. Theory and calculations

The basic biodegradability measure was the ratio of actually produced CO_2 arising during microbial breakdown to the theoretical quantity of CO_2 given by the carbon content present in samples, expressed as the percentage of mineralization (%) in accordance with (1):

$$\%\ \text{Mineralization} = \left[\left(n_{CO2(\ \text{sample}\ )} - n_{CO2(\ \text{blank}\ )}\right) / \text{ThCO}_2\right] \times 100 \qquad (1)$$

where $n_{CO2(sample)}$ is the quantity of CO_2 produced during breakdown of polymer materials (mmol), $n_{CO2(blank)}$ is the quantity of CO_2 produced during endogenous respiration of microorganisms (mmol), and $ThCO_2$ is theoretical production of CO_2 from total mineralization of the sample (mmol) (**eq. 2**)).

$$\text{ThCO}_2 = \left(m_{\text{sample}} \times \text{TOC}\right)/12 \times 44 \qquad (2)$$

where TOC is organically bound carbon in the tested material (Organic carbon in percentage - **Table 2**), m_{sample} is the weight of the tested sample (mg), 12 is the molecular weight of carbon and 44 is the molecular weight of carbon dioxide.

The second biodegradability measure was the relation of measured biological oxygen demand (corrected by a blank) versus theoretical oxygen demand, according to the following **equation (3)**:

$$\% \text{ Biodegradation } = \left[\left(\left(\text{BOD}_{\text{sample}} - \text{BOD}_{\text{blank}}\right) / \text{c}\right) / \text{ThOD}\right] \times 100 \qquad (3)$$

where BOD_{blank} and BOD_{sample} are experimentally found values of biological oxygen demand of the sample and blank, respectively (all in mg L^{-1}), ThOD is theoretical oxygen demand (in mg g^{-1}) and c is the concentration of the sample in the tested suspension (in g L^{-1}).

2.2.4. Acceleration factor

For a given material the acceleration factor (AF) was calculated as a ratio between the maximal mineralization rates at the two temperatures ($AF = k_{37}$ °C/k_{25} °c) according to the procedure described previously **[20]**.

2.3. Microscopy observation

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

For the fluorescent microscopy polymer samples were stained with Live/dead™ BacLight™ Bacterial Viability Kit according to the manufacturer instructions and observed with Olympus BX53F microscope equipped with a digital camera.

2.4. Microbial community analysis

At Lab2 the samples for the DNA analysis were taken after 1, 2, 3, 6 and 12 months of incubation (number before the letter "M" in the sample code). In the beginning, when it was possible, DNA was isolated from the surface of the material, later, when it was no longer possible to find the actual material, the medium was sampled (p or s, respectively, just after the material abbreviation in the sample code). DNA was isolated with DNeasy PowerSoil DNA extraction kit (Qiagen USA) and used for amplifying specific regions of fungi ITS2 (18S) and bacteria V3-V5 (16S) rRNA genes using primers F357 (5'-CCTACGGGAGGCAGCAG-3') and R926 (5'-CCGYCAATTYMTTTRAGTTT-3'), or ITS3F (5'-GCATC-GATGAAGAACGCAGC-3') and ITS4R (5'- TCCTCCGCTTATTGATATGC-3'), respectively, with barcodes and the universal overhang. Illumina sequencing adaptors were introduced in the second PCR, all in accordance with the Illumina instructions **[34]**. Products were evaluated by agarose electrophoresis, quantified with fluorimetric high sensitivity Acugreen kit (Bioline) and pooled into a library. The sequencing library was sequenced on MiSeq (Illumina) using v2 version of chemistry and 250 nt paired end reads settings in the external laboratory (SEQme s.r.o., Czech Republic). The data were further processed with DADA2 R package **[35]** and further visualized with phyloseq R package **[36]** and ComplexHeatmap **[37]** R packages. Taxonomy assigned for bacteria using SILVA 132 SSU NR 99 reference database **[38]**, and 8.3 release of UNITE reference database for fungi.

3. Results

3.1. Characterization of the tested materials

The materials that have been used in the current study are identical to those used in the previous study **[20]** namely, three commercially available polyesters and a non-commercial ICL-PN polyester network. The three commercial polyesters (PHB, PBS, and PBAT/PLA) are linear semi-crystalline polymers with a melting point well above 37 °C which are certified as biodegradable in soil and PLA is certified as biodegradable under industrial composting conditions. In contrast, ICL-PN polyester network is an amorphous aliphatic crosslinked polyester with a glass transition (Tg) at — 9 °C synthetized by our partner in an attempt to develop a material with slow biodegradation in soil. The general properties of the materials are listed in **Table 2**. All four polyesters have glass transition temperatures well below room temperature and since none of the materials has a melting point between 25 and 37 °C the materials do not change their internal morphology in this interval.

3.2. Biodegradation in freshwater environment at 25 °C and 37 °C

The absolute and relative biodegradation percentages of the reference and test materials are summarized in Table S1.

