

**CONSTRUCTING SUSTAINED-RELEASE HERBICIDE FORMULATIONS BASED ON
POLY-3-HYDROXYBUTYRATE AND NATURAL MATERIALS
AS A DEGRADABLE MATRIX**

**Short running title: POLY-3-HYDROXYBUTYRATE BASED SUSTAINED-RELEASE
HERBICIDE FORMULATIONS**

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Abstract

Slow-release formulations of three herbicides (metribuzin, tribenuron-methyl, and fenoxaprop-P-ethyl) were constructed as granules and pellets, with the active ingredients loaded into the matrix of biodegradable polymer poly(3-hydroxybutyrate) blended with the natural fillers (clay, wood flour, or peat). Differences in the structure and physicochemical properties of the formulations were studied as dependent on the type of the matrix; herbicide release and accumulation in soil were associated with the solubility of the herbicide. IR spectroscopy showed that no chemical bonds were formed between the components in the experimental formulations. The study conducted in laboratory soil microecosystems demonstrated that degradation of the formulations in soil was chiefly influenced by the shape of the specimens (granules or pellets) while the effect of the type of the filler was insignificant. The use of the granules enabled more rapid accumulation of the herbicides in soil: their peak concentrations were reached after three weeks of incubation while the concentrations of the herbicides released from the pellets were the highest after 5 to 7 weeks. Loading of the herbicides into the polymer matrix composed of the slowly degraded P(3HB) and natural materials enabled both sustained function of the formulations in soil (lasting between one and a half and three or more months) and stable activity of the otherwise rapidly inactivated herbicides such as tribenuron-methyl and fenoxaprop-P-ethyl.

Keywords: *P(3HB)*, blends, herbicides, physicochemical properties, degradation in soil, herbicide release

Introduction

Herbicides (from Latin: *herba* “herb” + *caedo* “killing; killer”) are chemicals that destroy unwanted plants. Herbicides represent the largest group of agrochemicals: 40-50% of all pesticides used by farmers are herbicides, and their diversity reaches about 40%. The reason for this is that weeds, which are represented by thousands of species, cause the greatest damage to agriculture. Most of them are annuals, which germinate from the seeds and live only one season. Perennial weeds, under favorable conditions, are able to reproduce every season. Weeds compete with crops for light, water, and nutrients. Therefore, farmers have to control them. Herbicide-based weed control is one of the major components of modern efficient agriculture. Herbicide application considerably enhances crop yields and, thus, contributes to food production for the growing human population. Moreover, as herbicides remove unwanted plants, they increase the efficiency of harvesters and dramatically decrease labor inputs. Thus, herbicides used in combination with agrotechnical measures remove weeds from the fields and contribute to achievement of high crop yields.

However, an inevitable side effect of using herbicides, as well as other pesticides, is accumulation of high concentrations of chemical compounds in soils. This not only poses hazard to human health but also causes the development of species resistant to herbicides, threatens the stability of agroecosystems, and endangers the long-term soil fertility. Thus, much research effort has been focused recently on development and use of new herbicide formulations, with the active ingredient loaded into matrices of various materials including polymeric ones.¹⁻³

Biodegradable polyesters of microbial origin - polyhydroxyalkanoates (PHA), which are considered as promising polymer materials, have been recently studied for construction of agrochemicals with prolonged effects. The use of poly(3-hydroxybutyrate) P(3HB) and 3-hydroxybutyrate-co-3-hydroxyvalerate copolymers, P(3HB-co-3HV) (the most common representatives of PHAs), for fabricating microspheres loaded with ametrine and atrazine herbicides was previously reported.⁴⁻⁶ Slow-release formulations of the “Zellek Super” herbicide loaded into films and microgranules of [P(3HB-co-3HV)] were prepared and investigated by our team showing effective suppression of model weeds.⁷ Other our research showed the efficacy of slow-release formulations in wheat plant communities contaminated with weeds.⁸ However, because of the relatively high cost of PHAs and the extensive use of pesticides in agriculture, a more effective approach is to blend available natural materials with PHAs rather than to use PHAs alone. Moreover, this approach corresponds to the concept of industrial ecology and “green” chemistry,

