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RESEARCH ARTICLE

Poly(3-hydroxybutyrate)/metribuzin formulations: characterization, controlled release properties, herbicidal activity, and effect on soil microorganisms

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Abstract Slow-release formulations of the herbicide metribuzin (MET) embedded in the polymer matrix of degradable poly-3-hydroxybutyrate [P(3HB)] in the form of microparticles, films, microgranules, and pellets were developed and tested. The kinetics of polymer degradation, MET release, and accumulation in soil were studied in laboratory soil microecosystems with higher plants. The study shows that MET release can be controlled by using different techniques of constructing formulations and by varying MET loading. MET accumulation in soil occurs gradually, as the polymer is degraded. The average P(3HB) degradation rates were determined by the geometry of the formulation, reaching 0.17, 0.12, 0.04, and 0.05 mg/day after 60 days for microparticles, films, microgranules, and pellets, respectively. The herbicidal activities of P(3HB)/MET formulations and commercial formulation Sencor Ultra were tested on the *Agrostis stolonifera* and *Setaria macrocheata* plants. The parameters used to evaluate the herbicidal activity were plant density and the weight of fresh green biomass measured at days 10, 20, and 30 after sowing. All P(3HB)/MET formulations had pronounced herbicidal activity, which varied depending on MET loading and the stage of the experiment. In the early phases of the experiment, the herbicidal effect of P(3HB)/MET formulations with the lowest MET loading (10 %) was comparable with that of the commercial formulation. The herbicidal effect of P(3HB)/MET formulations with higher MET loadings (25 and 50 %) at later stages of the experiment were stronger than the effect of Sencor Ultra.

Keywords Metribuzin · Degradable poly-3-hydroxybutyrate · Slow-release P(3HB)/MET formulations · Release kinetics · *Agrostis stolonifera* · *Setaria macrocheata*

Introduction

Weeds cause great damage to agriculture, and herbicides constitute the most extensively used group of pesticides (40–50 % of the total amount of the globally used pesticides), their commercial varieties accounting for about 40 % of all commercial pesticides. Weed control using herbicides is one of the major components of modern efficient agriculture. However, herbicides persist in the soil, posing a hazard to human health, leading to the emergence of herbicide-resistant weed species, threatening the stability of agroecosystems, and in some cases leaving the ground almost permanently barren.

S-1,3,5-triazines are commonly used broad-spectrum selective herbicides, which do not persist for a very long time in soil. Metribuzin (MET) is a pre-emergence and post-emergence herbicide based on the derivative of 1,2,4-triazine, which is used to treat soya, potato, and tomato. MET has a systemic effect against many undesirable plants in vegetable and grain crop fields, and both have a foliar action and can penetrate into plants through their roots; this is a pre- and post-

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emergence herbicide. MET inhibits plant photosynthesis. MET has been used by many researchers for constructing slow-release formulations based on various synthetic and natural materials: polyvinylchloride, carboxymethyl cellulose (Kumar et al. 2010a), acrylamide (Sahoo et al. 2014), methacrylic acid combined with ethylene glycol and dimethacrylate (Zhang et al. 2009), sepiolite (Maqueda et al. 2008), alginate (Flores-Céspedes et al. 2013), phosphatidylcholine (Undabeytia et al. 2011), kraft lignin (Chowdhury 2014), lignin/polyethylene glycol blends (Fernández-Pérez et al. 2011, 2015), chitin, cellulose, starch (Fernández-Pérez et al. 2010; Rehab et al. 2002), bentonite, and activated carbon (McCormick 1985). Release kinetics of metribuzin embedded in different materials was studied in laboratory systems (sterile water, soil) (Fernández-Pérez et al. 2010, 2015; Flores-Céspedes et al. 2013; Kumar et al. 2010a; Maqueda et al. 2008; McCormick 1985; Rehab et al. 2002; Sahoo et al. 2014; Zhang et al. 2009); the duration of MET release varied within a very wide range, between several tens of hours and several tens of days. Thus, by varying the shape of the carrier, the technique employed to construct it, and the material used, one can influence MET release kinetics and design controlled delivery systems for this herbicide. The majority of studies described experiments with MET released into water, and just a few authors used soil (Fernández-Pérez et al. 2010, 2015; Flores-Céspedes et al. 2013; Kumar et al. 2010a; Maqueda et al. 2008; McCormick 1985; Rehab et al. 2002; Sahoo et al. 2014; Zhang et al. 2009).

Much research effort has focused recently on constructing new formulations and investigating their behavior in the environment. The main purpose of such studies is to produce less toxic and more selective pesticides and reduce the rate of pesticide application. Degradable polymers of various origins are being tested as materials for constructing pesticide carriers. Special attention is being given to biodegradable polyesters such as polyhydroxyalkanoates (PHAs)—microbial polyesters that have many useful properties. These polymers are degraded in biological media by natural microflora to carbon dioxide and water under aerobic conditions and to water and methane under anaerobic ones. Production of PHAs is a rapidly developing branch of the industry of degradable bioplastics, and they are regarded as candidates to eventually replace synthetic polymers (Chen 2010; Ienczak et al. 2013; Kaur and Roy 2015; Volova et al. 2013a).

A search of the literature revealed few studies that reported the use of PHAs to construct slow-release eco-friendly pesticide formulations. One of the first studies reported the use of degradable films of poly-3-hydroxybutyrate as a matrix for embedding pesticides Ronilan (the active ingredient vinclozolin, or (RS)-5-vinyl-5-methyl-3-(3,5-dichlorophenyl)-1,3-vinyloxazolidine-2,4-dione) and Sumilex (the active ingredient procymidone, or N-(3,5-dichlorophenyl)-1,2-dimethylcyclopropane1,2-dicarboximide), which effectively suppressed phytopathogenic fungus *B. cinerea* (Savenkova et al. 2002). In a study conducted by our team, hexachlorocyclohexane and lindane were embedded in PHA to investigate polymer degradation kinetics and pesticide release into soil (Voinova et al. 2009; Volova et al. 2008). Suave et al. (2010) reported encapsulation of pesticide malathion in microspheres of poly-3-hydroxybutyrate/poly(ε-caprolactone). Other authors reported encapsulation of pesticides ametrine and atrazine in microspheres of the 3-hydroxybutyrate/3-hydroxyvalerate copolymer (Grillo et al. 2010, 2011; Lobo et al. 2011). In a more recent study, Prudnikova et al. (2013) described embedding of herbicide Zellek Super in poly-3-hydroxybutyrate (P(3HB)/3HV) microgranules and films to prepare slow-release formulations. In our recent study, polymer P(3HB) was used as a matrix in slow-release formulations of the herbicide MET. Physical P(3HB)/MET mixtures in the form of solutions, powders, and emulsions were used to construct different metribuzin formulations (films, granules, pellets, and microparticles). SEM, X-ray, and DSC proved the stability of these formulations incubated in sterile water in vitro for long periods of time (up to 49 days). Metribuzin release into water from the polymer matrix was also studied. By varying the shape of formulations (microparticles, granules, films, and pellets), we were able to control the release time of metribuzin, increasing or decreasing it (Volova et al. 2016).

The purpose of this study was to prepare and characterize controlled release metribuzin formulations and to study their microbiological and herbicidal activity.

Materials and methods

Chemicals

The herbicide used was MET [$C_8H_{14}N_4OS$] (State Standard Sample 7713-99—the state standard accepted in Russia (Blok-1, Russia), 99.7 % pure). As the control, we used Sencor Ultra, which contains metribuzin as the active ingredient (600 g/kg), purchased from Bayer CropScience.