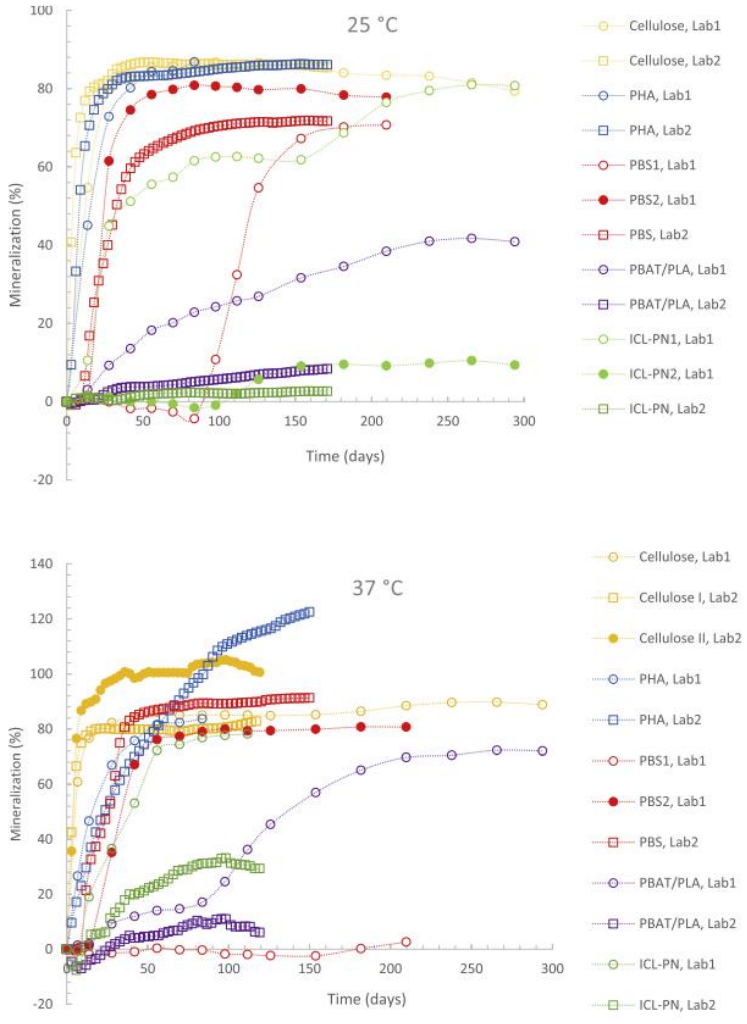

Fig.1. Biodegradation of the tested polymeric materials at two temperatures and two laboratories in freshwater test.

Fig. 1 shows the evolution of the average absolute mineralization at 25 °C and 37 °C, with the exception of materials that exhibited a high level of variation in biodegradation percentage or biodegradation course. For these materials the individual replicates are shown. According to the internationalstandards ISO 14851 (2019) and ISO 14852 (2021) a test is considered valid when the degree of biodegradation of the reference material is more than 60% at the end of the test. This requirement was easily fulfilled in the five tests, indicating sufficient general activity of the sludge inoculum. In Lab1 a biodegradation percentage of 79.4% (25 °C) and 88.8% (37 °C) was measured for cellulose after 294 days. The downwards biodegradation trend at 25 °C from 182 days onwards was caused by an increased activity in one of the blank vessels, causing a negative net signal and thus a decreasing biodegradation. In Lab2 a plateau in mineralization was reached for the reference material at 85.4% after 171 days (25 °C), at 82.8% after 117 days (37 °C, Cellulose I) and at 100.6% after 120 days (37 °C, Cellulose II).

The biodegradation rate and behavior of PHA was fast and similar tc the reference item (cellulose) at 25 °C. At 37 °C a slightly slower initial degradation was observed, but again high levels of mineralization were reached. The final percentages varied between 86.8% (Lab1) and 86.1% (Lab2) at 25 °C and 83.7% (Lab1) and 122.6% (Lab2) at 37 °C. According to the dissolved organic carbon value (DOC) determined at the end of the experiment the latter mentioned biodegradation percentage above 100% could be explained by the aerobic digestion of activated sludge used as an inoculum **[39]**. Carbon dioxide produced comes from two parallel processes, the direct oxidation of the tested sample, and the mineralization of the biomass present in the activated sludge inoculum **[40]**. With an easily biodegradable substrate as PHA, the accumulation of the active metabolizing cells can lead to the enhancement of the latter process after the exhaustion of the tested sample. The occurrence of this biomass mineralization is evident from the increase of DOC in PHA incubations **[39]** up to 101.9 mg L^{-1} at the end of the incubation, in comparison with 76.1 mg L^{-1} DOC in the cellulose incubation (the inorganic carbon was lower than 0.5 mg L^{-1}). In Lab2 all incubations where also followed by the oxygen consumption measurements (BOD). PHA (S1, Supplementary material) showed “two-step” biodegradation, the first stage corresponding to the mineralization of the tested sample and the second stage supposedly to that of the carbon from the biomass The real versus sample based theoretical oxygen consumption reached 124% for PHA and 100% for cellulose thus confirming the results from CO_2 measurements.

The biodegradation of PBS proceeded well throughout the experiment, with the exception of one replicate at 37 °C (Lab1). A fasl mineralization followed by a plateau was observed at both temperatures in Lab2, resulting in a biodegradation of 71.7% at 25 °C and 91.3% at 37 °C. In Lab1 the biodegradation behavior of PBS was more irregular. At 25 °C both replicates reached a high level of mineralization (74.2%), but one replicate started to degrade after two weeks, while the other replicate displayed a lag phase of 84 days. At 37 °C one replicate degraded until a percentage of 81.2%, while the other replicate showed no significant biodegradation (2.7%). Despite the latter PBS replicate, a good overall reproducibility was observed between the laboratories for the more easily degradable materials (cellulose, PHA and PBS).

