



Microbial communities responsible for the degradation of poly(lactic acid)/poly(3-hydroxybutyrate) blend mulches in soil burial respirometric tests

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Abstract

The microbial communities responsible for the degradation of poly(lactic acid)/poly(3-hydroxybutyrate) (PLA/PHB) blend foils were investigated in 1 year long laboratory soil burial experiments. Different PLA/PHB foils were tested: (a) PLA/PHB original transparent foil, (b) PLA/PHB carbon black filled foil and (c) PLA/PHB black foil previously exposed for 90 days to sun light. The microbiome diversity of these three types of foil was compared with that identified from soil/perlite sample at the beginning of experiment and that developed on a cellulose mat. Culture-dependent and culture-independent (DGGE-cloning) approaches together with PLA, PHB and PLA/PHB degradation plate assays were employed. The cultivation strategy combined with degradation tests permitted the isolation and evaluation of several PLA/PHB blend degrading microorganisms such as members of the genera *Bacillus*, *Paenibacillus*, *Streptomyces*, *Rhodococcus*, *Saccharothrix*, *Arthrobacter*, *Aureobasidium*, *Mortierella*, *Absidia*, *Actinomucor*, *Bjerkandera*, *Fusarium*, *Trichoderma* and *Penicillium*. The DGGE-cloning investigation increased the information about the microbial communities occurring during bioplastic degradation detecting several bacterial and fungal taxa and some of them (members of the orders *Anaerolineales*, *Selenomonadales*, *Thelephorales* and of the genera *Pseudogymnoascus* and *Pseudeurotium*) were revealed here for the first time. This survey showed the microbiome colonizing PLA/PHB blend foils and permitted the isolation of several microorganisms able to degrade the tested polymeric blends.

Keywords Poly(lactic acid)/poly(3-hydroxybutyrate) blend · Microbiome · Bacteria · Fungi · Polymer degradation plate assays

Introduction

The biopolymers for the production of mulching foils are considered, with respect to their ecologically friendly properties, as a valid alternative to other kind of plastic polymers,

as for example those derived from fossil fuels (Rudnik and Briassoulis 2011; Brodhagen et al. 2015; Dharmalingam et al. 2015; Muthuraj et al. 2017).

Two different families of biopolymers are represented by polylactic acid (PLA) and polyhydroxyalkanoates (PHAs). PLA is a thermoplastic produced from natural resources which combines biocompatibility, biodegradability and an excellent processability (Corneillie and Smet 2015). However, PLA also has some disadvantages as for example high crystallinity and brittleness, and slow biodegradability in soil (Shah et al. 2008; Corneillie and Smet 2015).

PHAs are a group of biopolyesters produced by several kinds of microorganisms as intracellular inclusions. There are different types of PHAs, the simplest and generally the most frequently studied PHA is poly(3-hydroxybutyrate) (PHB). The disadvantages of the PHB include brittleness,

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slow crystallization, poor thermal stability and poor melt processability (Mozejko-Ciesielska and Kiewisz 2016).

In order to improve the mechanical properties of mulching foils is a normal practice to prepare blends composed by different types of polymers. One solution is the production of blends combining PLA and PHB. In this way also the biodegradation characteristics of the new mulching foil can be modified and improved (Brodhagen et al. 2015).

Previously, many studies were aimed at elucidating the biodegradation properties of several PLA/PHB blends. The experimentation included respirometric test (Abdelwahab et al. 2012), composting conditions (Arrieta et al. 2016) and soil environment (Weng et al. 2013). Although, all these investigations produced important data regarding the changes of the properties of blends after a period of incubation in soil buried conditions, they didn't indicate which kind of microorganisms are the main actors during the degradation of PLA/PHB blends.

In literature, several studies identified the cultivable soil microbial community responsible for bioplastic degradation. They were oriented either to PLA (Karamanlioglu et al. 2014) or PHB polymer (Mergaert et al. 1993; Boyandin et al. 2013; Volova et al. 2017).

To our knowledge the microbial communities that contribute to the degradation of PLA/PHB blends in soil were not studied until now and also no culture-independent strategies were applied to have a better view of such microbial populations. The aim of this study was the investigation of bacterial and fungal assemblages, through culture-dependent and culture-independent approaches, responsible for the degradation of different kinds of PLA/PHB mulching foils (T8—PLA/PHB foil white; T12—PLA/PHB foil black; T21—PLA/PHB foil black exposed 90 days to sunlight) before and after 1 year of incubation in respirometric reactors.

Materials and methods

Polymeric foils

Commercial blend PLA/PHB Nonoil® pellets were provided by Panara s.r.o. (Nitra, Slovakia; Nonoil contained PLA, PHB polymers and citrate ester as plasticizer). Delivered pellets were converted into transparent film and 1 wt% containing carbon black (CB, N220, supplied by Corax®) composite film using film blowing technology as is described in details by Mosnáčková et al. (2017). The samples named as cellulose (T6) for cellulose foil (used as control), PLA/PHB_w (T8) for original transparent foil, PLA/PHB_b (T12) for original carbon black filled foil and PLA/PHB_b 90D sun (T21) for outdoor weathering foil exposed 90 days to sun light.

Soil samples and isolation of microorganisms

Soil was collected in an agricultural region at South Slovakia near Danube River. The pH of the soil was determined according to the standard STN ISO 10390 and was 7.5. Its moisture hold capacity (MHC) represents the moisture in soil that keeps inside against the gravity and was established on 0.85. Expanded Perlite EP 200 for agricultural purposes has grain diameter 0.5/4 mm and was purchased from LB Minerals, a.s. (Košice, Slovakia).

The sieved agriculture soil was mixed with perlite in equivalent amounts (SSP; totally 200 g). This soil mix was using in order to bury polymeric foils in several respirometric reactors as schematically showed in Fig. 1.

10 g (wet weight) portion of this soil mixture (SSP), at the beginning of experimentation were mixed in sterile 250 ml Erlenmeyer flasks with 90 ml of a 0.9% (w/v) NaCl solution and incubated at room temperature 24–26 °C in a shaker at 90 rpm for 2 h. The obtained suspension were filtered through Whatman 1 filter paper (Merck, Darmstadt, Germany), and these filtered soil suspension were inoculated directly onto different agar media for the isolation of bacteria and fungi.

After 1 year of incubation, in respirometric reactors, the polymeric foils (T6, T8, T12 and T21) were taken off and immersed in 3 ml of physiological solution in 50 ml Erlenmeyer flasks. In this way the soil and dust attached to foils were spread in the solution. Each suspension was serially diluted and inoculated in specific agar plate for the growth of bacteria and fungi.