focused on deriving new available materials from renewable sources.⁹ The data on producing PHA blends and composites with natural materials are scant. Just a few studies report using fillers such as clay and its derivatives,¹⁰ plant fibers, lignin and holocellulose from lignocellulosic biowaster,¹¹ wood shavings and sawdust.¹² Such blends are primarily considered as material for the manufacture of furniture and construction materials. In the study addressing the use of PHA blends for loading pesticides, PHAs were used in composition with sawdust for embedding metribuzin.¹³ This successful experience defined the purpose of the present study – to develop and investigate slow-release formulations of herbicides, which differ in their structure, solubility, and the types of weed species that they kill, loaded into a degradable matrix of P(3HB) blended with available natural materials (peat, clay, and sawdust). Metribuzin, tribenuron-methyl, and fenoxaprop-P-ethyl are studied as herbicides.

Experimental

Materials

P(3HB) polymer samples were synthesized using the *Cupriavidus eutrophus* B10646 strain and proprietary technology.¹⁴ Polymer was extracted from cells with chloroform, and the extracts were precipitated using hexane. The extracted polymers were re-dissolved and precipitated again 3-4 times to prepare homogeneous specimens. The polymer had the following properties: degree of crystallinity 75%, melting point 176°C, thermal decomposition temperature 287°C, molecular weight (Mw) 590 kDa, and polydispersity index 5.8.

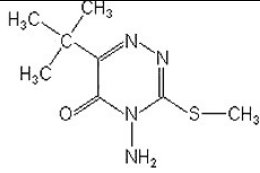
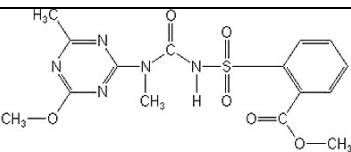
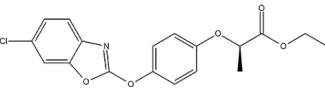
Three natural materials were used as fillers: peat, wood flour, and clay. High-moor peat “Agrobalt-N”, state registration 0428-06-209-139-0-0-01, was produced by OOO “Akademiya tsvetovodstva”, Russia); degree of crystallinity 9%; onset of thermal decomposition 130°C.

Wood flour was produced by grinding wood of birch (*Betula pendula* Roth) using an MD 250-85 woodworking machine (“StankoPremyer” Russia). Then it was dried at 60°C for 120 h until it reached constant weight, and 0.5 mm mesh was used to separate the particle size fraction; degree of crystallinity 26%; onset of thermal decomposition 220°C.

Clay was taken from the mine “Kuznetsovskoye”, the Krasnoyarskii Krai, Russia. Its composition: loss on ignition – 6.92 %; SiO₂ – 60.1 %; Al₂O₃ – 19.17 %; Fe₂O₃ – 6.72 %; CaO – 2.02 %; MgO – 2.12 %; SO₃ – 0.65 %; Na₂O – 0.88 %; K₂O – 1.45 %; degree of crystallinity 53%.

Table 1 – Structure of herbicides

Herbicide	Structural formula	Use
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<p>Metribuzin (4-amino-6-tert-butyl-3-methylthio-1,2,4-triazin-5(4H)-one)</p>		<p>Effectively protects soybean, maize, cereal, potato, and tomato crops from annual dicots and grass weeds</p>
<p>Tribenuron-methyl (methyl ester of 2-(6-methyl-4-methoxy-1,3,5-triazin-2-yl(methyl) carbamoylsulfamoyl) benzoic acid)</p>		<p>Effectively protects cereal crops from dicots and grass weeds</p>
<p>Fenoxaprop-P-ethyl (R)-2-(4-(6-chloro-2-benzoxazolyl)-phenoxy-propanoate)</p>		<p>Effectively protects cereal crops and vegetables from various weeds (barnyard grass, oat grass, bristle grass)</p>