The mineralization of test items PBAT/PLA and ICL-PN differed more strongly between the laboratories, both in rates and levels of biodegradation. In Lab1, PBAT/PLA degraded at a slow rate during the first 3 months until a biodegradation of approximately 25% was reached at both temperatures. Subsequently, an acceleration in biodegradation was observed at mesophilic temperature (37 °C), while at ambient temperature (25 °C) a slow but steady rate was maintained. After 294 days a biodegradation of 40.9% and 72.0% was measured, respectively, at 25 °C and 37 °C. 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 Lab2 PBAT/ PLA degraded considerably slower, reaching only 8.4% mineralization at 25 °C and 6.0% at 37 ° C at the end of the experiment.

The degradation of ICL-PN varied from limited to partial to substantial. At ambient temperature (25 °C) limited biodegradation was observed, with the exception of one replicate in Lab1. This replicate reached a plateau in biodegradation at 80.8%. The other replicate showed only 9.3% mineralization and in Lab2 an average biodegradation of 2.6% was reached. At 37 °C ICL-PN degraded at a good rate in Lab1, while a moderate rate was maintained in Lab2. At the end of the mesophilic experiment a biodegradation percentage of 78.2% (Lab1) and 29.4% (Lab2) was measured. In our opinion, ICL-PN has the potential to fully degrade, but specific conditions and/or micro-organisms may be needed to achieve high levels of biodegradation.

3.3. Kinetics of biodegradation at 25°C and 37°C

The S-shaped logistic curve characteristic for many natural processes was used previously to fit the biodegradation curves in soil **[20].** In the case of the freshwater experiments the same model function was used but the fitting was more difficult because the mineralization curves were often less regular and, in some cases, profound differences were observed between the replicate incubations of the same sample. In the latter mentioned cases, data from only one of the replicates (the one with faster mineralization) was evaluated instead of the average data used normally. Maximal reaction rates were estimated and the acceleration factors, expressing the possibility to accelerate the biodegradation testing in the freshwater environment by increasing the temperature, were calculated for the individual materials and laboratories and listed in **Table 3**.

Resulting acceleration factors reflected higher variability of the data and with the exception of cellulose tended to oscillate around the value of 1 and thus did not conclusively indicate an acceleration of the process with temperature. In some cases, the resulting AF was even lower than 1. The high value of ICL-PN AF (Lab2) was a product of the extremely low mineralization rate for this material at 25 °C.

3.4. Microscopic observation of the polymer samples

SEM **(Fig. 2)** was used to observe biodeterioration of the materials during the experiment and eventual formation of biofilm on their surface (Lab2). PHA and PBS films disintegrated during the first months, consequently, only PBAT/PLA and ICL-PN could be retrieved and observed. ICL-PN exhibited profound and gradual deterioration while in the case of PBAT/PLA the changes were evident but less substantial. On the other hand, a microbial biofilm was not observed and only individual cells sparsely distributed could be seen. To further investigate the possible microbial activity on the surface of the samples, fluorescence microscopy and Live/Dead staining was employed. In these technique the active metabolizing cells are stained green while the death cells should appear red **(Fig. 3)**.

In agreement with SEM the microbial activity and the number of cells seen was rather low, but on some pictures, it was discernible that some microorganisms were indeed present, and that the microbial activity was concentrated into cavities formed in the material during its biodeterioration and well visible in SEM pictures.

Table 3 Maximal mineralization rates and acceleration factors

Material	Lab1 k_{MAX}, 25°C $days^{-1}$	k_{MAX}, 37°C $days^{-1}$	AF	Lab2 k_{MAX}, 25°C $days^{-1}$	k_{MAX}, 37°C $days^{-1}$	AF
Cellulose	4.51	10.4	2.30	9.37	24.6	2.62
PHA	3.73	3.62	0.97	6.00	2.81	0.46
PBS	2.03	1.88	0.93	1.36	2.77	2.03
PBAT/PLA	0.28	0.45	1.60	nd	nd	nd
ICL-PN	1.22	1.43	1.17	0.04	0.56	14.0