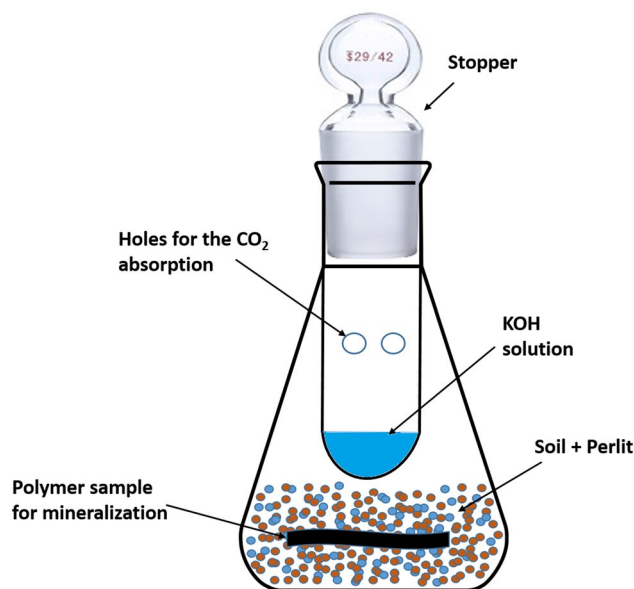


Fig. 1 Respirometric reactor used for polymers mineralization tests

For the isolation of bacteria SM1 agar (Tan et al. 2006), Bennett's Agar (BA; Himedia; Mumbai, India), Actinomycete Isolation Agar (AIA; Himedia) and LB10 agar (peptone 1 g l⁻¹, yeast extract 0.5 g l⁻¹, NaCl 1 g l⁻¹, agar 15 g l⁻¹; Grivalský et al. 2016) were used. The fungi were isolated on Potato Dextrose Agar (PDA; Himedia) and Sabouraud agar (SAB; Himedia). Both bacteria and fungi were also isolated on Filtered Soil Suspension agar (FSS) which was prepared using 1000 g (wet weight) of the same agriculture soil mixed with 2 l of a 50 mM NaOH solution and incubated overnight at laboratory temperature. Then, this mixture was filtered through Whatman 1 filter paper (Merck) and centrifuged 60 min at 18,000 rpm. The pH value of filtered soil suspension supernatant was adjusted to 7–7.5. For the preparation of FSS medium 1000 ml of filtered soil suspension and 1.5 g of agar were mixed and boiled for three times at 100 °C during 24 h.

In order to inhibit the growth of fungi and bacteria some media were supplemented either with 80 mg l⁻¹ cycloheximide (LB10 and FSS for bacteria) or with 100 mg l⁻¹ chloramphenicol (PDA, SAB and FSS for fungi), respectively.

The pure bacterial colonies were maintained on LB10 or on AIA plates, while the fungi on SAB slants.

Mineralization test under laboratory controlled conditions

Mineralization test was carried out according to standards ASTM D5988-12 and STN 17556-2012 in soil under aerobic conditions in the reactor schematically showed in Fig. 1. Organic carbon content in tested PLA/PHB foil determined by elemental analysis was 52.15%. Polymer foil with size 7 cm × 7 cm with thickness 0.1 mm (weight approximately 0.45 g) was buried in mixed perlite and soil equivalent amount (totally 200 g) in 500 ml Erlenmeyer flask. Flask was closed by perforated glass tube fulfilled with 20 ml of KOH 0.5 M solution and equipped by rubber stopcock. CO₂ produced by mineralization was absorbed in the KOH solution. Samples of the trap solution were opened periodically at least every 10 days for 1 h to tend free respiration of microorganisms in the soil and to establish the amount of absorbed CO₂. 1 ml of KOH solution from the trap was titrated with 0.05 M HCl with phenolphthalein as indicator. For new period the new 20 ml of KOH was added to the trap. All samples were processed by triplicate. Flasks with only perlite and soil were used as control and empty flasks were used as blank, respectively, to measure production of the CO₂ by the microorganisms itself and to measure CO₂ present in the air, respectively.

DNA extraction of isolated microorganisms, clustering and identification

Bacterial chromosomal DNA was isolated using the InstaGene Matrix (Biorad, Hercules, CA, USA) following the instructions of the manufacturer. The isolated strains were clustered by ITS PCR. The internal transcribed spacer (ITS) between 16S and 23S rRNA genes was amplified and the produced amplicons were separated by Qiaxcel electrophoresis (Qiagen, Hilden, Germany) according to Bučková et al. (2018). Bacterial representatives of each ITS cluster were identified by the sequencing of their 16S rRNA genes using the primers 27F and 685R (Puškárová et al. 2016). The final PCR mixture (25 µl) consisted 50 pmol of each primer, 200 µmol l⁻¹ of dNTP, 1.5 U BioTherm Taq DNA polymerase (Genecraft, Köln, Germany), 1× PCR buffer, and 6 µl of template. The PCR program consisted of initial denaturation at 95 °C for 1 min, 30 cycles (95 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min 30 s) and a final polymerization step at 72 °C for 8 min.

The purified fungal isolates were selected on the basis of the different morphology and polymer degradation properties ("PLA/PHB blend, PHB and PLA plate degradation assays" section). For DNA extraction from fungal isolates, the isolates were inoculated in SAB broth at 28 °C until growth; they were then separated from the broth by filtration through sterile filter paper, followed by DNA extraction with Ron's fungal DNA mini kit (Bioron, Ludwigshafen, Germany), according to the instructions of the manufacturer. The ITS region of rDNA was amplified with the primers ITS1 and ITS4 (Sclocchi et al. 2017). The 25 µl PCR mixture contained 50 pmol of each primer, 200 µmol l⁻¹ of dNTP, 1.5 U HotStar Taq plus DNA polymerase (Qiagen), 1× PCR buffer and 3 µl of the extracted DNA. The PCR program consisted of an initial denaturation at 94 °C for 5 min, followed by 30 cycles (denaturation at 94 °C for 30 s, annealing at 54 °C for 45 s, extension at 72 °C for 1 min) and a final polymerization step at 72 °C for 10 min.

The resulting PCR products from both fungal and bacterial isolates were purified using ExoSAP-IT (Affymetrix, Cleveland, Ohio, USA) and sequenced at a commercial facility (GATC-Biotech, Konstanz, Germany). The resulting sequences were directly compared with those in GenBank using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and were subsequently deposited in GenBank under the accession numbers MH155488–MH155548 (bacterial isolates) and MH161229–MH161273 (fungal isolates).

Direct DNA extraction from soil and polymer foil samples

One and half milliliters of soil suspension used to inoculate the agar plates (SSP) and also of the polymer foil

suspensions of the respirometric reactors T6, T8, T12, T21 were used to extract the DNA by the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, USA), according to the protocol of the manufacturer and repeatedly eluted to a final volume of $2 \times 30 \mu\text{l}$ of TE buffer.

Culture-independent analysis: denaturing gradient gel electrophoresis and clone library

First PCR amplification

Bacterial 16S rDNA and eukaryotic ITS fragment were amplified by two steps, a portion of the PCR product of the first step being used for the construction of clone libraries, and another portion in the second amplification step, a semi-nested PCR facilitating fingerprint analysis based on denaturing gradient gel electrophoresis (DGGE).