Experimental techniques

Preparation of blends of P(3HB) and natural materials and fabrication of pellets and granules

The polymer and fillers were pulverized by impact and shearing action in ultra-centrifugal mill ZM 200 (Retsch, Germany). To achieve high fineness of polymer grinding, the material and the mill housing with the grinding tools were preliminarily cooled at -80 °C for about 30 min in an Innova U101 freezer (New Brunswick Scientific, U.S.). Grinding was performed using a sieve with 2-mm holes at a rotor speed of 18000 rpm. The fractional composition of the powders was determined using vibratory sieve shaker AS 200 control (Retsch, Germany). Polymer + filler + fungicide (50:30:20 wt.%) powders were mixed in planetary mixer SpeedMixer DAC 250 SP (Hauschild Eng., Germany), for 1 min at 1000 rpm. The resulting homogenous mixtures were used to prepare pellets and granules. Pellets were prepared by cold pressing at a pressure of 36 bar using automatic press “Usilennyi Superpress” (Byelorussia). Granules were prepared by mixing polymer paste wetted with ethanol in screw granulator Fimar (Italy).

A study of the properties of the experimental formulations

The starting herbicides and the experimental specimens, which differed in the composition of the blended matrix, were analyzed using FTIR spectroscopy. IR spectra were taken in the 400-4000 cm^{-1} range using a “NICOLET 6700” FT-IR spectrometer (Thermo Scientific, U.S.) and a Smart Orbit accessory, by the attenuated total reflection (ATR) technique.

Thermal analysis of the starting materials and experimental herbicide formulations were performed using a DSC-1 differential scanning calorimeter (METTLER TOLEDO, Switzerland). Powdered samples (4.0 ± 0.2 mg each) were placed into the aluminum crucible and compressed prior to measurement. Every sample was measured at least 3 times. The samples were heated once, from -20 °C to 320 °C. Glass transition temperature (T_g), crystallization temperature (T_c), melting point (T_{melt}), and thermal degradation temperature (T_{degr}) were determined from peaks in thermograms using the “StarE” software.

X-Ray structure analysis and determination of crystallinity of the starting materials and experimental herbicide formulations were performed employing a D8 ADVANCE X-Ray powder diffractometer equipped with a VANTEC fast linear detector, using CuK α radiation (Bruker, AXS, Germany). The degree of crystallinity was calculated as a ratio of the total area of crystalline peaks to the total area of the radiograph (the crystalline + amorphous components). The error in determination of the degree of crystallinity, which was calculated based on multiple measurements, was 2% or less.

The surface microstructures of the specimens (pellets and granules) were analyzed before and after incubation in soil using scanning electron microscopy (a TM-3000 Hitachi microscope with the QUANTAX 70 program, Japan). Prior to microscopy, the samples were sputter coated with platinum (at 10 mA, for 40 s), with an Emitech K575XD Turbo Sputter Coater (Quorum Technologies Limited, U.K.).

Degradation of herbicide formulations in soil

Biodegradation of the experimental herbicide formulations was studied in laboratory, using agro-transformed soil collected at field laboratory “Minderlinskoye” of the Krasnoyarsk State Agrarian University (56°N , 92°E). Soil was placed into plastic containers (100 g soil in each). Herbicide formulations were placed into fine-meshed gauze bags and incubated in soil at a temperature of 25 °C and constant moisture content of 50%. The experiment lasted 83 days. Three samples of each type were collected periodically to investigate biodegradation of the polymeric matrix and herbicide release. Mass loss was measured to estimate degradation. Three specimens of each type were periodically taken out of the soil, rinsed with distilled water, dried to a constant weight, and weighed on the analytical balance of accuracy class 4 (Metler, Toledo). The mass (X) was determined as follows:

$$X_{\%} = \frac{Y_1 - Y_2}{Y_1} \times 100,$$

where Y_1 and Y_2 denote the average mass of the samples (mg) before and after testing, respectively.