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

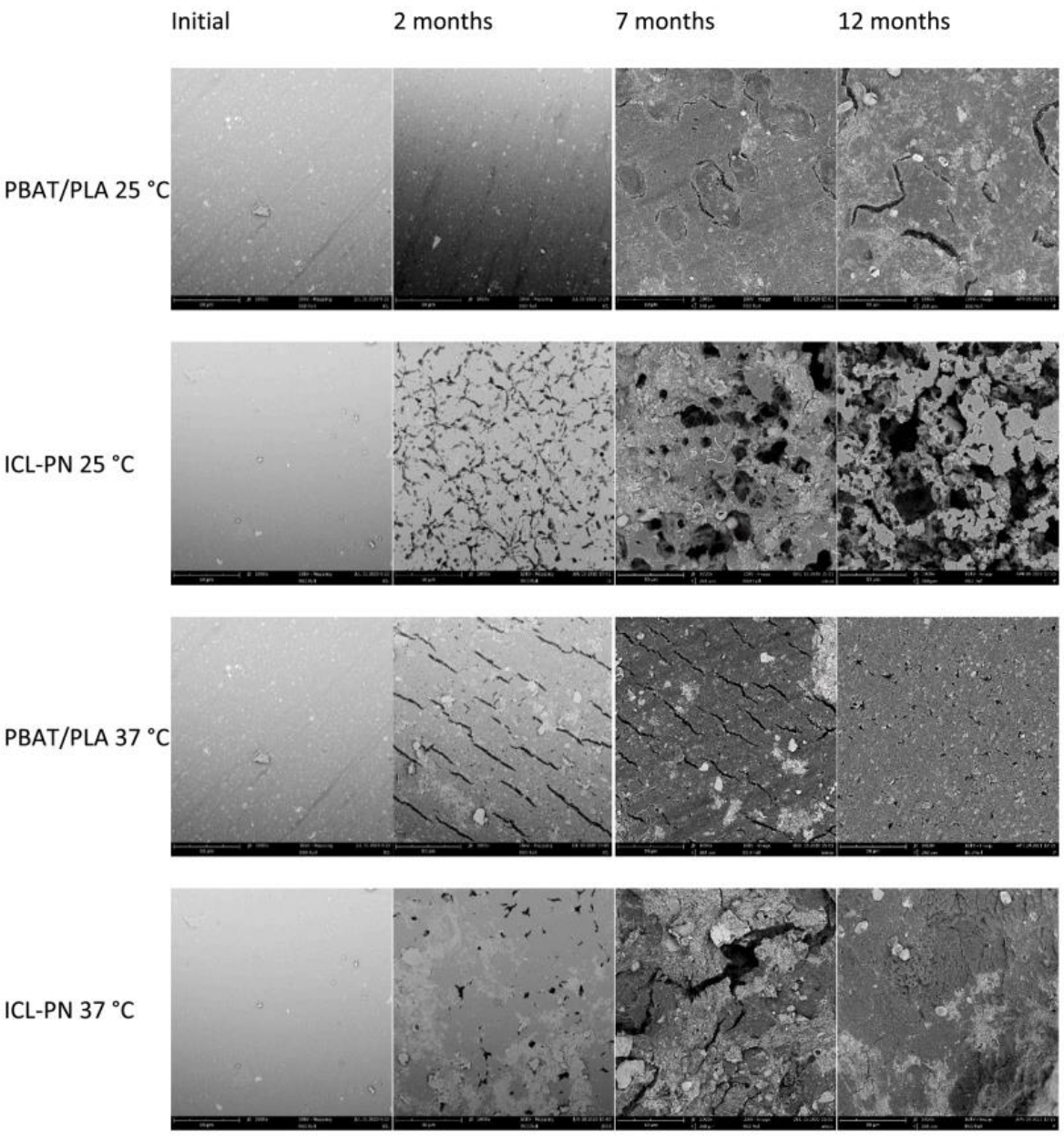

Fig. 2. Scanning electron microscopy of the selected polymers samples during their biodegradation (Lab2).

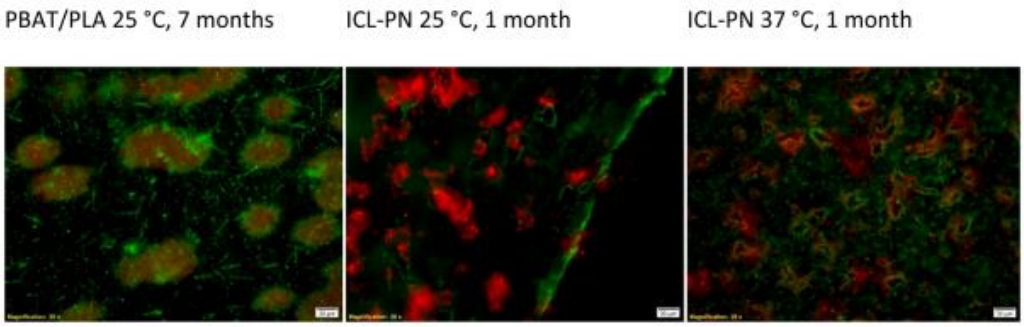

Fig. 3. Fluorescent microscopy of the selected polymer samples (Lab2). Live/Dead staining.

3.5. Microbial community analysis during the biodegradation

Universal bacterial and fungal primers were used to investigate microbial communities with help of 16S and 18S rDNA sequencing. In general, at 25 °C the microbial community (MC) at Class level was (Fig. S2, Supplementary material) characterized by high abundance of Gammaproteobacteria (e.g., Genera Curvibacter, Limno2bacter, Undibacterium), with the important presence of Bacteroidia (Genus Prevotella) and Alphaproteobacteria (Genus Bradyrhizobium). All these organisms are rather common in the municipal wastewater sludge environment **[41, 42]**. In the incubation with polymer materials, the development of other genera could be seen after the first month of incubation, namely Acti-nobacteria (Actynomyces, Rothia) as important degraders of synthetic polymers **[43]**, Bacilli (Streptococcus sp.), Fusobacteria (Fusobacter sp.), Negativicutes (Veillonella sp.), and an increase in Bacteroidia presence (Prevotella sp.). This is even more evident on the Order level **(Fig. 4)**. This shift thus could be influenced by the polymer biodegradation and solid surface colonization, especially in the case of Actinobacteria. However, the feature disappeared after the second month regardless of the further presence (e.g., PBAT/PLA) or almost complete mineralization (e.g., PHA) of the particular polymer and from this time point the similar shift can also be observed in the blank incubation suggesting that MC transformation is independent of the polymer presence and polymer related processes.