Production of P(3HB)

Polymer P(3HB) was used as a degradable matrix for embedding the herbicide. The polymer was synthesized in the Laboratory of Chemoautotrophic Biosynthesis at the Institute of Biophysics SB RAS by using bacterium *Cupriavidus eutrophus* B10646, batch-cultured under strictly aseptic conditions in a 7.5-L BioFlo/CelliGen115 fermentation system ("New Brunswick," USA) following the procedure developed by Volova et al. (2013b, 2014). Polymer was extracted from cells with chloroform, and the extracts were precipitated using hexane. The extracted polymers were redissolved and precipitated again three to four times to prepare homogeneous specimens. The purity of the polymer was

determined by chromatography of methyl esters of fatty acids on an Agilent 7890A chromatograph mass spectrometer (Agilent Technologies, USA).

Preparation of sustained-release MET formulations

P(3HB) was used as the matrix for preparing films, pellets, microgranules, and microparticles. Each polymer matrix was loaded with 10, 25, and 50 % (*w*/*w*) metribuzin. The choice of these loadings was based on metribuzin concentrations in the formulations commonly used in agriculture (0.180–0.96 kg/ha). Three concentrations of Sencor Ultra added to the soil in the control corresponded to metribuzin concentrations in the experimental formulations, i.e., 3, 7.5, and 15 μg MET/g soil. The techniques employed to construct P(3HB)/MET blends and their properties are described in detail elsewhere (Volova et al. 2016).

Films were prepared as follows: a chloroform solution containing 2 % (*w*/*v*) of P(3HB) was mixed with the metribuzin solutions (the polymer/herbicide mass ratios in the film were 90:10, 75:25, and 50:50). The polymer/metribuzin solution systems were placed onto the MR Hei-Standart magnetic stirrer (Heidolph, Germany) operated at a speed of 300 rpm for 2–3 h (until completely dissolved). The homogeneous polymer/metribuzin solution was filtered and poured into the degreased mold under a bell glass (to protect it from draught and dust). The films stayed under the bell glass for 24 h at room temperature, and then, they were placed into a vacuum drying cabinet (Labconco, USA) for 3–4 days, until complete solvent evaporation took place. The films were then weighed on an analytical balance. The film thickness was measured with an EDM-25-0.001 digital micrometer (LEGIONER, Germany). The films were 25 ± 0.3 μm thick. Squares of 25 mm^2 in area (5 mm × 5 mm) were then cut from the film.

Polymer microgranules loaded with metribuzin were prepared from a solution of the herbicide and P(3HB) in chloroform. The system was mixed to achieve homogeneity, by using a Silent Crusher high-speed homogenizer (Heidolph, Germany). A Pumpdrive 5001 peristaltic pump (Heidolph, Germany) was used to drop the polymer/metribuzin solutions into a sedimentation tank that contained hexane, where the polymer was crystallized and granules formed. Polymer concentration in the solution = 10 % (*w*/*v*), needle size = 20G, and thickness of the precipitate layer (h) = 200 mm. Three types of microcapsules containing different proportions of herbicide were prepared. In the first batch of microcapsules, the polymer/herbicide mass ratio was 90:10; in the second, the mass ratio was 75:25; and in the third, the mass ratio was 50:50. The average diameter of the granules with the MET encapsulation efficiency close to 100 % was 2–3 mm.

MET-loaded pellets were prepared as follows: the polymer was ground in a ZM 200 ultracentrifugal mill (Retsch, Germany). The fractional composition of the polymeric powder was determined by using an AS 200 control analytical sieve shaker (Retsch, Germany); apparent density of the fractions was determined with PT-TD 200 Touch (Retsch, Germany). Samples of the P3HB and MET powders were weighed on an analytical balance, mixed at polymer/metribuzin ratios of 90:10, 75:25, and 50:50, and then homogenized with a laboratory stirrer for 2 min. Pellets were prepared from the P3HB/MET powder by cold pressing, using a laboratory bench-top hand-operated screw press (Carl Zeiss Jena, Germany) under pressing force of 6000 F. Pellets prepared from polymer powder and MET were 3 mm in diameter and 1 mm thick.

Microparticles were prepared by the emulsion technique. Polymer emulsion was prepared as follows. The oil phase, represented by a 2 % P(3HB) solution with different proportions of MET in chloroform, was combined with the aqueous phase–polyvinyl alcohol (PVA) solution (Sigma, USA, M_w 30 kDa)–and mixed for 24 h at a speed of 750 rpm, until complete solvent evaporation took place. Microparticles 10 μm or more in diameter were prepared with an MR Hei-Standard magnetic stirrer (Heidolph, Germany), taking into account the previously determined effects of the type of the emulsion and agitation speed on the particle diameter. After solvent evaporation, microparticles were collected by centrifugation (Centrifuge 5810 R, 5417 R, Eppendorf, Germany, 10,000 rpm), rinsed, and freeze-dried (Alpha 1-2 LD plus, Christ®, Germany).

A physicochemical study

Initial substances in the form of powders (MET and P(3HB)) and MET formulations constructed as films, granules, pellets, and microparticles were examined by using state-of-the-art physicochemical methods. Thermal analysis was performed with a DSC-1 differential scanning calorimeter (Mettler Toledo). Samples of films, powders, granules, and pellets (4.0 ± 0.2 mg) were placed in aluminum crucibles and heated at 5 °C/min. The melting point (T_{melt}) and thermal decomposition temperature (T_{degr}) were determined from exothermic peaks on thermograms, using the StarE software. The measurement error was ±1.5 °C between −20 and 200 °C and ±2.5 °C between 200 and 300 °C.

X-ray structure analysis and determination of the degree of crystallinity (C_x, %) of films, powders, or pellets were performed using an X-ray spectrometer (D8 Advance, Bruker Corporation, Bremen, Germany) (graphite monochromator on a reflected beam) in a scan-step mode, with a 0.04 °C step and exposure time 2 s, to measure intensity at point. The instrument was operated at 40 kV × 40 μA. The measurement accuracy was 2 %.

Morphology of the microparticles and films was studied by electron microscopy, using an S-5500 scanning electron microscope (Hitachi, Japan). Samples of granules and pellets

were examined under a TM 3000 electron microscope (Hitachi, Japan). Platinum sputter coating of the specimens was conducted in an Emitech K575XD Turbo Sputter Coater (Quorum Technologies Limited, UK).

Molecular weight and molecular weight distribution of the initial P(3HB) and MET and of P(3HB) and MET in the formulations were investigated by gel permeation chromatography with an Agilent Technologies 1260 Infinity system (Germany), using Agilent PS-H EasiVial calibration standards, enabling separation of polymers with a wide range of molecular weights: 200–3,000,000 Da. The measurement accuracy was 2 %.

The MET formulations were investigated by methods corresponding to their geometries (films, granules, pellets, microparticles). The film thickness was measured with a digital micrometer (LEGIONER EDM-25-0.001, Germany). Granules were examined by determining their size and morphology. The sizes and ξ-potential of microparticles were measured with a Zetasizer Nano ZS (Malvern, UK). Size range maximum (diameter) was between 0.3 nm and 10 μm. Size range suitable for measurement (diameter) of zeta potential was between 3.8 nm and 100 μm. Conductivity accuracy was +/−10 %.