For describing herbicide release kinetics from different formulations, we used the Korsmeyer – Peppas model (formula 3):

$$M_t / M_{\infty} = Kt^n,$$

where M_t is the amount of the herbicide released at time t , 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. For the case of cylindrical pellets, $0.45 \geq n$ 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 $M_t / M_{\infty} < 0.6$ should only be used.¹⁵

A microbiological study

Analysis of microflora in the samples of field soil was conducted using conventional methods of soil microbiology.¹⁶ Soil suspension dilutions from 10^3 to 10^7 were performed and plated on selective media in triplicate. The abundances of eco-trophic bacterial groups and their ratios were determined as follows: copiotrophs were counted on nutrient agar (HiMedia, India); prototrophs on starch and ammonia agar (SAA); oligotrophs on soil extract agar (SA), and nitrogen-fixing bacteria on Ashby's medium. The mineralization coefficient was determined as the ratio of prototrophic to copiotrophic bacteria. The oligotrophic coefficient was the ratio of oligotrophic to copiotrophic bacteria. The plates were incubated for 3-7 days at 30°C for bacteria and at 25°C for fungi. Pure cultures of dominant bacteria were isolated and identified by the MALDI time-of-flight mass spectrometry method using a MALDI-TOF mass spectrometer (Bruker, Germany).

A short duration acute experiment was done to study the effect of free herbicides on soil microorganisms 24 h after application. Herbicide concentration was 0.1 mg/g of soil. The effect of slow-release formulations of herbicides on soil microflora was investigated after 83 d of incubation of formulations in soil microecosystems.

Herbicide release systems

Herbicides were extracted from soil with chloroform; then chloroform was removed, and herbicide concentrations in soil were determined by chromatography. A 10-g soil sample was extracted with 50 ml chloroform while agitating it on a shaker for 24 h at a temperature of 25°C. 25

ml of the resulting extract was concentrated using a rotary evaporator, and the residue was re-dissolved in 2 ml acetonitrile.

Herbicide concentrations were analyzed using high-performance liquid chromatography, with an Agilent 1200 Infinity chromatographic system (Agilent Technologies, U.S.) equipped with a gradient pump, autosampler, a column thermostat, and a diode matrix detector. An Eclipse XDB-C18 column was used. Chromatography was performed in the gradient of water acidified with 0.1% acetic acid and acetonitrile, with acetonitrile concentration gradually increased from 45% to 60%. Chromatography lasted 12 min. Detection was done at the wavelengths located at the absorption maxima of the herbicides: at 298 nm for metribuzin, at 220 nm for tribenuron-methyl, and at 280 nm for fenoxaprop-P-ethyl. The reference values were recorded at a wavelength of 360 nm. The range of the concentrations detected was between 1 µg/ml and 500 µg/ml or higher. Calibration curves were plotted by using high-purity compounds: state standard reference samples (GSO) of metribuzin (GSO 7713-99) and tribenuron-methyl (GSO 8628-2004) and a standard reference sample (SOP) of fenoxaprop-P-ethyl (SOP 52-06). Calibration solutions of the active ingredients of concentrations 0.1, 1, 10, 100, 500, and 1000 µg/ml were used.

Statistics

Statistical analysis of the results was performed by conventional methods, using the standard software package of Microsoft Excel, STATISTICA 8. Arithmetic means and standard deviations were found. The statistical significance of results was determined using Student's *t*-test (significance level: $P \leq 0.05$). Statistical analysis of surface properties of the samples was performed by using embedded methods of the DSA-4 software.

3. Results and discussion

3.1. Characterization of slow-release herbicide formulations

Blends of powdered P(3HB) and natural materials (fillers) mixed with herbicide powders (metribuzin, tribenuron-methyl, or fenoxaprop-P-ethyl) were used to fabricate slow-release formulations shaped as compacted pellets, 6 mm in diameter and 3 mm high, and granules, 2 mm in diameter and 2 to 4 mm long. Photographs and SEM images of the pellets and granules are shown in Figures 1 and 2A.