The first step involved PCR with primers 27F and 685R (Lane 1991) oriented to 16S rRNA gene. For amplification of ITS region of yeasts and fungi, primers ITS1 and ITS4 were used (White et al. 1990). The PCR mixture (25 μl) contained 50 pmol of each primer, 200 $\mu\text{mol l}^{-1}$ of dNTP, 1.5 U SuperHot-*Taq* DNA polymerase (Bioron) and $1 \times$ PCR buffer. 3 μl of extracted DNA were used as a template in the first amplification. The temperature programme consisted of initial denaturation at 94 °C for 5 min, 30 cycles (94 °C for 30 s, 54 °C for 45 s, 72 °C for 1 min) and a final polymerization step at 72 °C for 10 min. For each DNA target (16S rDNA and ITS), four reactions of 25 μl (100 μl altogether) were produced. The four reactions of each DNA target were mixed together and the specificity of amplification was checked by agarose gel electrophoresis.

Semi-nested PCR and DGGE fingerprinting

The PCR product of the first step (2 μl) was used as a template in the second amplification, a semi-nested PCR for each DNA target. The 16S rDNA was re-amplified with primers 518f and 685R-GC (Puškárová et al. 2016). Primers ITS1f-GC and ITS2 were used for the semi-nested amplification of ITS fragment (Pangallo et al. 2014; Puškárová et al. 2016). The PCR conditions were the same as above. Four semi-nested PCR products (four reactions) for each DNA target were pooled, checked by electrophoresis in agarose gel, and precipitated with 96% ethanol, resuspended in 20 μl H₂O and the precipitate (10 μl) was analysed by DGGE in 8% polyacrylamide gel (acryl amide-bisacrylamide 37.5:1) with the denaturation gradient of 25–55% for separation of 16S rDNA amplicons and 20–50% for separation of ITS amplicons (100% denaturant contained 7 mol l⁻¹ urea and 40% (v/v) formamide). DGGE was run on DCode System (Bio-Rad) in $0.5 \times$ TAE (20 mmol l⁻¹ Tris, 10 mmol l⁻¹

acetate, 0.5 mmol l⁻¹ Na₂ EDTA; pH 8.0) at 200 V and 60 °C for 3 h for bacteria, or for 5 h for fungi.

Construction of clone libraries and sequencing

The rest of the PCR products from the first amplifications were used for the construction of bacterial 16S rDNA and eukaryotic ITS clone libraries. Briefly, the PCR products were purified by QIAquick PCR purification kit (Qiagen), ligated to pGEM-T Easy vector (Promega, Madison, Wisconsin, USA), transformed to *E. coli* XLI-Blue, and spread to LB plates with ampicillin (100 $\mu\text{g ml}^{-1}$), X-Gal (0.1 mmol l⁻¹) and IPTG (0.2 mmol l⁻¹). A number of about 120 white colonies from each clone library was checked by vector-specific PCR with primers SP6 (5'-ATT TAG GTG ACA CTA TAG AAT AC-3') and T7 (5'-TAA TAC GAC TCA CTA TAG GG-3').

One hundred positive clones of each library were analysed by DGGE at conditions described above, using bacterial primers 518f and 685R-GC, or ITS primers ITS1f-GC and ITS2. Profiles of individual clones were compared with each other and with the profile of the whole community. Clones with different profiles were sequenced using primers SP6 and T7. The obtained sequences were compared with those present in the GenBank database (nucleotide collection database and, in the case of sequences identified as uncultured bacterium or fungus, the 16S ribosomal RNA sequences database or the exclusion of the uncultured/environmental sample sequences, respectively) by BLAST search (National Center for Biotechnology Information, Bethesda, Maryland, USA). The sequences were deposited in GenBank under accession numbers MH155549–MH155592 (bacterial clones) and MH161274–MH161325 (eukaryotic clones).

PLA/PHB blend, PHB and PLA plate degradation assays

In order to assess the degradation abilities of isolated strains for biodegradable polymers three different media containing respectively PLA/PHB blend, PHB and PLA (provided by Panara s.r.o. and Polymer Institute, SAS) were prepared. One gram of PLA/PHB, PHB or PLA granules were dissolved in 30 ml of dichloromethane. To each solution were added 150 ml of a basal medium composed of 1 g l⁻¹ KH₂PO₄, 1 g l⁻¹ (NH₄)₂SO₄, 0.2 g l⁻¹ MgSO₄·7H₂O, 0.01 g l⁻¹ FeCl₃, 0.05 g l⁻¹ NaCl, 0.05 g l⁻¹ CaCl₂, 0.25 g l⁻¹ Yeast extract, 0.1 g l⁻¹ Triton X and 15 g l⁻¹ of agar according to Grivalský et al. (2017). Then, media were stirred at 70 °C until dichloromethane was evaporated, which takes approximately 20 h. The media were sterilized by autoclaving and poured on Petri dishes. The assays were performed for each isolate in triplicate and the positive reaction was displayed as a zone of clearance around the assayed microbial colonies.

Results

Mineralization of PLA/PHB (CB) blends before and after outdoor weathering

Cellulose mat (T6) was used as reference material in order to check the microbial activity of the soil. It can be seen in Fig. 2 that cellulose sample started to mineralize almost immediately, it grew linearly and reached approximately 70% of mineralization in a year. In mineralization tests hydrolytic degradation of the PLA/PHB foils can not be excluded, but it can be assumed that measured evolved CO₂ can be produced exclusively by microorganisms, which use this polymer for feeding. In this context, initial pre-degradation by sunlight could affect velocity of followed mineralization. According to Fig. 2 all polymer samples undergo mineralization faster compared to control soil sample without polymer foil, suggesting that polymer samples increase microbial activity. However, this difference was small and mineralization was slow for all investigated polymers including cellulose.

PLA/PHB_w (T8) polymer sample exhibited slightly higher mineralization compared to PLA/PHB_b (T12) polymer composite. The mineralization seemed to be slightly faster for the sunlight exposed sample PLA/PHB_b 90D (T21), but generally for all of the samples the difference could be considered to be within the experimental error (Fig. 2).

Surprisingly to this promising results, after withdrawal samples lose only approximately 8–12% of its original weight (Fig. 3). Only cellulose sample exhibit approximately 50% weight loss. This is in contradiction to the mineralization tests, which showed five times overestimation. While

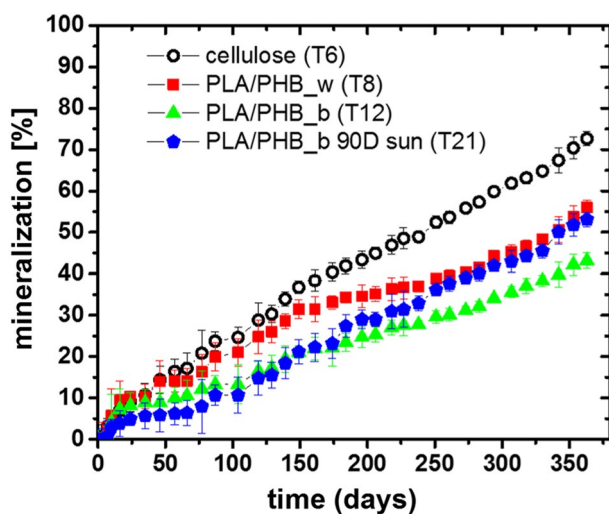


Fig. 2 Degree of mineralization of PLA/PHB mulching foils in controlled laboratory experiment in soil and perlite at 20 °C