Laboratory ecosystems with higher plants

Herbicidal activity of the P(3HB)/MET formulations loaded with 10, 25, and 50 % MET was studied in the laboratory ecosystems with higher plants. Plastic containers were filled with field soil, which was used to grow two species of weeds: perennial creeping bentgrass (*Agrostis stolonifera*) and foxtail (*Setaria macrocheata*). P(3HB)/MET formulations and plant seeds were simultaneously buried in the soil. Plants were grown in a Conviron A1000 environmental chamber (Canada) under stable ambient conditions: lighting under a 12L:12D photoperiod, a temperature of 25–28 °C, and humidity of 65 %. Two groups were used as controls: in the positive control, the herbicide Sencor Ultra with metribuzin concentrations corresponding to those of the experimental formulations was buried in the soil; in the negative control, no herbicide was added. A long-term experiment (60 days) was conducted, with the condition and growth of the plants photo-monitored every week. Plant growth and productivity were evaluated by measuring fresh green biomass. The green biomass of the weeds was cutoff near the ground every 7 days and weighed on the analytical balance of accuracy class 1 (Ohaus Discovery, Switzerland); the weighed portion of the plants (g) per area (m^2) was calculated. The density of the weeds was calculated based on the number of plants in a 54-cm^2 container, converted to m^2. P(3HB/MET) fungicidal activity was evaluated based on the time of plant death and the number of dead plants.

Soil characterization

The agrogenically transformed soil (the village of Minino, the Krasnoyarsk Territory, Siberia, Russia) was placed into 250-mm^3 plastic containers (200 g soil per container). The soil was cryogenic-micellar agro-chernozem with high humus content in the 0–20-cm layer (7.9–9.6 %). The soil was weakly alkaline (pH 7.1–7.8), with high total exchangeable bases (40.0–45.2 mequiv/100 g). The soil contained nitrate nitrogen N-NO_3, 6 mg/kg, and P_2O_5, 6, and K_2O, 22 mg/100 g soil (according to Machigin).

A microbiological study

Microbial analysis of the soil in laboratory systems was conducted by using generally accepted methods. The number of ammonifying and copiotrophic bacteria (CFU/g soil) was determined on fish-peptone agar (FPA), the number of mineral nitrogen-assimilating prototrophic bacteria was determined on starch and ammonia agar (SAA), nitrogen-fixing bacteria were counted on Ashby's medium, oligotrophs were counted on soil extract agar (SA), and the number of micromycetes was determined on the wort agar (WA) (Netrusov et al. 2005). Mineralization coefficient was determined as a ratio between microorganisms assimilating mineral nitrogen and ammonifying bacteria. Oligotrophy coefficient was determined as a ratio of oligotrophic to ammonifying bacteria. Pure cultures of bacteria were isolated from soil samples and tested by conventional methods, based on their cultural and morphological properties and using standard biochemical tests mentioned in identification keys (Brenner et al. 2005; Dworkin et al. 2006). Dominant microorganisms were identified using MIKROLATEST® ID identification kits and 16S rRNA gene sequence analysis. Soil microscopic fungi were identified by their micro- and macromorphological features (the structure and color of colonies, the structure of mycelium, the particularity of anamorph and teleomorph stages) (Sutton et al. 2001; Watanabe 2002).

The soil had high mineralization and oligotrophy coefficients (1.52 and 11.74, respectively), indicating soil maturity and low contents of available nitrogen forms. The number of copiotrophic bacteria was 16.3 ± 5.1 million CFUs/g soil—1.5 and 11.7 times lower than the number of prototrophic and oligotrophic bacteria, respectively, while the number of nitrogen fixing bacteria was very high (26.1 ± 4.7 million CFUs/g soil).

A study of MET release from P(3HB)/MET formulations and degradation of P(3HB)

After 10, 20, 30, 45, and 60 days of the experiment, specimens were taken out of the soil and MET concentration in the soil

was measured. The amount of metribuzin released (RA) was determined as percentage of the metribuzin encapsulated in the polymer matrix, using the following formula (Eq. 1):

$$RA = r/EA \times 100\ \% \quad (1)$$

where EA is the encapsulated amount (mg), and r is the amount released (mg).

For describing herbicide release kinetics from different formulations, we used the Korsmeyer–Peppas model (Eq. 2):

$$M_t/M_\infty = Kt^n \quad (2)$$

Here, M_t is the amount of the herbicide released at time t, and M_∞ is the amount of the herbicide released over a very long time, which generally corresponds to the initial loading. K is a kinetic constant, and n is the diffusional exponent. At $n = 0.5$, herbicide is released via diffusion, in accordance with the Fickian diffusion mechanism. At $n = 1$, the release mechanism is described as the case II transport, determined by relaxation processes and transitions in the carrier rather than by diffusion laws. This type of release occurs when the diffusion layer is dissolved and the matrix is partly destroyed and degraded. Values of n between 0.5 and 1 indicate the superposition or anomalous release. For the case of cylindrical pellets, $0.45 \le n$ corresponds to a Fickian diffusion mechanism, $0.45 < n < 0.89$ to non-Fickian transport, $n = 0.89$ to case II (relaxation) transport, and $n > 0.89$ to super case II transport. To find the exponent n, the portion of the release curve where $Mt/M_\infty < 0.6$ should only be used (Peppas and Narasimhan 2014; Ritger and Peppas 1987).

To measure residual (undegraded) polymer, the specimens (three in a bag) were removed from the soil, thoroughly rinsed in distilled water, dried to constant weight, and weighed on the analytical balance of accuracy class 1 (Ohaus Discovery, Switzerland). Evaluation of polymer biodegradation was based on the mass loss of the specimen.

MET analysis

Metribuzin was isolated from soil in accordance with the Procedural Guidelines (MUK 4.1.1405-03) (Procedural Guidelines 2006). MET was extracted with acetone from the total mass of the soil three times. Before extraction, the soil was air-dried for 48 h. The extracts were placed into the separating funnel, and distilled water, NaCl, a 10 % aqueous solution of KOH, and dichloromethane were added to the funnel. The lower, dichloromethane, layer was passed through anhydrous sodium sulfate. Extraction and filtration were performed two more times. The solvent was removed from the filtrate using a Rotavapor R-210 rotary evaporator (Switzerland), and the residue was dissolved in the known volume of acetone; MET was determined by gas chromatography. MET content in formulations was determined as follows. The formulation was dissolved in chloroform. Polymer was precipitated with a double volume of hexane. Solvents containing MET were passed through anhydrous sodium sulfate. The solvents were removed using a Rotavapor R-210 rotary evaporator (Switzerland), and the residue was dissolved in the known volume of acetone. Detection and determination of MET was performed using a gas chromatograph equipped with a mass spectrometer (7890/5975C, Agilent Technologies, USA), using a capillary column, under varied temperature. The chromatography conditions were as follows: an HP-5MS capillary column, 30 m long, and 0.25 mm in diameter; carrier gas—helium, flow rate 1.2 mL/min; sample introduction temperature 220 °C; initial temperature of chromatography 150 °C; temperature rise to 310 °C at 10 °C/min; transfer line temperature 230 °C, ion source temperature 150 °C, electron impact mode at 70 eV, fragment scan from m/z 50 to m/z 550 with a 0.5-s cycle time. The peak corresponding to metribuzin was detected by mass spectrometer. The State Standard Sample No. 7713-99 was used. It is the state standard accepted in Russia: 99.7 % pure. Calibration curve was prepared by using a wide range of concentrations of metribuzin in acetone (0.1–4.2 μg/μL). The range of linear detection was obtained for a wide variety of concentrations: between 0.1 and 4.2 μg/μL. The standard error of the method was no more than 3 %.