Figure 1 – Photographs of the formulations of herbicides (tribenuron-methyl) loaded into a P(3HB)/natural material degradable matrix.

SEM images of formulations show differences in their surface microstructure, which are mainly associated with the type of the filler. P(3HB)/clay and, especially, P(3HB)/peat specimens had loose surfaces, with numerous defects, namely, pores and voids (shown by arrows), in contrast to P(3HB)/wood flour specimens. The surface of P(3HB)/wood flour specimens was more solid, with a few fractures and polymer flakes, except the specimen with fenoxaprop-P-ethyl (Fig. 2 A).

Figure 2 – SEM images of slow-release herbicide formulations with the active ingredient loaded into the matrix of degradable P(3HB) and natural materials: 1 – tribenuron-methyl, 2 – fenoxaprop-P-ethyl, 3 – metribuzin; pre-incubation formulations. B – after incubation in soil.

After the specimens had been incubated in soil for 83 days, their surfaces differed significantly, with the greatest differences observed between the P(3HB)/wood flour and P(3HB)/clay specimens. On the surface of the specimens with fenoxaprop-P-ethyl and metribuzin, there were large, 100 to 300 μm , pores.

Physicochemical properties of the starting herbicides and the slow-release formulations are listed in Table 2 and Fig.2 B.

Table 2 – Physicochemical properties of the free herbicides and experimental formulations

Herbicide	Molecular weight, M, kDa	Degree of crystallinity, C_x , %	T_{melt}
P(3HB)	585110	75	176
Starting materials and herbicides			
Metribuzin	214.3	95	126.9
Fenoxaprop-P-ethyl	361.8	92	88.7
Tribenuron-methyl	395.4	95	136.9
Clay		53	200*
Peat		9	130*
Wood flour		26	220*
Properties of the blends used to prepare formulations			
Composition	C_x , %	T_{melt} °C	T_{degr} °C (onset of sample decompositi on)
P(3HB)/wood flour/metribuzin	65	127.9; 162.8	261.4

P(3HB)/peat/metribuzin	61	126.7; 164.9	271.5
P(3HB)/clay/metribuzin	79	127.2; 161.6	262.7
P(3HB)/wood flour/fenoxaprop-P-ethyl	61	88.7; 171.5	262.7
P(3HB)/peat/fenoxaprop-P-ethyl	56	88.5; 169.3	277.3
P(3HB)/clay/fenoxaprop-P-ethyl	74	88.5; 168.5	265.7
P(3HB)/wood flour/tribenuron	61	133.1; 164.1	265.3
P(3HB)/peat/tribenuron	60	128.4; 164.2	271.5
P(3HB)/clay/tribenuron	77	132.6; 163.7	249.8

(*) - start of T_{degr} .

The herbicides used in this study differed in their molecular weight and thermal degradation temperature (M_w and T_{degr}); also, they showed higher degrees of crystallinity than P(3HB) and the free herbicides. In the three-component (polymer/filler/herbicide) experimental formulations, the crystalline phase prevailed over the amorphous phase (Table 2), and the degree of crystallinity in all specimens varied between 60 and 77%, except the C_x of P(3HB)/peat/fenoxaprop-P-ethyl – 56%. The temperature properties of the experimental specimens differed depending on the type of the herbicide used. All thermograms showed two peaks, corresponding to melting of the pesticide and polymer, suggesting that the components separated and there was no chemical bonding between the matrix and the herbicide. The melting point of P(3HB) in the blend was, however, lower than that of the pure polymer. The most dramatic decrease in the melting point was observed for the formulations containing metribuzin and tribenuron: to 161 - 164°C. For the formulations with metribuzin, the onset of thermal decomposition occurred at a temperature between 261 and 271°C, and for those with fenoxaprop-P-ethyl between 262 and 277°C. The earliest onset of thermal decomposition – at a temperature of 250°C – was observed for a tribenuron-containing specimen (P(3HB)/clay/tribenuron).