For the 37 °C incubation (Fig. S3 Supplementary material,5) the general dominance of Gammaproteobacteria, Alphaproteobacteria and Bacteroidia had copied the situation at 25 °C. However, the influence of the polymer materials and their biodegradation even in the first months was more difficult to see at both Class and Order **Fig. 5** levels. The MC shift appears to be quasi-identical in the blank and incubations with the materials.

The MCs were also compared with the help of principal component analysis. Several ordering methods were tested and nonparametric DCA method was found as the most capable to distribute individual samples (S5, supplementary materials). It is evident that the two temperatures were not systematically separated which proves that MCs at both temperatures are similar.

PCA comparison further emphasize the idea that especially at 37 °C the MCs were more influenced by the evolution and ageing of the initial sludge inoculum and less by the material biodegradation. This could be the consequence of the well mixed water environment where the MC components are readily transported and homogenized and where the organic carbon of the material tested is not the dominant fraction of the total organic carbon present. In the Lab2 setup there was approximately 1:1 ratio between carbon from the sample and from the inoculum.

In Lab1, working with the identical materials, incubation media were sampled and processed at the very end of the experiments only, but independent parallel samples were taken from two independent incubations allowing to see eventual variability in MC development. Lab2 samples from the end of the experiments (12 months) were incorporated in the picture for a comparison. At 25 ° C MCs were again dominated by Gammaproteobacteria, Bacteroidia and Alphaproteobacteria **(Fig. 6)**. The difference from the Lab2 could be seen in the higher presence of Fus-sobacteria or Spirochaetia at least in some samples. There is a satisfactory similarity between the Lab1 parallel samples, except for ICL-PN, where some degree of difference is apparent. This could eventually help to explain the observed differences in the mineralization of this sample between the parallel incubations.

The picture is more complex at 37 °C (Fig. S6, Supplementary material) with important variability between some parallel samples which could probably suggest lower long-term stability of such systems at the increased temperature and explain discrepancies in the observed mineralization rates.

Principal component analysis shows proximity of samples with similar MCs (Fig. S7, S8 Supplementary material). The two laboratories are separated along the horizontal axis which suggest a difference between MCs which probably resulted from the different sources of inocula. Clearly also, MCs of parallel incubation of ICL-PN at 25 °C that behaved very differently (Lab1) are well separated. On the other hand, parallel PBS samples at 37 °C showing also very distinct mineralization patterns (Lab1) are grouped very closely together proving similar MCs seen also in Fig. S6 and thus, overall MC differences cannot serve as an explanation of this result.

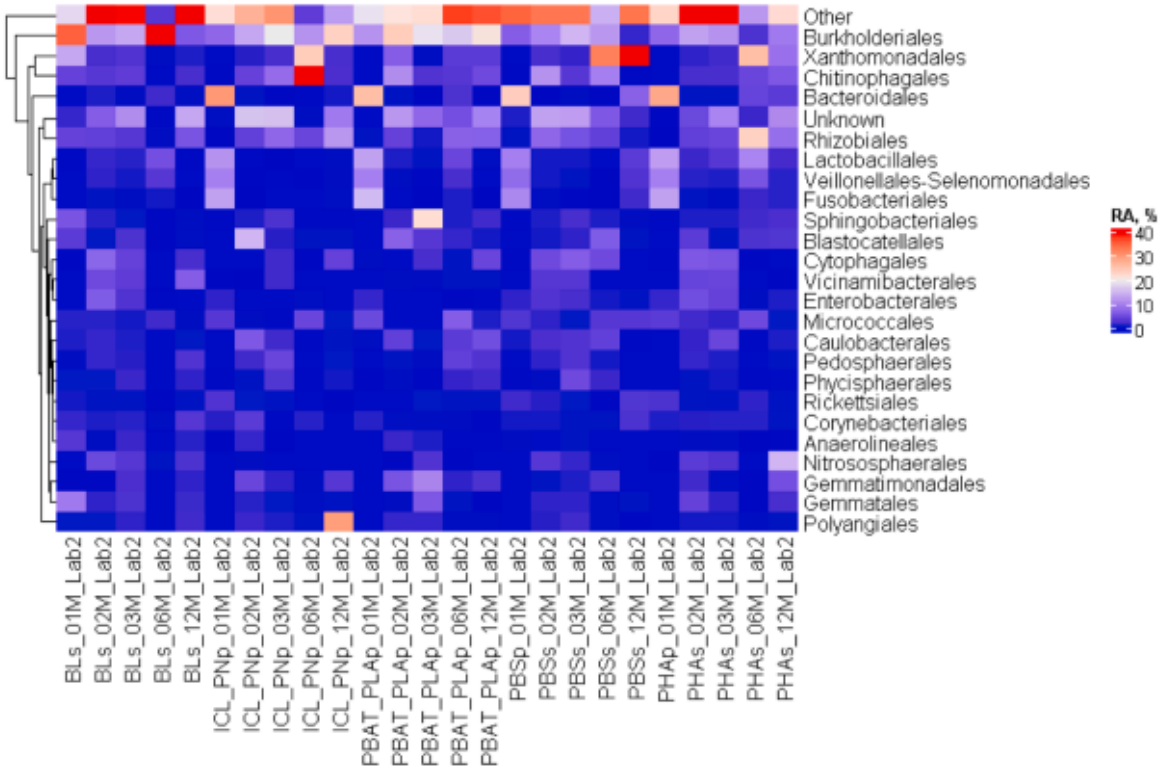

Fig. 4. Bacterial communities during polymer biodegradation at 25 °C represented as heatmap at Order level (Lab2). RA, relative abundance.