Statistical analysis

Three replicates of the experimental data were averaged, and their standard deviations were calculated by using the standard software package of Microsoft Excel. To compare the means of all release data and to assess statistical significance between them, either one-way analysis of variance (ANOVA) or an unpaired two-tailed t test was carried out at 5 % significance level.

Results and discussion

Characterization of slow-release MET formulations P(3HB)/MET

Formulations of metribuzin embedded in the P(3HB) matrix, with different MET loadings, were prepared (Suppl. Figs. 1 and 2). The size of microparticles was influenced by MET loading: the average sizes of 10 and 25 %-loaded microparticles were similar to each other—54 μm; as the loading was increased to 50 %, the average diameter of the particles increased to 70.7 μm. However, no relationship was found between the value of the ξ-potential, which is an important parameter of particles characterizing their stability in solutions, and the loading of microparticles with metribuzin; ξ-potential of the particles with different MET loadings varied within a

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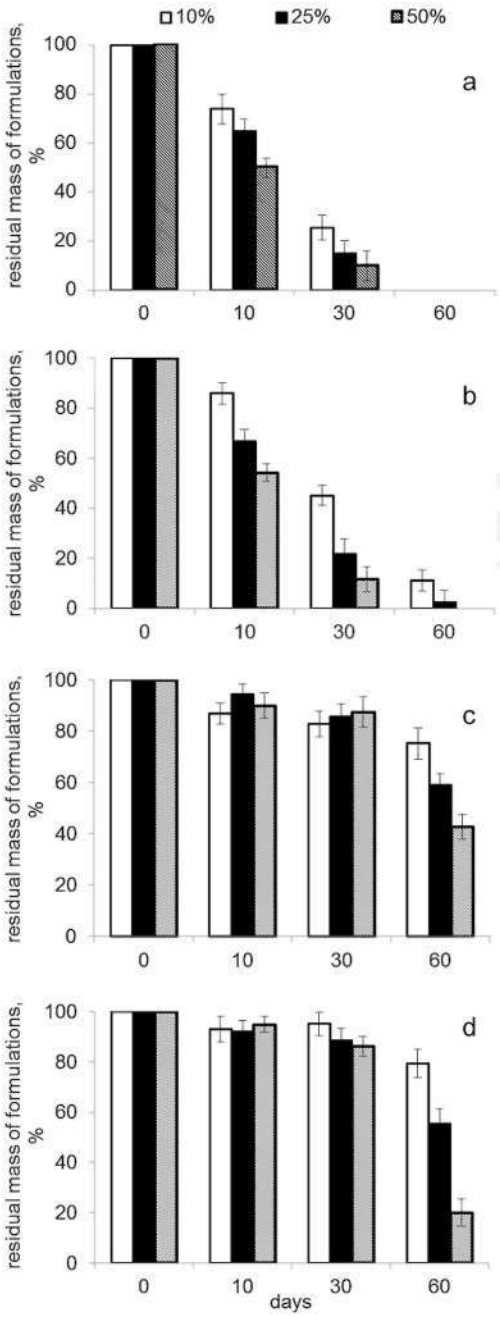
narrow range, between −26.2 and −33.2 mV. The yield of the particles from emulsions with different metribuzin loadings was rather high, more than 60 %, but MET encapsulation efficiency was low, no more than 33 %.

Measurements of the initial substances (polymer and MET) and the experimental P(3HB)/MET formulations by DSC and X-ray (Suppl. Table) did not reveal any significant effect of MET on the physicochemical properties and, hence, performance of the polymer. The results of measurements showed that the blending of the components did not cause their chemical binding, but produced a physical P(3HB)/MET mixture. X-ray structure analysis of P(3HB)/MET formulations showed an about 10 % decrease in the degree of crystallinity (C_x) of the pellets, microgranules, and microparticles compared with the initial polymer (74 %) and MET (90 %); the decrease in the C_x of the films was more significant, reaching 51 %. Thus, the embedding of metribuzin into the polymer influenced crystallization of the polymer, making it somewhat more amorphous.



Q4/Q5 **Fig. 1** Degradation dynamics of P3HB/MET microparticles (**a**), films (**b**), granules (**c**), and pellets (**d**) incubated in soil

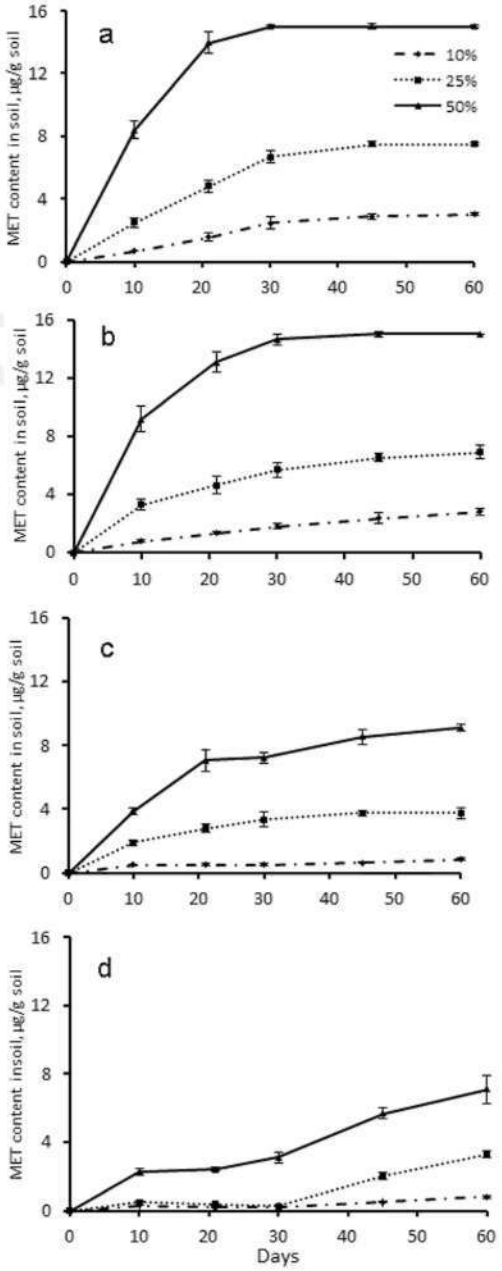


Fig. 2 Cumulative release of MET into soil from microparticles (**a**), films (**b**), microgranules (**c**), and pellets (**d**) with MET loadings of 10, 25, and 50 % of the polymer weight in laboratory soil microecosystems with high plants

Molecular weight properties of P(3HB) used to construct P(3HB)/MET formulations of various shapes were compared with those of the initial polymer (Suppl. Table). The chromatograms of P(3HB), MET, and P(3HB)/MET formulations did not show any changes in the weight average and number average molecular weights (M_w and M_n) and polydispersity caused by preparation of the formulations.

Kinetics of MET release from P(3HB)/MET formulations and degradation of P(3HB)

All microparticles, irrespective of the amount of metribuzin loading, were almost completely degraded after 30–40 days of incubation in soil (Fig. 1); the average degradation rates of the microparticles with the 10, 25, and 50 % MET loadings were 0.15, 0.17, and 0.18 mg/day, respectively.