To determine the type of interactions between the composite polymer matrix and herbicides loaded into it, we used IR spectroscopy (Fig. 3), which showed that no chemical bonds formed between the herbicide and the matrix, i.e. their interactions were of physical nature. Figure 2 shows IR spectra of fenoxaprop-P-ethyl loaded into degradable matrices with different compositions.

Figure 3 – IR spectra of the experimental formulations of fenoxaprop-P-ethyl loaded into the degradable matrix of P(3HB) and natural fillers

The IR spectra contained absorption bands characteristic of the herbicide and materials of the matrix. The spectra showed distinct stretching vibrations of the carbonyl groups C=O of the polymer (1700-1760 cm^{-1}) and superposition of vibrations of the fenoxaprop-P-ethyl C=O groups as well as asymmetric stretching of CH₃- and CH₂-groups (2994, 2974, 2936 cm^{-1}). Stretching and skeletal vibrations of the benzene ring (1422 cm^{-1} , 1504 cm^{-1} , 1568 cm^{-1}) were visible. The vibrations at 1192, 1134 cm^{-1} were regarded as asymmetric and symmetric vibrations of the C-O-C bond. The spectra of the specimens containing clay had the bands characteristic of inorganic compounds; the peak at 511 cm^{-1} was regarded as the Si-O-Si stretching vibration. The absorption frequency at 1635 cm^{-1} indicated deformation vibrations of H-O-H, suggesting water sorption on the surface of the mineral. The 3615 and 3392 cm^{-1} absorption band corresponded to AlO-H stretching and symmetric vibrations for the bound water.

The presence of the 1687 cm^{-1} absorption band, characteristic of the H vibrations in the OH-group, broad absorption bands in the 2900 – 3100 cm^{-1} range, and the shift of the C=O vibrations from 1735 and 1790 cm^{-1} , characteristic of pure fenoxaprop-P-ethyl and P(3HB), to 1720 cm^{-1} were indicative of the weak hydrogen bonds in the specimens.

3.2. Degradation of slow-release herbicide formulations in soil

Studies of biodegradation of the experimental herbicide formulations were performed in laboratory soil microecosystems. The soil in which the specimens were incubated was a heavy loamy granular black soil with a density of 0.80 to 1.24 g/cm^3 ; pH 7.3, with a low content of ammonium (35 mg/kg) and nitrate (9.2 mg/kg) nitrogen; the phosphorus and potassium contents were 280 and 250 mg/kg , respectively. As the main factor of biodegradation was P(3HB) soil microflora and the content of P(3HB) in the formulations was 50%, special attention was paid to microbiological research. The total abundance of copiotrophic bacteria in the starting soil was $(1.1 \pm 0.9) \cdot 10^6$ $\text{CFU} \cdot \text{g}^{-1}$ of air-dry soil, but oligotrophic and prototrophic bacteria prevailed over copiotrophs, amounting to $(76.7 \pm 12.4) \times 10^6$ and $(5.3 \pm 1.8) \times 10^6$ $\text{CFU} \cdot \text{g}^{-1}$, respectively. Hence, the oligotrophy and mineralization coefficients were high, 6.76 and 4.72, respectively, suggesting, along with the high abundance of nitrogen fixers, soil depletion and shortage of available forms of nitrogen.

The specimens of herbicide loaded polymer/filler carriers were degraded gradually. The residual masses of the pellets and granules at the end of the experiment were 45-70% and 60-80%, respectively, of their starting masses (Fig. 4). The composition of the matrix was also a factor, although a minor one, that affected mass loss of the slow-release herbicide formulations. Peat-containing pellets and clay-containing granules were degraded at faster rates throughout the experiment.