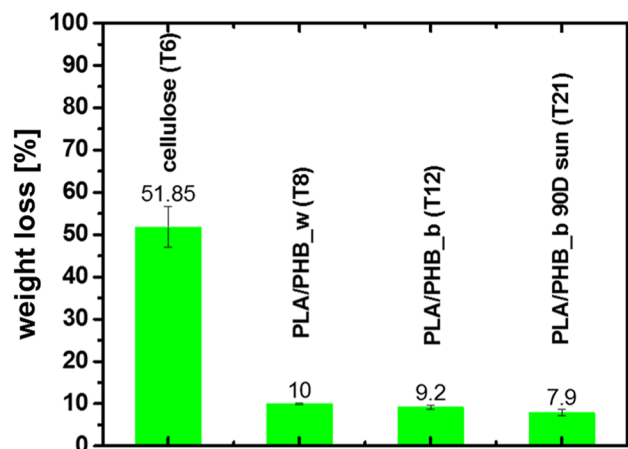


Fig. 3 Mineralization of the PLA/PHB foils evaluated according to weight changes before and after 1 year of mineralization in soil and perlite at 20 °C

disintegration of polymer foils was expected at such degree of mineralization, all withdrawal foils were still self-holding. Only some of the samples exhibited visible cracks, which are usually a cause of loss of mechanical properties such as decreasing of elongation at break and increasing of brittleness of polymer foils. These cracks were most visible for PLA/PHB_b 90D (T21; Fig. 4).

Cultivation analysis

Bacteria belonging to three phyla (*Firmicutes*, *Actinobacteria* and *Proteobacteria*) were isolated from soil sample (SSP) at the beginning of the mineralization process and from the cellulose foil (T6) after mineralization test. Members of only *Firmicutes* and *Actinobacteria* were recovered from the different PLA/PHB foils (T8, T12 and T21) after 1 year of incubation in the respirometric reactors. The most isolated bacteria were the members of the genus *Bacillus*. They were present already in the SSP sample and repeatedly isolated from all polymeric foils (Fig. 5; Table S1).

The bacterial community isolated from transparent foil (PLA/PHB_w; T8) was dominated mainly by *Bacillales* and *Streptomycetales* members. Bacteria recovered from PLA/PHB black foils were represented also by different kinds of *Actinobacteria*: *Streptomyces*, *Rhodococcus* and *Arthrobacter* (Fig. 5; Table S1). For the isolation several types of culture media were used (SM1, BA, AIA, FSS and LB10), but in our case the best isolation media were represented by LB10, FSS and AIA. We were not able to isolate any microorganisms from polymeric samples using BA and SM1 media. In addition the specificity of SM1, BA and AIA for the isolation of *Actinobacteria* was partially respected, in fact only by SM1 medium this group of bacteria was exclusively isolated. In the other media (AIA and BA) also

Fig. 4 Different appearance of PLA/PHB black foils exposed for 90 days on sunlight after 1 year of mineralization

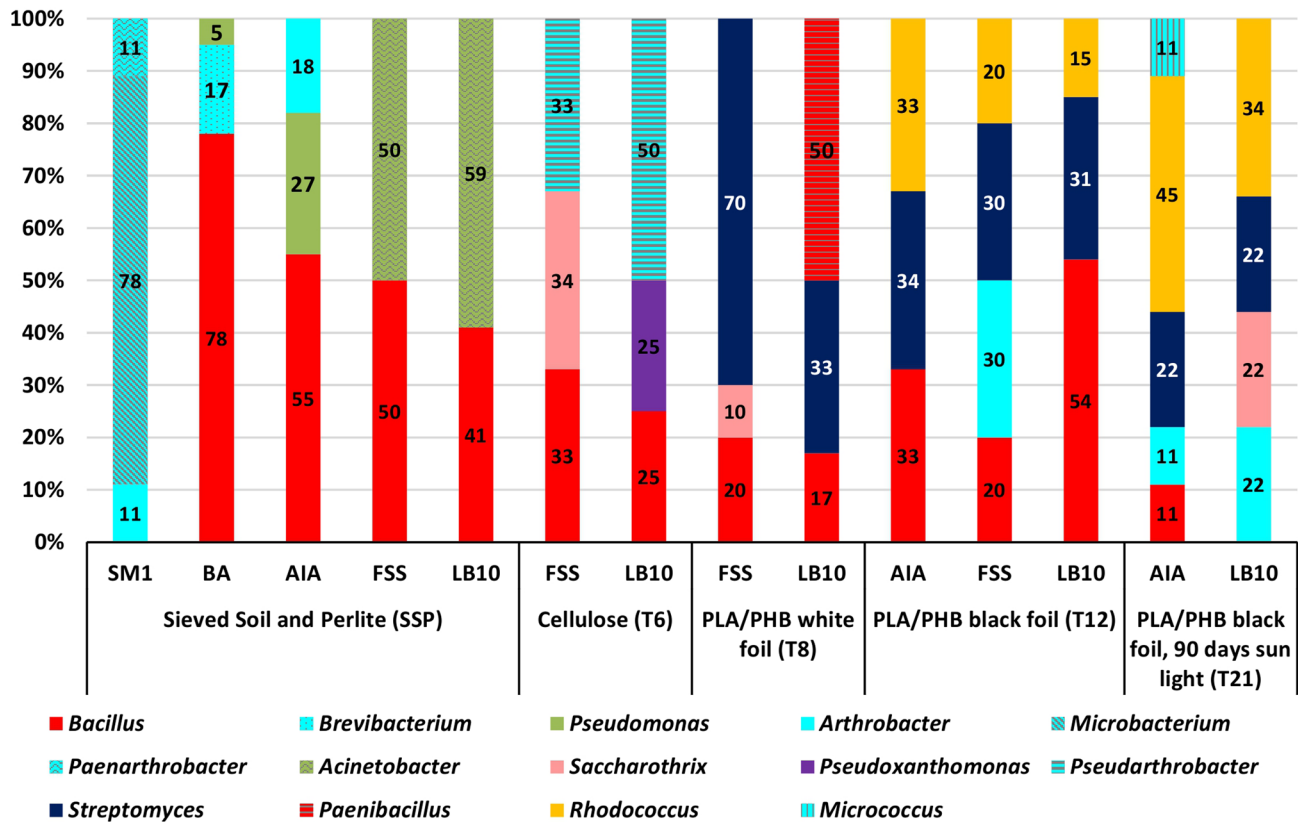
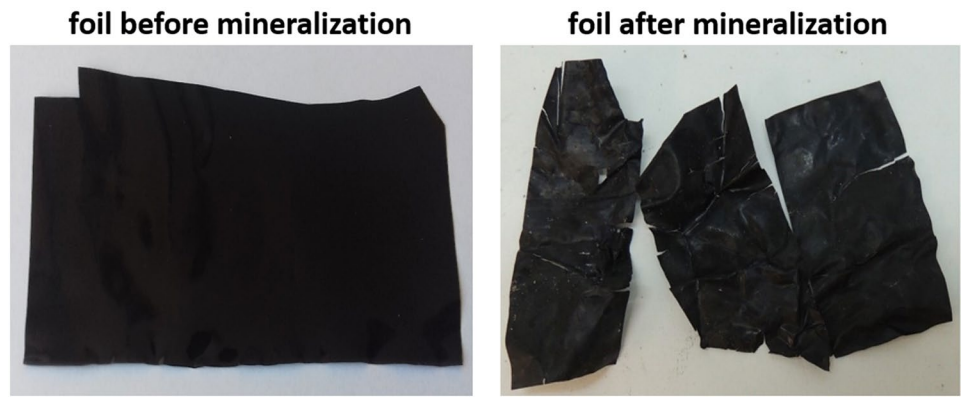