Films showed the second highest degradation rate. After 1 month of incubation in soil, their residual mass was about 25, 15, and 10 % of the initial mass of the films with 10, 25, and 50 % metribuzin loadings, respectively. The degradation rates of the films loaded with 10, 25, and 50 % of metribuzin were slower than those of the microparticles: 0.09 ± 0.004, 0.10 ± 0.005, and 0.18 ± 0.01 mg/day, respectively. Degradation rates of the granules were even lower: after 60 days of incubation in soil, their residual mass was 80, 60, and 40 % of the initial mass, and the average degradation rates were 0.02 ± 0.003, 0.04 ± 0.002, and 0.06 ± 0.002 mg/day at metribuzin loadings of 10, 25, and 50 %, respectively. Similar mass loss dynamics was observed for the pellets, with the average degradation rates of 0.02 ± 0.001, 0.04 ± 0.002, and 0.08 ± 0.003 mg/day. The higher degradation rate of the films compared with microgranules made of P(3HB-co-3HV) and loaded with herbicide Zellek Super was determined in our previous study (Prudnikova et al. 2013). As the polymeric matrix was degraded, molecular weight of the polymer decreased, while its polydispersity and degree of crystallinity increased, suggesting preferential disintegration of the amorphous phases of the polymer.

The dynamics of degradation of the polymer matrix, which determines MET release, influenced herbicide concentration in soil (Fig. 2). The highest concentrations of MET were released from microparticles and films, which were comparable with metribuzin concentration in soil from Sencor Ultra, and were measured after 20–30 days of incubation of the formulations loaded at 50, 25, and 10 % MET. Concentrations reached about 15, 6.9–7.5, and 2.8–3 μg/g soil, respectively. For microgranules, MET concentration in the soil was somewhat lower—7 ± 0.68, 3 ± 0.48, and 0.5 ± 0.03 μg/g soil after 20–30 days of incubation of the formulations loaded at 50, 25, and 10 % MET; by the end of the experiment, the highest MET concentration in soil had reached 9.1 ± 0.19 μg/g soil in the experiment with the 50 % MET loading. Similar MET release and concentrations in soil were obtained for pellets. Amount of the MET released from these forms was the lowest in the initial phase (about 30 days), reaching 0.2–4.2 μg/g soil by the end of the experiment. Thus, MET release to soil was determined by the loading degree and shape of the formulation. The 100 % release of MET was observed from the microparticles, which were

Table 1 Constants characterizing metribuzin release, according to equation $M_t/M_\infty = Kt^n$, from the experimental P(3HB)/MET formulations of different geometries, loaded at 10, 25, and 50 % MET, incubated in laboratory soil ecosystems with plants

Type of P(3HB)/MET formulation; MET loading, %	K (/h)	n	R^2	t_{50} (days)
Kinetics of metribuzin release from microparticles				
10	0.0013	0.98	0.99	21
25	0.0024	0.91	0.99	21
50	0.0021	0.99	0.99	10
Kinetics of metribuzin release from films				
10	0.103	0.27	0.92	30
25	0.005	0.77	0.96	21
50	0.0002	1.10	0.96	10
Kinetics of metribuzin release from microgranules				
10	0.020	0.24	0.82	60
25	0.019	0.47	0.99	45
50	0.021	0.46	0.91	45
Kinetics of metribuzin release from pressed pellets				
10	0.010	0.28	0.97	60
25	0.0001	0.99	0.99	60
50	0.0004	0.85	0.99	60

M_t the amount of the herbicide released over time t, M_∞ the amount of the herbicide corresponding to the initial loading, K a kinetic constant, which contains structural and geometric data on the formulation, n the parameter characterizing the mechanism of the release of the herbicide, t_{50} the time of release of more than 50 % herbicide

completely degraded during the experiment. Granules and pellets were degraded at slower rates, which affected MET release. MET release to soil occurred with the slowest rate (22–23 % of the loaded amount) from 10 % loaded granules and pellets, which were only 20–25 % degraded. We showed the relationship between herbicide release rate and the level of loading and the type of the form in a previous study, in which we investigated P(3HB-co-3HV) microgranules and films loaded with the herbicide (Prudnikova et al. 2013).

Constant K and exponent n, characterizing kinetics of metribuzin release from the experimental P(3HB)/MET formulations, which were obtained by using the Korsmeyer-Peppas model, are given in Table 1. The time

Table 2 The density of plants and weight of fresh green biomass of the weeds grown in the laboratory microecosystems with slow-release P(3HB)/MET formulations

Type of P(3HB)/MET formulation; MET loading (%)	10 days	20 days	30 days	10 days	20 days	30 days
Biomass of *Agrostis stolonifera* (g/m^2)				Density of *Agrostis stolonifera* (number/m^2)		
Microparticles						
10	9.03 ± 0.47	4.08 ± 0.28	–	3536 ± 424	1597 ± 175	–
25	6.40 ± 0.37	3.26 ± 0.15	–	2506 ± 223	1276 ± 166	–
50	4.70 ± 0.20	1.49 ± 0.06	–	1840 ± 145	583 ± 52	–
Films						
10	20.07 ± 1.60	9.24 ± 0.55	4.36 ± 0.19	7859 ± 865	3618 ± 290	–
25	14.41 ± 1.01	7.94 ± 0.48	–	5642 ± 670	2209 ± 199	–
50	12.41 ± 0.75	–		4859 ± 435	–	–
Microgranules						
10	17.10 ± 1.33	10.42 ± 0.83	3.12 ± 0.22	6696 ± 870	4080 ± 326	1221±
25	15.41 ± 0.93	7.16 ± 0.58	–	6030 ± 540	2803 ± 252	–
50	11.07 ± 0.78	4.86 ± 0.23	–	4334 ± 475	1903 ± 133	–
Pellets						
10	17.10 ± 1.19	10.24 ± 0.72	5.36 ± 0.27	6696 ± 604	4009 ± 480	2098 ± 187
25	15.41 ± 0.93	9.94 ± 0.70	3.02 ± 0.13	6030 ± 840	3892 ± 500	1182 ± 154
50	12.41 ± 0.87	8.40 ± 0.59	1.12 ± 0.05	4859 ± 401	3289 ± 462	438 ± 31
Control (−)	72.81 ± 5.10	152.0 ± 10.63	198.0 ± 15.02	46,296 ± 6020	46,296 ± 6940	60,306 ± 8443
Control (+)	21.28 ± 1.26	10.64 ± 0.84	5.32 ± 0.32	8333 ± 750	6481 ± 713	2090 ± 187
Biomass of *Setaria macrocheata* (g/m^2)				Density of *Setaria macrocheata* (number/m^2)		
Microparticles						
10	29.63 ± 1.76	–	–	741 ± 81	–	–
25	16.42 ± 1.15	–	–	370 ± 33	–	–
50	–	–	–	–	–	–
Films						
10	29.63 ± 1.58	7.61 ± 0.52	–	741 ± 67	185 ± 23	–
25	16.40 ± 1.15	–	–	555 ± 61	–	–
50	29.63 ± 1.47	–	–	741 ± 89	–	–
Microgranules						
10	22.81 ± 1.80	16.44 ± 1.15	7.61 ± 0.47	555 ± 50	370 ± 48	185 ± 17
25	16.40 ± 1.14	8.21 ± 0.56	–	370 ± 44	185 ± 16	–
50	16.40 ± 0.97	8.21 ± 0.50	–	370 ± 38	185 ± 17	–
Pellets						
10	29.63 ± 2.30	22.83 ± 1.37	16.44 ± 0.97	741 ± 89	555 ± 50	370 ± 30
25	28.41 ± 1.96	22.83 ± 1.29	16.44 ± 0.81	741 ± 70	555 ± 48	370 ± 36
50	29.63 ± 2.07	14.81 ± 1.08	–	741 ± 65	370 ± 40	–
Control (−)	326.48 ± 28.37	482.55 ± 37.61	557.4 ± 50.1	7962 ± 955	7962 ± 916	7962 ± 876
Control (+)	29.62 ± 1.67	22.7 ± 1.57	–	741 ± 59	555 ± 58	–