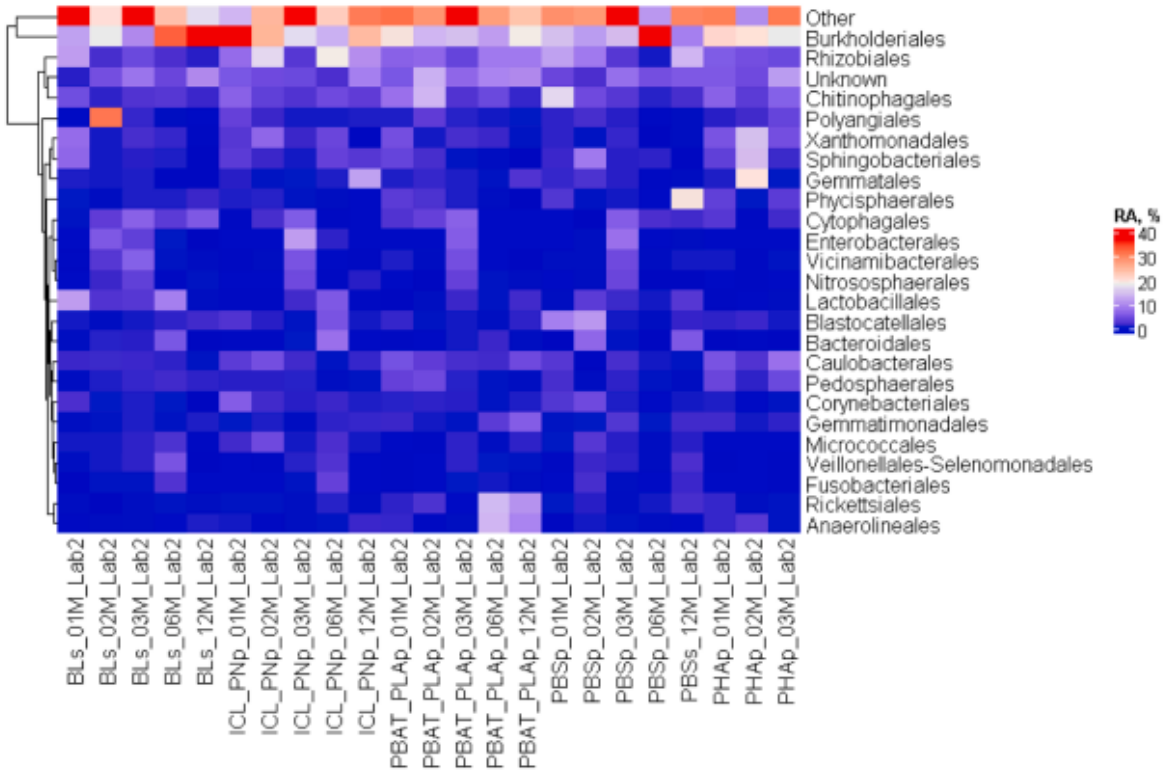

Fig. 5. Bacterial communities during polymer biodegradation at 37 °C represented as heatmap at Order level (Lab2). RA, relative abundance.