Fig. 5 Bacterial isolates occurring in soil at the beginning of experiment and on different polymeric foils after 1 year of incubation in respirometric reactors

members of other bacterial groups, mainly *Bacillus*, grew very well.

The fungal community isolated from soil (SSP) was composed by few more taxa (9 genera) compared to the bacterial community (7 genera). Members of the genera *Phialophora*, *Cladosporium* and *Trichoderma* were the prevalent fungi isolated from soil. *Penicillium* spp. were isolated on all the three agar media used.

The fungal analysis after 1 year of incubation in the respirometric reactors showed different changes in the

composition of community depending on polymeric substrate. In fact, from the cellulose mat were isolated only *Fusarium* and *Penicillium* (Fig. 6; Table S2). The *Fusarium* were isolated from all PLA/PHB foils together with members of *Mucorales* (*Absidia* and *Actinomucor*). The largest fungal diversity, constituted by *Fusarium*, *Penicillium*, *Aspergillus*, *Actinomucor*, *Bjerkandera* and *Mortierella*, was found in the PLA/PHB foil previously exposed to sun light for 90 days (Fig. 6; Table S2).

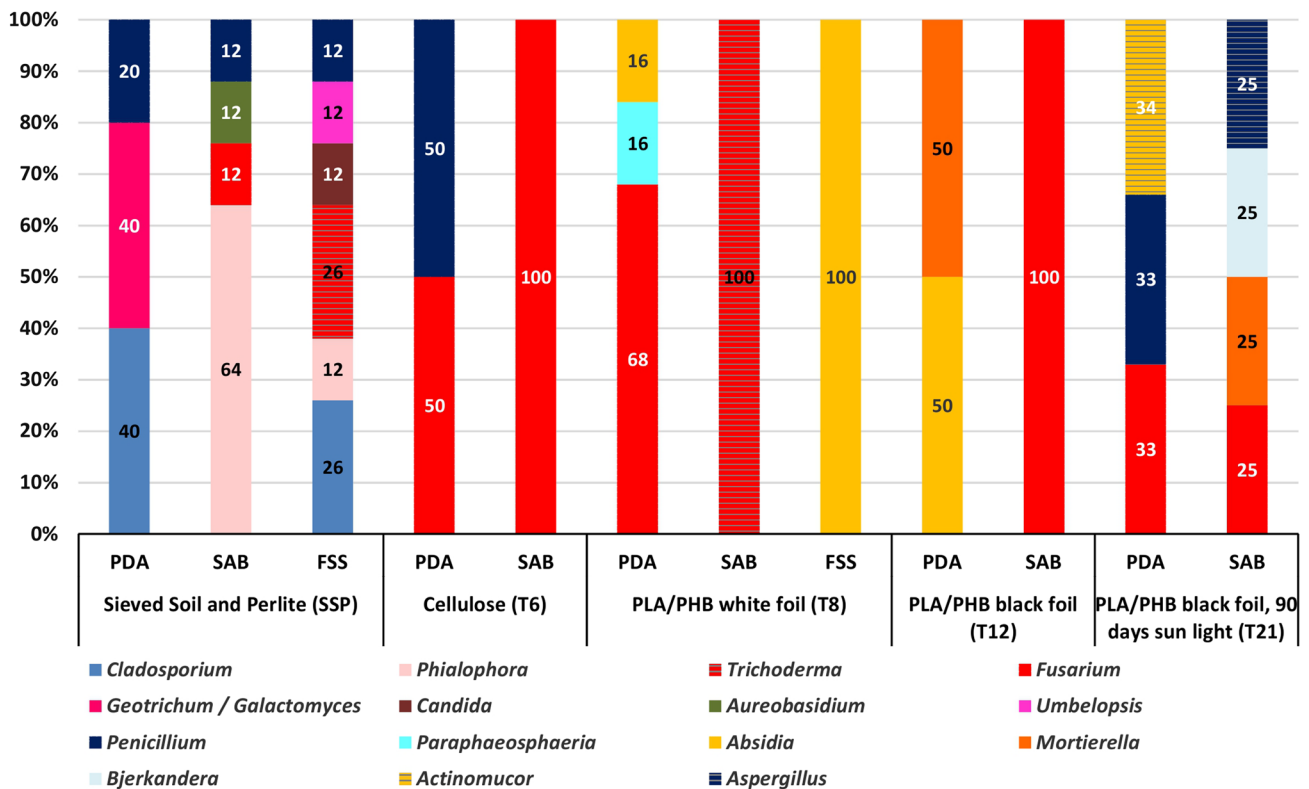


Fig. 6 Fungal isolates occurring in soil at the beginning of experiment and on different polymeric foils after 1 year of incubation in respirometric reactors

Polymers degradation abilities of isolated strains

The hydrolytic agar media assays were performed in order to evaluate the degradation power of bacterial and fungal isolates.

The 68% of bacteria recovered from soil were able to degrade at least one of the tested polymeric substrates (PHB, PLA/PHB blend and PLA). This percentage increased when the bacteria from polymeric foils were assayed: 86% from cellulose mat, 94% from film T8 and 79% from T12. The bacteria from sample T21 showed at least one degradation property (Table S1).

Several isolates of the genus *Bacillus* demonstrated to be valuable polymers degraders. They were isolated mainly from soil. Among bacteria isolated from the different polymeric foils the best degradation abilities were exhibited by *Actinobacteria* of the genus *Streptomyces*, *Rhodococcus*, *Saccharothrix* and the species *Arthrobacter oryzae* (Table 1; Table S1). Many bacterial isolates displayed very rapid and extensive positive reaction for each assayed substrate (Table 1).

Each selected and identified fungal isolate expressed at least positive reaction for two of the tested substrates. Numerous fungal isolates displayed high PLA/PHB blend degradation ability often associated with a relevant

hydrolytic activity for PHB and PLA. The best fungal degraders encompassed *Cladosporium subcinereum*, *Trichoderma harzianum*, *Aureobasidium pullulans*, *Absidia* sp., *Penicillium* sp., *Bjerkandera adusta*, *Actinomucor elegans*, *Aspergillus fumigatus* and several members of the genera *Fusarium* and *Mortierella* (Table 1; Table S2).