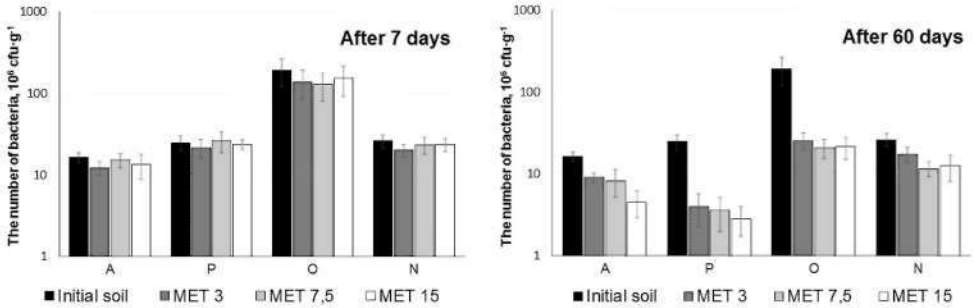


Fig. 3 The effect of free metribuzin on the total counts of microorganisms in plant-free soil: ammonifying (A), prototrophic (P), oligotrophic (O), and nitrogen-fixing (N) bacteria

when metribuzin is released with the highest rate is characterized by parameter t^{50}—the time needed for the herbicide content of the specimen to reach $M_t/M_\infty \leq 0.5$. Metribuzin release from microparticles was characterized by the anomalous case II transport. The values of the exponent at different loadings varied between 0.91 and 0.99. Constant K, which contains diffusion coefficient and structural and geometric data on the formulations, varied between 0.0013 and 0.0024/h as the loading was increased. Metribuzin embedded in microgranules was released via diffusion, in accordance with the Fickian diffusion mechanism. The mode of metribuzin release from the films and pellets differed depending on the loading. At the 10 % loading, the values of the exponent were 0.27 for films and 0.28 for pellets. At higher loadings, metribuzin release was characterized by the superposition of the case II transport. Constant K decreased as exponent n increased. This relationship was reported by Akbuga (1993), Quadir et al. (2003), and Sato et al. (1997).

Changes in constant K for films and pellets suggested structural inhomogeneity of the specimens that had the same geometry but different MET loadings. That was also supported by SEM images, which showed druses of larger areas, and by the increased degradation rates of the formulations with greater MET loadings. Degradation of films and pellets led to a change in the mechanism of metribuzin release.

The time when metribuzin is released with the highest rate is characterized by parameter t_{50}. This parameter varies depending on the shape of the specimen and loading: as the loading is increased, the value of t_{50} is decreased (Table 2). Thus, variations in this parameter suggest the possibility of controlling the herbicide release rate by choosing the proper technique to produce the formulation and by varying its shape.

Microbiological study

As microorganisms play an essential role in pesticide cycling and transformation, we studied the effect of metribuzin on the structure of microbial communities in laboratory systems. Studies of toxicity of metribuzin showed that suppression of plant growth was due to the toxic effects on symbiotic microorganisms *Rhizobium* sp. MRL3, *Bradyrhizobium* sp. MRM6, and *Pseudomonas aeruginosa* PS1 isolated from the soil under lentil, mung bean, and mustard, respectively (Ahemad and Khan 2011a, b).

Free metribuzin was added to the plant-free soil at concentrations of 3, 7.5, and 15 μg MET/g soil. In 7 days after application, no significant effect on the number of microorganisms was identified, but long-term effects of the herbicide (after 60 days) decreased the number of bacteria at all concentrations used. The number of ammonifying bacteria decreased by a factor of 1.8–3.6, prototrophic bacteria by a factor of 6.2–8.7, and nitrogen fixing bacteria by a factor of 1.5–2 compared with the herbicide-free soil (Fig. 3).

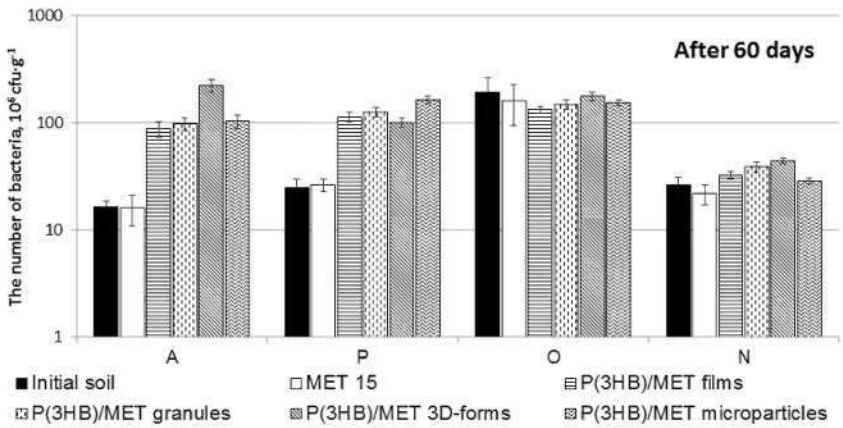


Fig. 4 The effect of different forms of metribuzin on the total counts of microorganisms in the rhizosphere (*Setaria pumila*): ammonifying (A), prototrophic (P), oligotrophic (O), and nitrogen-fixing (N) bacteria

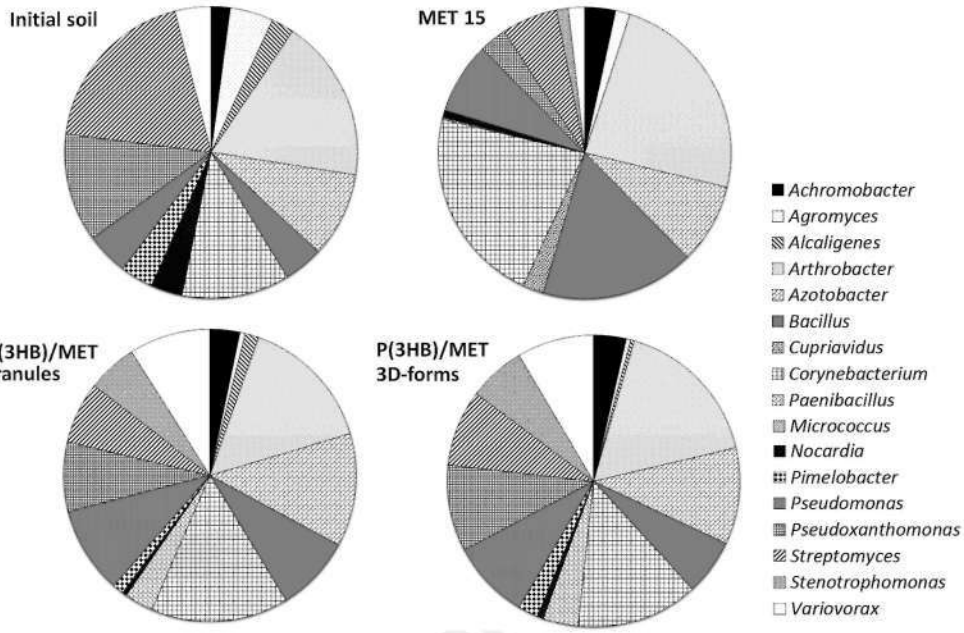


Fig. 5 The effect of different forms of metribuzin on taxonomic composition of bacterial community in the rhizosphere (*Setaria pumila*)

Incubation of P(3HB)/MET formulations with different concentrations of metribuzin in plant-free soil increased the total number of bacteria by a factor of 2.7–3.4 compared with the initial soil. Thus, the growth of microorganism populations in the soil was influenced by the presence of the additional substrate, P(3HB), and slow release of metribuzin from formulations, which reduced the time of exposure to high herbicide concentrations. Ultimately, that reduced the inhibitory effect of metribuzin on soil microflora.