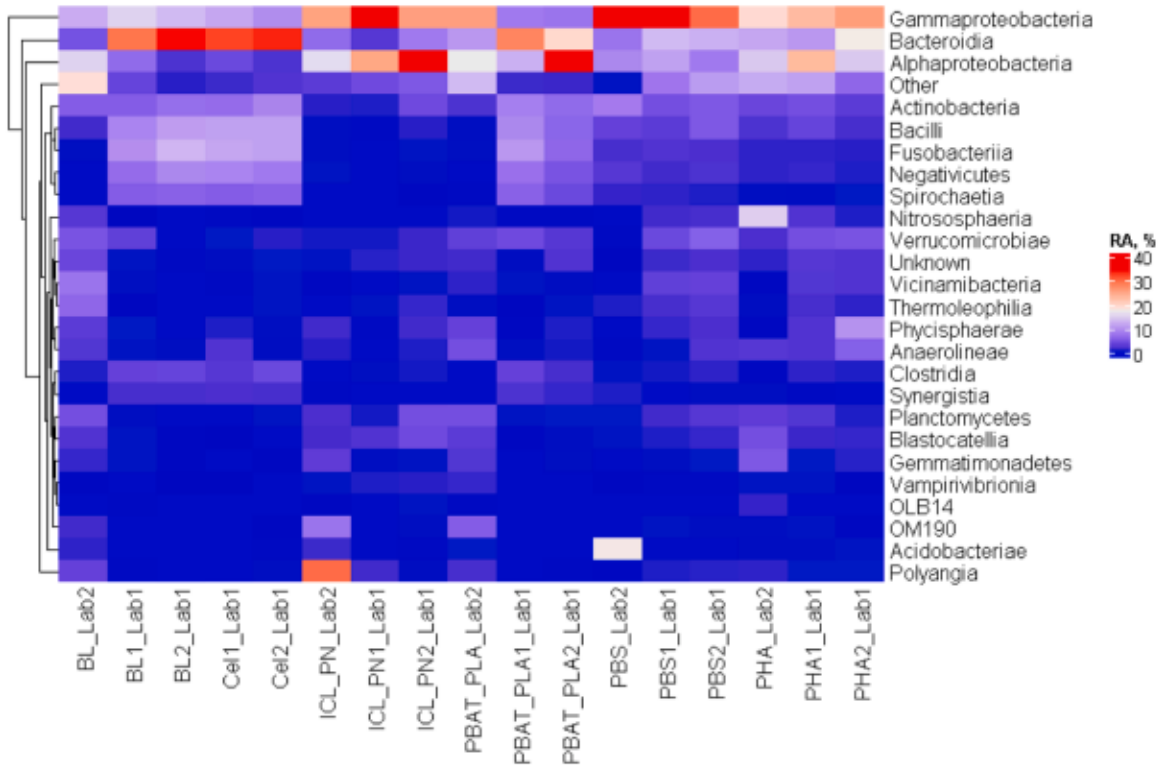

Fig. 6. Bacterial communities at the end of the biodegradation experiment at 25°C represented as heatmap at Class level (Lab1 and Lab2).

Fungi MC normally is only marginally developed in the wastewater sludge environment, which was well reflected by the low or even zero number of the retrieved fungi sequences at the beginning of the incubation **(Fig. 7)**. However, during the course of the experiment an apparent development of the fungi community was evident both from the number of retrieved sequences and from the alpha diversity of the fungi MC (**Fig. 7** and S9, supplementary materials). The tendency was very similar at both temperatures and visible in blank incubations as well as in the incubations with polymers. This provides additional evidence of the "ageing" of MCs during the course of the test which could complicate and distort eventual long term biodegradation experiments made in similar setups.

4. Discussion

The goal of the current study in freshwater environment was to examine the feasibility of a prolonged freshwater biodegradation test for insoluble polymer materials. As in the previous study on soil biodegradation **[20]**, an accelerated version of the test that would enable to judge biodegradability in a shorter time frame by increasing the temperature of the test from 25 °C to 37 °C was also investigated. In soil, the AF varied from about 1.5 to about 4, which led to the conclusion that an accelerated test at 37 °C could be used to evaluate the ultimate biodegradation potential under conditions that were representative for ambient temperature conditions (provided that there is no phase transition in the polymer sample between 25 °C and 37 °C). The only process that could eventually continue is the cold crystallization. However, we believe that further cold crystallization is unlikely as the materials were stored sufficiently long before the start of the experiments (more than one year) at room temperature. Also, in our previous study **[20]** with the identical materials a potential effect of the cold crystallization was not observed or did not seem to have an impact on biodegradation. There, a very good agreement between the laboratories and rather regular acceleration at the higher temperature was achieved.

In freshwater, hardly any acceleration was observed when the test temperature was increased to 37 °C. AF oscillated around 1 for most materials, with the exception of cellulose which showed an AF of 2.5. The biodegradation data show that the basic test set-up of ISO 14851 (2019) and ISO 14852 (2021) can support prolonged testing, beyond the typical test period of 2 to 6 months at 25 °C, but adjustments should be made to make the methodology for testing of slowly degradable polymers more robust. Some recommendations learned during the study and suggestions for future improvements are given below:

Sludge inoculum quantity and quality. Lab2 encountered more problems with MC aging and biomass degradation than Lab1 which could be connected to a higher load of inoculum biomass at the start of the incubation. Sampling inoculum from multiple and diverse locations, as done in Lab1, could also be helpful to maximize its biodiversity (e.g., ASTM D5988 for soil biodegradation applies a similar approach). Please note, that even though the bulks of MCs from the two laboratories and probably from other wastewater treatment plants seem similar, some minority organisms can be important for the biodegradation of the particular polymer. There were clear differences between MCs in the two Labs, which in general could complicate reproducibility of such test between laboratories.