Culture-independent analysis

The DGGE-cloning approach permitted a more complete analysis of the bacterial and fungal microbiomes in soil and associated to polymeric foils.

Twenty-seven bacterial orders were revealed, the biggest number of taxa were detected from soil sample, with 12 orders comprising 14 genera, followed by the cellulose mat, with 10 orders including 13 genera. The number of taxa detected from PLA/PHB foils was very similar (Fig. 7; Table S3).

The operational taxonomic units (OTUs) of the order *Acidobacteria* (subdivision 6) were the most spread, indeed they were detected in each analyzed samples (Fig. 7). The second most diffuse OTUs belonged to the orders *Cytophagales* (absent only in the T8 sample) and *Anaerolineales* (not detected in soil; Fig. 7). The OTUs of the orders *Gemmatimonadales* and *Burkholderiales* occurred in soil, on

Table 1 The most effective bacterial and fungal PLA/PHB blend degraders based on polymer agar assays

Sample	Strain	PHB	PLA/PHB blend	PLA
SSP—sieved agriculture soil mixed with perlite	SSP BA 1 <i>Bacillus</i> sp.	+	+++	+
	SSP BA2 <i>Bacillus</i> sp.	–	+++	–
	SSP BA3 <i>Bacillus</i> sp.	+++	+++	+++
	SSP BA13 <i>Bacillus subtilis</i>	++++	+++	+++
	SSP BA14 <i>Bacillus subtilis</i>	++++	+++	+++
	SSP BA17; SSP BA18 <i>Bacillus subtilis</i>	–	+++	–
	SSP AIA3 <i>Bacillus subtilis</i>	+++	+++	+
	SSP FSS5; SSP FSS6; SSP FSS7; SSP FSS8; SSP FSS9 <i>Bacillus subtilis</i>	+++	+++	+++
	SSP LB10-5 <i>Bacillus subtilis</i>	++++	++++	+++
	SSP AIA4 <i>Bacillus pseudomycooides</i>	++	+++	+
	SSP AIA1 <i>Pseudomonas</i> sp.	–	+++	–
	SSP LB10-15 <i>Acinetobacter ursingii</i>	–	+++	–
	SSP LB10-17 <i>Acinetobacter ursingii</i>	–	+++	+++
	SSP FSS15-f <i>Cladosporium subcinereum</i>	+++	+++	+++
	SSP FSS11B-f <i>Trichoderma harzianum</i>	+++	+++	+++
	SSP SAB12-f <i>Aureobasidium pullulans</i>	+++	+++	+++
	T6—cellulose mat	T6 FSS29 <i>Bacillus idriensis</i>	+++	++++
T6 PDA2-f <i>Penicillium</i> sp.		++	+++	+
T6 PDA3-f <i>Fusarium oxysporum</i>		++	++++	++
T6 PDA4-f <i>Fusarium</i> sp.		+	++++	+
T6 SAB1-f <i>Fusarium solani</i>		++	++++	+++
T6 SAB3-f <i>Fusarium</i> sp.		++++	++++	+
T8—(PLA/PHB_w) original PLA/PHB transparent foil	T8 FSS77 <i>Bacillus</i> sp.	++	++++	+
	T8 LB10-50 <i>Paenibacillus</i> sp.	++++	++++	++++
	T8 LB10-49 <i>Streptomyces</i> sp.	–	+++	+
	T8 FSS38 <i>Streptomyces albobinaceus</i>	++	+++	–
	T8 FSS40 <i>Streptomyces cyaneogriseus</i>	+++	++++	++++
	T8 PDA2-f <i>Fusarium</i> sp.	++	+++	+
	T8 PDA4-f <i>Fusarium oxysporum</i>	++	+++	++
	T8 PDA5-f <i>Fusarium oxysporum</i>	+++	+++	++
	T8 PDA6-f <i>Fusarium oxysporum</i>	++	+++	+
	T8 SAB1-f <i>Trichoderma harzianum</i>	–	++++	++++
	T8 PDA8-f <i>Absidia</i> sp.	+++	++++	++
T12—(PLA/PHB_b) original carbon black PLA/PHB filled foil	T12 LB10-60 <i>Bacillus megaterium</i>	+++	+++	++++
	T12 LB10-65 <i>Bacillus licheniformis</i>	++	+++	++++
	T12 LB10-67 <i>Streptomyces ciscaucasicus</i>	++	+++	+++
	T12 PDA3-f <i>Mortierella lignicola</i>	++++	+++	–
	T12 SAB1-f <i>Fusarium equiseti</i>	++++	++++	+++
T21—(PLA/PHB_b 90D sun) carbon black PLA/PHB foil exposed for 90 days to sun light	T21 AIA10; T21 AIA11 <i>Rhodococcus</i> sp.	++++	++++	++++
	T21 AIA12; T21 LB10-54 <i>Rhodococcus</i> sp.	++++	+++	++++
	T21 LB10-60 <i>Rhodococcus</i> sp.	–	++++	+
	T21 LB10-53 <i>Saccharothrix</i> sp.	–	+++	–
	T21 AIA56 <i>Streptomyces lateritius</i>	++++	++++	+++
	T21 LB10-70 <i>Streptomyces lateritius</i>	–	++++	+
	T21 AIA59; T21 LB10-71 <i>Arthrobacter oryzae</i>	+++	++++	++
	T21 SAB1-f <i>Bjerkandera adusta</i>	+++	++++	+++
	T21 SAB2-f <i>Mortierella</i> sp.	++	++++	+++
	T21 PDA1-f <i>Actinomucor elegans</i>	+++	+++	++++
	T21 SAB3-f <i>Aspergillus fumigatus</i>	+	+++	–
	T21 SAB5-f <i>Fusarium solani</i>	++	++++	++
	T21 PDA5-f <i>Fusarium equiseti</i>	++++	+++	+++

++++: very rapid and extensive positive reaction; +++: quick and extensive positive reaction; ++: good positive reaction; +: weak positive reaction; –: negative reaction