Figure 4 – Mass loss kinetics of slow-release formulations of herbicides loaded into degradable matrices (polymer/filler) with different compositions: I– metribuzin, II – tribenuron-methyl, III – fenoxaprop-P-ethyl.

The photographs (Fig. 5) and SEM images (Fig. 2 B) show that as the specimens were degraded, their surfaces became less uniform and looser; the number of defects and voids increased, especially in the specimens with the matrix containing peat and clay and loaded with fenoxaprop-P-ethyl and metribuzin. Nevertheless, all forms were able to function in the soil for a long time, despite their biodegradation.

Figure 5 – Photographs of slow-release herbicide formulations with the active ingredient loaded into degradable polymer/filler matrices of various compositions: 1– fenoxaprop-P-ethyl, 2 – tribenuron-methyl, 3 – metribuzin

Degradation kinetics of the majority of the experimental slow-release herbicide formulations was as follows: $\ln M_i = \ln M_0 + kt$, where M_0 ; M_i are the starting mass of the specimen and the mass of the specimen after degradation, g, k is degradation rate constant, and t is time, h. The validity of this equation for describing degradation kinetics of all the specimens was supported by the high accuracy of approximation (between 0.91 and 0.98) (Table 3). The highest values of degradation rate constant (0.00746 and 0.0094) were found for the specimens with metribuzin loaded into the clay- and peat-containing matrices and tribenuron loaded into the clay- and peat-containing matrices (0.0074 and 0.0077).

Table 3 – Degradation rate constant for formulations with herbicides loaded into the polymer/filler matrices of different compositions

Herbicide formulations	Degradation rate constant, k	Approximation accuracy, R^2
Pellets		
P(3HB)/wood flour/metribuzin	0.0064	0.93
P(3HB)/peat/metribuzin	0.0094	0.98
P(3HB)/clay/metribuzin	0.0074	0.92
P(3HB)/wood flour/tribenuron	0.0069	0.96
P(3HB)/peat/tribenuron	0.0074	0.94
P(3HB)/clay/tribenuron	0.0077	0.97

P(3HB)/wood flour/fenoxaprop-P-ethyl	0.0060	0.96
P(3HB)/peat/fenoxaprop-P-ethyl	0.0052	0.95
P(3HB)/clay/fenoxaprop-P-ethyl	0.0040	0.91
Granules		
P(3HB)/wood flour/metribuzin	- 0.0070	0.97
P(3HB)/peat/metribuzin	- 0.0100	0.98
P(3HB)/clay/metribuzin	- 0.0082	0.96
P(3HB)/wood flour/tribenuron	- 0.0042	0.99
P(3HB)/peat/tribenuron	- 0.0052	0.99
P(3HB)/clay/tribenuron	- 0.0071	0.99
P(3HB)/wood flour/fenoxaprop-P-ethyl	- 0.0033	0.93
P(3HB)/peat/fenoxaprop-P-ethyl	- 0.0062	0.93
P(3HB)/clay/fenoxaprop-P-ethyl	- 0.0039	0.91

Thus, herbicide formulations were capable of functioning in soil longer than the experimental period of 83 d.

As the available literature contains no data on degradation behavior of slow-release herbicide formulations in the PHA/filler matrix, it does not seem possible to compare the results obtained in this study with the literature data. However, PHA degradation behavior in natural media has been thoroughly studied by different authors, including our team. Those studies showed that degradation occurred at different rates depending on the polymer composition, shape of the specimen (film or 3D construct), climate and weather conditions, and microbial community composition. The time over which the polymer loses 50% of its mass may vary between 68.5 and 270 days in Siberian soils,¹⁷ between 16 and 380 days in tropical soils of Vietnam,¹⁸ between 73 and 324 days in the brackish lake (Shira),¹⁹ between 127 and 220 days in the seawater of the South China Sea,²⁰ and between 17 and 65.9 days in freshwater Siberian recreational water bodies²¹. These data support the choice of PHAs for constructing slow-release formulations.