Different results were obtained in experiments with MET added to the soil ecosystems with higher plants. The addition of metribuzin as Sencor Ultra (3, 7.5, and 15 μg MET/g soil) did not significantly change the total counts of soil bacteria and fungi, i.e., MET did not either inhibit or stimulate the growth of microorganisms even at the highest concentration. The MET added as a component of P(3HB)/MET formulations stimulated the development of soil microflora: by the end of the experiment (60 days), the total counts of organotrophs and nitrogen-

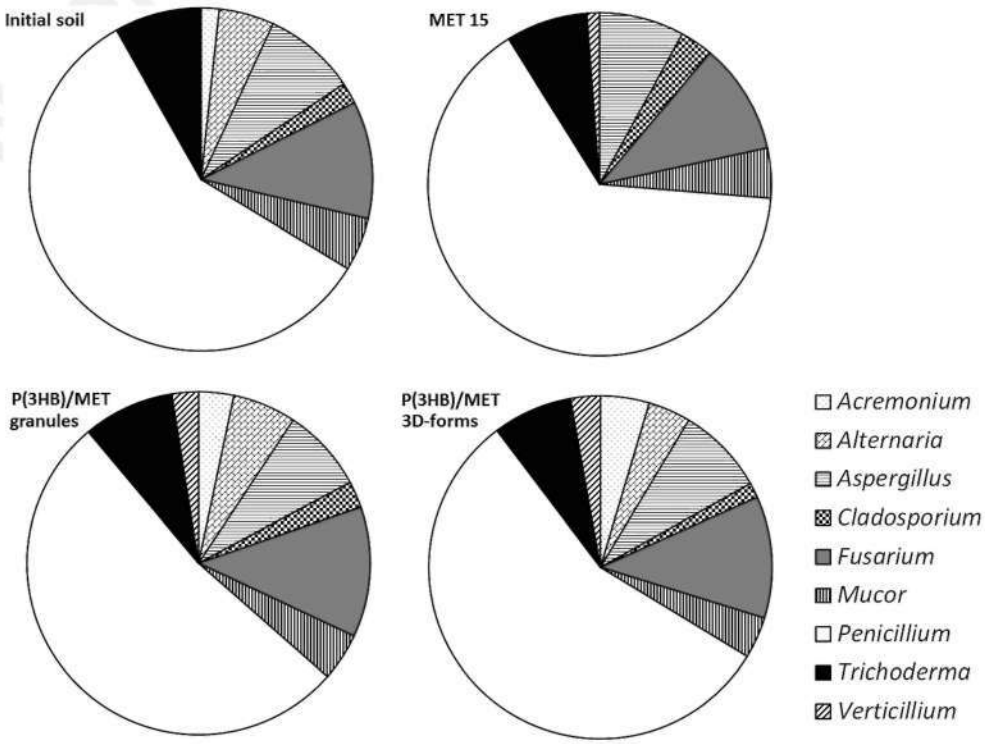


Fig. 6 The effect of different forms of metribuzin on taxonomic composition of soil fungi in the rhizosphere (*Setaria pumila*)

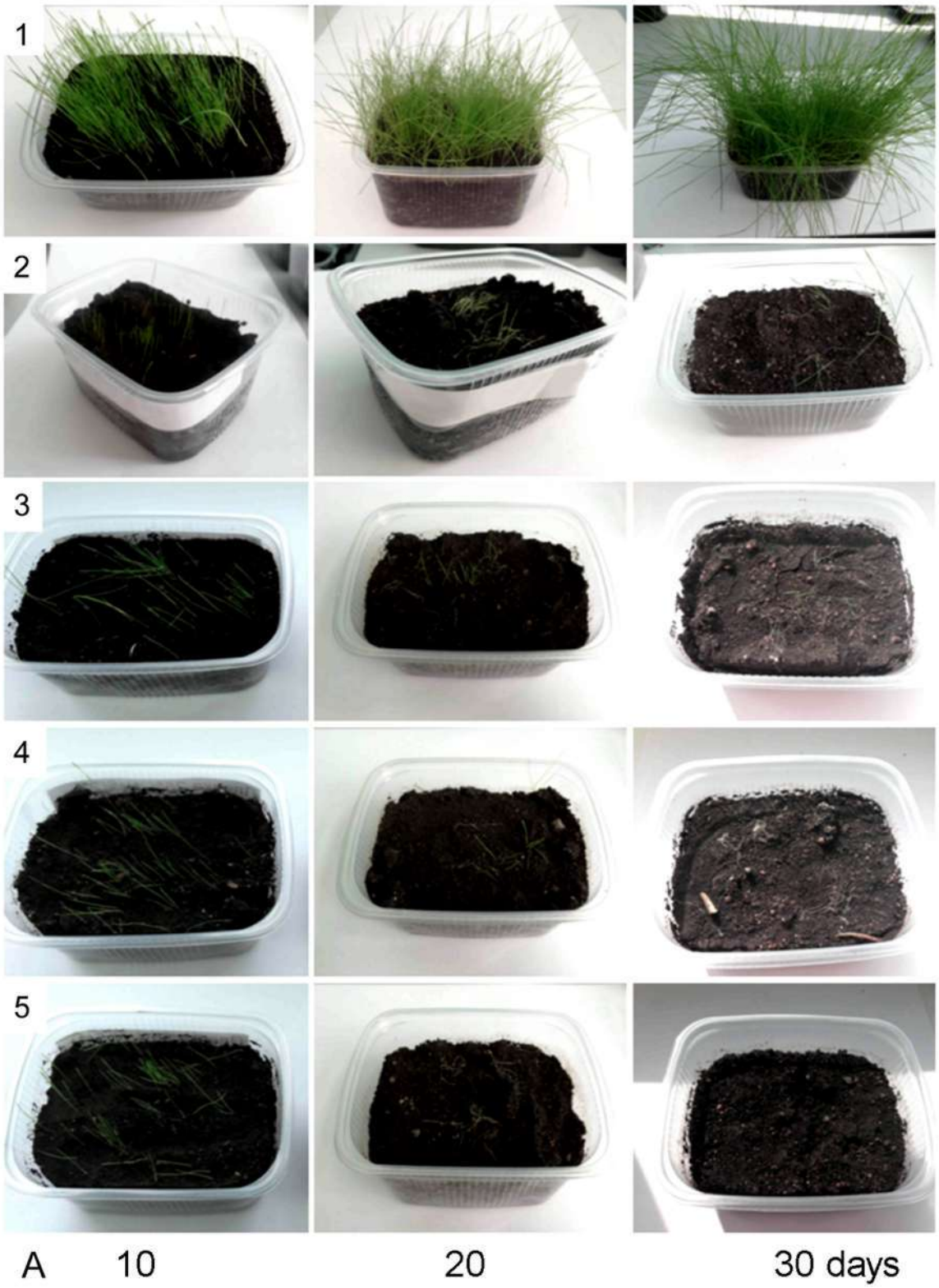


Fig. 7 Photographs of *Agrostis stolonifera* (**a**) *Setaria macrocheata* (**b**) grown under laboratory conditions using P(3HB)/MET films with MET loadings of 10 (*3*), 25 (*4*), and 50 % (*5*) of the polymer weight relative to negative control (*1*) and positive control (*2*)

fixing bacteria had increased by a factor of 1.5–13.8 compared to their counts in the initial soil (Fig. 4). Thus, soil oligotrophy coefficient decreased by a factor of 8–15.