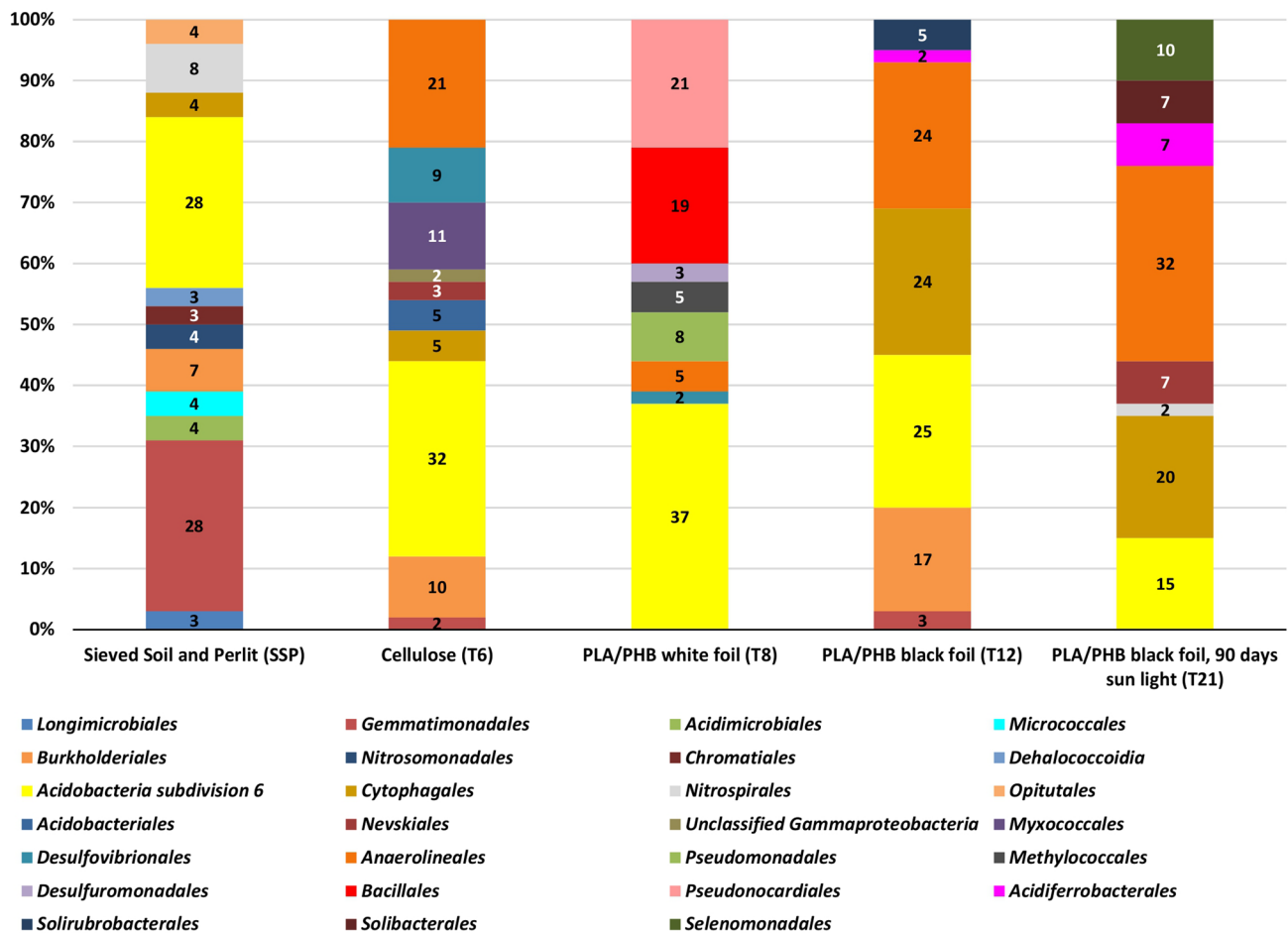


Fig. 7 Bacterial microbiome detected in soil and on polymeric foils by DGGE-cloning strategy

cellulose mat and PLA/PHB black foil (T12). The two PLA/PHB black foils (T12 and T21) displayed quite similar bacterial microbiomes. By contrast, the bacterial community of PLA/PHB transparent foil (T8) was different and composed by characteristic OTUs belonging to the orders *Pseudonocardiales*, *Bacillales*, *Pseudomonadales*, *Methylococcales* and *Desulfuromonadales*, that were not detected on the black foils (Fig. 7, Table S3).

Twelve different fungal orders together with four orders (*Ulvales*, *Chlamydomonadales*, *Sporodotrichida* and *Stichotrichida*) belonging to other eukaryotic organisms were identified by DGGE-cloning strategy. The highest number of fungal genera (9) of 8 different orders were identified in soil samples. From the polymeric samples the number of detected taxa sharply decreased, in fact only four orders were identified for each of investigated foils (Fig. 8; Table S4).

The eukaryotic community of cellulose mat was composed mostly by fungi, the other eukaryotic organisms representing only the 2%. The most detected OTUs belonged to the orders *Agaricales* (44%) and *Leotiomyces incertae sedis* (37%). The latter order was common to all analyzed samples (Fig. 8).

The OTUs of *Pleosporales* order were the second most diffused, although they reached lower percentages with respect to *Leotiomyces*. They were detected in all samples except the PLA/PHB transparent foil (T8). The foil T8 showed a different fungal microbiome in comparison with the black foil samples. OTUs of the order *Hypocreales* were identified only here (Fig. 8).

The two black foils (T12 and T21) showed very similar fungal microbiomes composed by the orders *Pleosporales*, *Mortierellales* and *Leotiomyces*. The *Leotiomyces* community of the sample T12 exhibited also OTUs belonging to the genus *Pseudogymnoascus*, which was not detected on foil T21 (Table S4). The differences between the fungal microbiomes of the PLA/PHB black foils regarded also the orders *Pezizales* (2%) and *Thelephorales* (37%) identified on foil T12 and T21, respectively (Fig. 8).