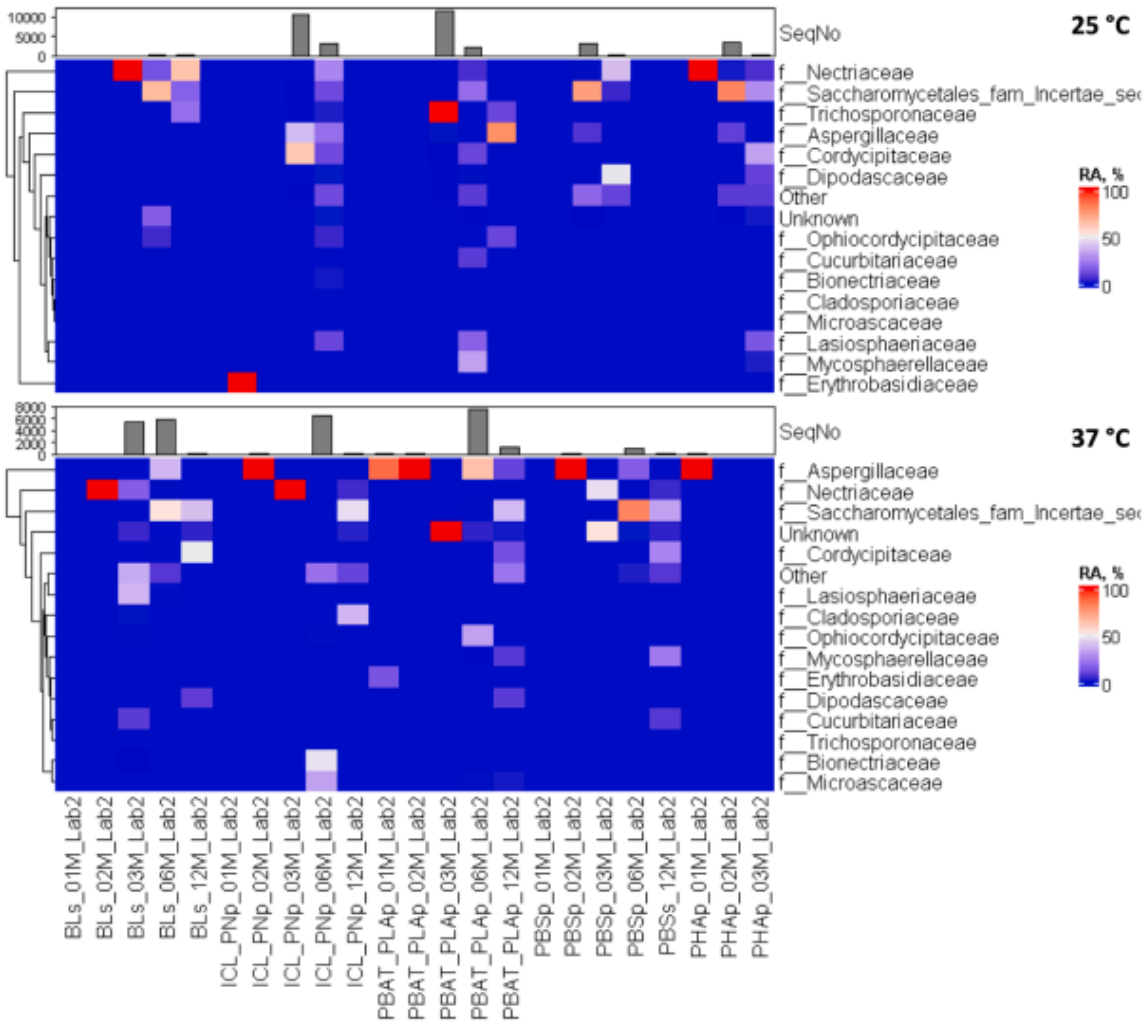

Fig. 7. Fungi communities during polymer biodegradation at 25 °C and 37 °C represented as heatmaps at Family level (Lab2). SeqNo, number of retrieved fungi sequences.

Reinoculation. As it is seen from the development of MCs in Lab2 incubations, most pronounced changes in MCs that could be assigned to the biodegradation of polymer materials could be distinguished after the first month which went parallel with the most active polymer mineralization. Periodic re-inoculation (for example every 2-3 months) with small amounts of fresh activated sludge may prolong the inoculum activity and counteract the ageing of MCs.

Pre-exposed inoculum. Pre-exposed inocula can be obtained from laboratory biodegradation tests or from samples collected from locations where relevant environmental conditions exist (e.g., contaminated areas or industrial wastewater treatment plants). Research on the biodegradation of cellulose acetate has shown that the addition of just 4% of preexposed test medium from a former biodegradation test can significantly shorter the lag phase and reduce the variation between the replicates **[44]**. Here, if applied the approach could eliminate the differences between replicates (e.g., PBS and ICL-PN Lab1 at 25 °C, PBS Lab1 at 37 °C) or even between laboratories. Such practice does not need to be regarded as artificial because it also takes place in nature, where over time the most active strains are selected and adapt to a given substrate. At the moment, this option is not allowed by the

EChA Annex XV Restriction report on intentionally added microplastics, while the use of pre-exposed inoculum is accepted for OK biodegradable WATER certification at TUV AUSTRIA Belgium.

Incubation temperature. The study rather clearly showed that the lower temperature (25 °C) is more suitable for long-term freshwater tests. At 37 °C the acceleration of the mineralization appeared to be insignificant, and the tests were complicated by fungi development, inoculum autodigestion (Lab2), and higher variability between incubation replicates.

The abovementioned recommendations are intended to reduce the variability between parallel incubations within a test and between laboratories. Lab 1 (OWS nv) and Lab 2 (UTB) have more than twenty years of experience in the field of biodegradability testing and use well-validated test set-ups. In the previous study on soil **[20]** the results showed good agreement between the two independent laboratories, yet in freshwater this seems to be less straightforward. It was shown that the test can be prolonged beyond 6 months, but the experienced difficulties make it unreasonable to extend the aqueous biodegradation test in the actual settings up to 24 or 48 months (i.e., 4 to 8 times the currently allowed maximum duration) and thus make it difficult to prove quantitative aqueous biodegradation of slowly biodegradable substances introduced into soil, as described in the EU fertilizer regulation 2019/1009. We think that the investigated freshwater biodegradation test is not well suited for slowly biodegradable polymeric materials used in e.g., agricultural applications.

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