The study of taxonomic diversity of soil microorganisms showed that the proportions of species in the microbial community had also changed with the introduction of MET.

Fig. 7 (continued)

Morphological, physiological, and biochemical studies and molecular genetic examination of the 16S and 28S rRNA gene fragments showed that the initial soil microbial community was dominated by actinobacteria (24 %, including 19 % of *Streptomyces*), *Arthrobacter* (18 %) and *Corynebacterium* (12 %) species; *Pseudoxanthomonas* were the major Gram-negative bacilli (12 %) (Fig. 5). By the end of the experiment (60 days), the composition of bacteria influenced by Sencor Ultra had changed due to an increase in the percentages of *Corynebacterium* (19–22 %), *Arthrobacter* (20–24 %), and

Bacillus (15–17 %). The percentage of *Pseudomonas* increased to 5–8 %, but the total percent of Gram-negative bacilli decreased by 10–12 % and actinobacteria decreased too (by 8–15 %). The addition of P(3HB)/MET formulations increased the percentage of Gram-negative bacilli, including *Pseudomonas*, *Pseudoxanthomonas*, *Stenotrophomonas*, and *Variovorax*, to 31–33 %. The proportion of spore forming bacteria (*Bacillus* and *Paenibacillus*) also increased (Fig. 5).

In all soil samples, the major microscopic fungi were *Penicillium* species, which constituted 52–65 % (Fig. 6). Fungi of the genera *Fusarium*, *Trichoderma*, and *Aspergillus* constituted 8–11 % of the population of microscopic fungi in the initial soil and did not change significantly either. In the experiment with P(3HB)/MET formulations, an increase in the percentages of *Acremonium* and *Verticillium* was observed.

We assume that the stimulating effect of P(3HB)/MET was caused by poly-3-hydroxybutyrate, which was a supplementary growth substrate for soil microflora, and that was a stronger factor than the inhibitory effect of MET.

Herbicidal activity of experimental P(3HB)MET formulations

The weeds *A. stolonifera* and *S. macrocheata* were used to study the herbicidal activity of the experimental slow-release formulations of metribuzin embedded in the polymer matrix of P(3HB)/MET. All P(3HB)/MET formulations had comparable effects on the plants (Table 2). In a previous study, we also showed that formulations of the herbicide Zellek Super shaped as microgranules and films successfully suppressed the growth of *A. stolonifera* (Prudnikova et al. 2013). Moreover, the effectiveness of MET embedded in carboxy methyl cellulose-kaolinite composite (CMC-KAO) against weeds growing in wheat crops was shown in the field experiment by Kumar et al. (2010b).

The herbicidal effect of the experimental P(3HB)/MET formulations on the plants was comparable with or, in some cases, stronger than the effect achieved in the positive control. Analysis of the parameters of MET effect on the plant density and the weight of fresh green biomass (Table 2) showed that all experimental P(3HB)/MET formulations exhibited herbicidal activity. Photographs in Fig. 7 show *A. stolonifera* and *S. macrocheata* plants at 10, 20, and 30 days after sowing and herbicide application.

In the positive control, at 10 days after sowing, the plant density and the weight of the biomass of *A. stolonifera* were 8333 ± 750 plants/m^2 and 21.28 ± 1.26 g/m^2, at 20 days 6481 ± 713 plants/m^2 and 10.64 ± 0.84 g/m^2, and at 30 days 2090 ± 187 plants/m^2 and 5.32 ± 0.32 g/m^2, respectively. That was almost five to six times lower than in the negative control. For *S. macrocheata*, the difference was even more considerable.

The inhibitory effect of the experimental P(3HB)/MET formulations varied depending on the MET loading and the duration of the experiment. At 10 days after sowing, the number of *A. stolonifera* plants and their biomass in the experiment with films, microgranules, and pellets loaded with MET at 10 % were comparable with the positive control, but in the ecosystems with the microparticles, which were degraded in the soil at the highest rate, these parameters were lower by more than a factor of two. P(3HB)/MET formulations with higher MET loadings had more pronounced herbicidal effects: at 10 days after sowing, the biomass was lower than in the positive control by a factor of between 1.7 and 4.1 in the ecosystems with microparticles and films and by a factor of between 1.3 and 1.8 in the experiments with microgranules and pellets. At 20 days, a considerable number of plants in all treatments were dead, and the green biomass was reduced much more dramatically than in the positive control. At 30 days, all plants were dead in the treatments and positive control. Similar results were obtained for *S. macrocheata* plants. The herbicidal activity of the P(3HB)/MET formulations also increased with the increase in the MET loading and with the duration of the experiment (Table 2). At 10 days after sowing, the plant density and the weight of fresh biomass were either comparable with or lower than the corresponding parameters in the positive control, depending on the MET loading and type of formulation. At 20 days, in the ecosystems with P(3HB)/MET microparticles and films, almost all plants were dead; in the ecosystems with microgranules and pellets, the herbicidal effects were less pronounced but stronger than in the positive control (by a factor of 1.5–2.8).

Conclusions

Kinetics of degradation of the polymeric matrix and MET release from slow-release P(3HB)/MET formulations prepared as films, pellets, microparticles, and microgranules were studied in laboratory soil microecosystems with higher plants. The weight loss of formulations and MET concentration in the soil were measured. The study showed that MET release could be regulated by the process employed to fabricate the formulations and by the amount of MET loaded into the polymeric matrix; MET accumulation in soil occurred gradually, as the polymer was degraded. Analysis of soil microbial community showed that all P(3HB)/MET formulations stimulated the development of saprotrophic microorganisms, including the typical rhizosphere bacteria *Pseudomonas* and *Bacillus*. In addition, the number of nitrogen-fixing bacteria had increased compared to their counts in the initial soil. The herbicidal activity of the experimental MET formulations against *A. stolonifera* and *Setaria pumila* plants was compared with the herbicidal activity of the commercial formulation Sencor Ultra (positive control), while intact plants, which were not treated with MET, were used as the negative control. Results of these experiments showed that all P(3HB)/MET formulations exhibited

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pronounced herbicidal activity. The inhibitory effect of the experimental P(3HB)/MET formulations depended on the MET loading and duration of the experiment. In the early stages and at the lowest (10 %) loadings, the effect of P(3HB)/MET was comparable to that of commercial Sencor Ultra. The effects of experimental formulations with higher MET loadings and in longer experiments were superior to the effect of Sencor Ultra. Thus, degradable poly-3-hydroxybuturate can be regarded as a promising material for designing slow-release formulations of the herbicide metribuzin for soil applications.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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