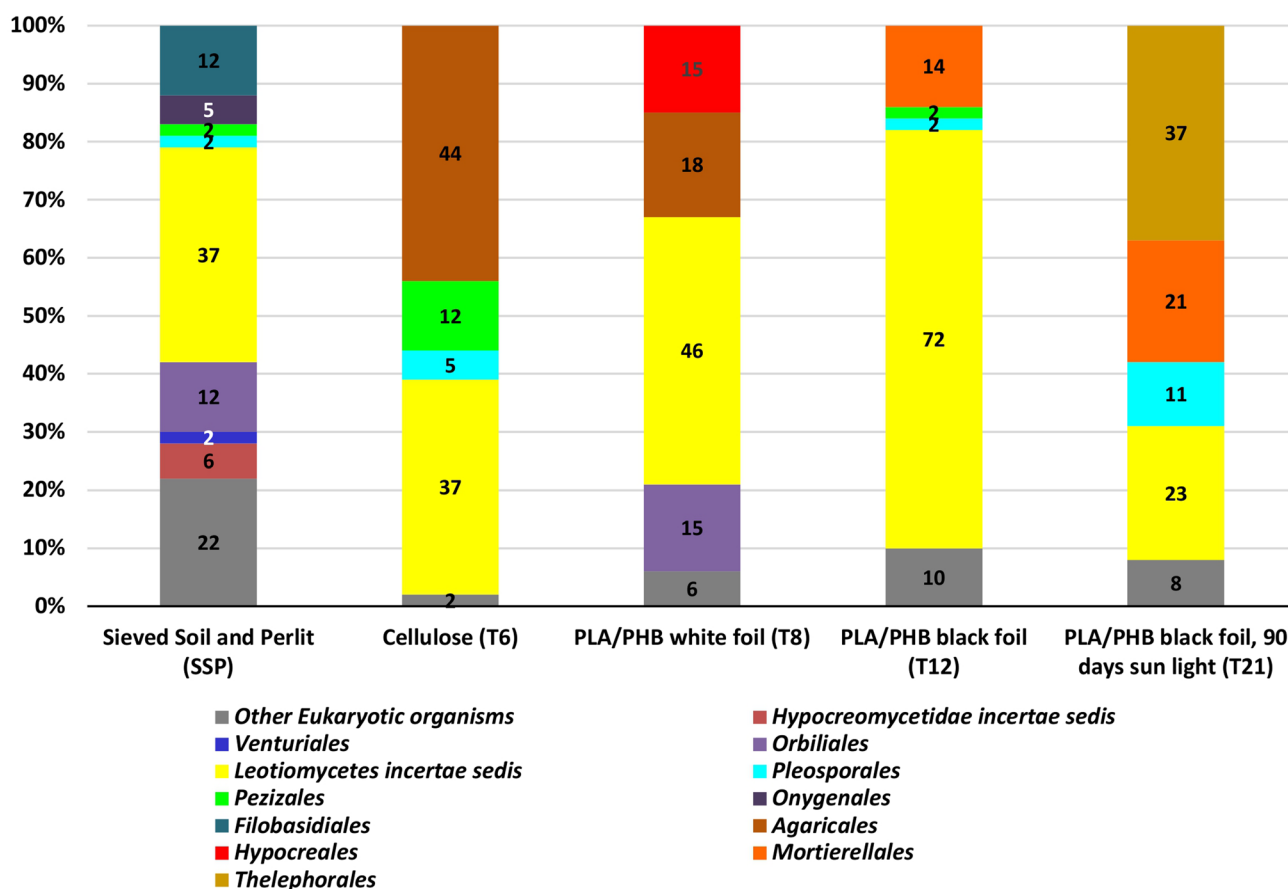


Fig. 8 Eukaryotic microbiome detected in soil and on polymeric foils by DGGE-cloning strategy

Discussion

Our investigation showed the change of the microbial community responsible for the biodegradation of different polymeric foils (one cellulose mat and 3 PLA/PHB blend films) after 1 year of incubation into respirometric reactors.

Culture-dependent and culture-independent strategies were combined together with different agar degradation assays in order to characterize the degrading microbiome.

Both types of analysis showed how the microbiome changed on the basis of available substrate. The cellulose degrading microbiome is different with respect to the microbial communities isolated and detected from the PLA/PHB foils. Moreover, the microbiomes of the two black foils (T12 and T21) have more affinity between them than with the microbiota of transparent film (T8).

Many *Bacillus*, *Paenibacillus*, *Streptomyces*, *Rhodococcus*, *Saccharothrix* and *Arthrobacter* isolates displayed excellent abilities to degrade the three types of PLA/PHB foils. In the literature, it has been reported that some of these bacteria contributed to the degradation of bioplastics or mulching foils (Akutsu-Shigeno et al. 2003; Tokiwa et al.

2009; Brodhagen et al. 2015), but in these studies, only PLA or PHB were tested and not a PLA/PHB blend.

As far as fungal isolates are concerned, several of them were able to degrade PLA/PHB blend. This confirmed the findings of previous studies (Kim and Rhee 2003; Ghosh et al. 2013; Brodhagen et al. 2015) where *Fusarium*, *Trichoderma*, *Cladosporium*, *Penicillium* and *Aspergillus* isolates hydrolyzed either PLA or PHB. To our knowledge, in this study we have demonstrated for the first time the ability of various members of the genera *Aureobasidium*, *Mortierella*, *Absidia*, *Actinomucor* and *Bjerkandera* to degrade PLA, PHB and PLA/PHB blend polymers.

One of the novelty of our study included also the use of a culture-independent method (DGGE-cloning approach) to study the bacterial and eukaryotic microbiota responsible for PLA/PHB blend degradation. Until now only few attempts were tried for studying the PLA and PHB microbial degraders by culture-independent methods and such attempts regarded very different environments (Kamiya et al. 2007; Yagi et al. 2014; Qiu et al. 2017). Moreover, they were oriented either to fungal or to bacterial community.

Our DGGE-cloning analysis revealed a complex microbiome complementary to that identified by the cultivation

strategy. A low correspondence was observed between isolated microorganisms and OTUs; the analogies were related only to limited bacterial and fungal orders. Both methods detected the presence of *Bacillales* and *Pseudonocardiales* on transparent PLA/PHB foil (T8). Importantly, the *Bacillales* members detected by both strategies were *Bacillus* and *Paenibacillus*, which again confirms that these two genera are implicated in the degradation of the PLA/PHB blend. On the other hand, the *Pseudonocardiales* species identified were different: *Saccharothrix* were isolated by cultivation and *Pseudonocardia* were identified by cloning.

The fungal analogies between culture-dependent and culture-independent analyses confirmed also the importance, during the biodegradation of PLA/PHB foils, of the members belonging to the genus *Fusarium*, detected by both strategies on sample T8, and *Mortierella* species revealed by the two identification approaches only on both black foils (T12 and T21). A previous study evidenced the ability of the fungus *Mortierella subtilissima* to degrade polyethylene films (Nowak et al. 2011).

Pleosporales is another taxon identified by both methods, mainly by DGGE-cloning, although the coincidence between the isolated and detected species did not match. Members of this order were associated in the degradation of PLA mulches in soil environment (Moore-Kucera et al. 2014).

We believe that other taxonomic orders detected in high percentages by DGGE-cloning, such as *Acidobacteria* (subdivision 6), *Cytophagales*, *Burkholderiales*, *Anaerolineales* and *Selenomonadales*, *Leotiomyces* (incertae sedis), *Agaricales* and *Thelephorales*, also have a major role in PLA/PHB blend degradation. The bacterial orders *Acidobacteria*, *Cytophagales* and *Burkholderiales* have already shown their abilities to degrade different bioplastic mixtures, but not the PLA/PHB blend, in specific denitrification systems (Horiba et al. 2005; Shen et al. 2013; Chu and Wang 2017; Xu and Chai 2017). The orders *Anaerolineales* and *Selenomonadales* have so far demonstrated their capacity to colonize PLA/PHB foils only in this study.

Regarding the fungal taxa, it seems that only the genera *Coprinus* (order *Agaricales*) and *Geomyces* (*Leotiomyces* incertae sedis) previously showed an ability to degrade nylon (Nomura et al. 2001) and a biodegradable mulching film (Moore-Kucera et al. 2014), respectively. We could find no information in the literature about the bioplastic degradation activities of the genera *Pseudogymnoascus* and *Pseudeurotium* (*Leotiomyces* incertae sedis) or the order *Thelephorales*.

With our PLA/PHB foils experimental trial we did not assess the optimal conditions for reaching a mineralization rate of 60–70% (STN EN ISO 17556; Gómez and Michel 2013) that would reasonably represent complete biodegradation, but evidence suggests that 50% values are sufficient to

prove that biodegradation was successful (Bayer and Lamed 1992; Weytjens et al. 1994; Solaro et al. 1998). In addition, our microbiological analysis (including culture-dependent and culture-independent approaches, and biodegradation plate assays) permitted the identification of PLA/PHB degraders. The obtained data showed the main actors of the PLA/PHB blend degradation and produced information for future studies focused on the development and modulation of new PLA/PHB foils with improved biodegradability.

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