3.3. The effects of herbicides on soil microflora

Soil application of the pesticides loaded into the degradable matrix containing poly-3-hydroxybutyrate provided microorganisms with a supplementary substrate. On the other hand, the herbicide released during degradation of the matrix could exert a negative effect on the microflora. Soil microbial communities are the main component in detoxification and transformation of pesticides and self-purification of the environment. Pesticides applied to soil interact with microorganisms, usually producing an adverse effect on soil biota.^{22,23} Du et al.²⁴ demonstrated that

the trifluralin herbicide decreased species diversity of soil bacteria. The 2,4-D herbicide changed the structure of the bacterial rhizosphere community of *Acacia caven*.²⁵ Other data, however, suggested that slow-release formulations of herbicides loaded into carriers, unlike their free forms, did not inhibit the growth of soil microbiota and stimulated enzymatic activity of soil. For instance, application of controlled release formulations of diuron and alachlor, loaded into phosphatidylcholine-montmorillonite complex, enhanced dehydrogenase, phosphatase and β -glucosidase activity in soil.²⁶ The addition of compost or sewage sludge to soil reduced the negative effects of herbicides on soil microorganisms.²⁷

To study the impact of herbicides on soil microflora, special tests were carried out. The effects of pure metribuzin, fenoxaprop-P-ethyl, and tribenuron-methyl herbicides at a concentration of 0.1 mg/g soil on the species composition of bacteria were studied in short-term experiments. Figure 6 shows results of the microbiological study of the starting field soil (control) and the soil 24 h after the application of the three herbicides.

Figure 6 – Species composition of the dominant bacteria in the starting soil and 24 h after application of the herbicides

The microbial community of the starting soil was dominated by Gram-positive bacteria (61.8%), primarily *Bacillus* species such as *B. horikoshii* (26.3%) and *B. pumilus* (17.4%). The second most abundant genus was *Agrococcus* (7.9%). *Micrococcus luteus*, *Psychrobacillus psychrodurans*, and *Streptomyces avidinii* were minor species, constituting no more than 5%. The Gram-negative bacteria were dominated by *Stenotrophomonas rhizophila* (27.6%), and *Pseudomonas kilonensis* constituted 5.3%. Microbiological analysis showed that the herbicides caused a 9.4-15.8-fold decrease in the total abundance of organotrophic bacteria in 24 h. The herbicides had a selective effect on the species composition of the microbial community: tribenuron-methyl and metribuzin increased the proportion of Gram-negative bacteria to 47.9 and 53.0%, respectively (Fig. 6). A considerable increase was observed in the proportion of *Pseudomonas*: *P. brassicacearum* (15.0-31.6%) and *Pseudomonas kilonensis* (21.2-39.4%). On the other hand, soil application of fenoxaprop-P-ethyl caused the most substantial decrease in the species diversity, resulting in the absolute predominance of *Bacillus*. Hence, herbicides accumulated in soil inhibit the growth of organotrophic bacteria and change the proportions of the major species in the microbial community.

The study of the effects that the experimental herbicide formulations incubated in soil for period of the experiment ~~an extended period of time (83 days)~~ exerted on soil microflora produced the following results. Analysis of the abundance of organotrophic bacteria showed that after 83 days of incubation of the slow-release herbicide formulations in the soil, the total abundance of

copiotrophic bacteria increased by a factor of 4-10 compared to the control, without herbicide application. The addition of polymer/wood flour formulations loaded with metribuzin or tribenuron-methyl increased the abundance of copiotrophs but reduced the mineralization and oligotrophy coefficients. The likely reason for this was the input of supplementary substrate for the microorganisms: P(3HB), its degradation products, and wood flour components including humic acids. By contrast, soil application of the polymer/clay herbicide formulations caused an increase in the abundance of oligotrophic bacteria and higher oligotrophy coefficients compared to formulations with the other fillers. This may be attributed to the input of organic matter – polymer degradation products – and an increase in the amount of the available organics.

Examination of the species composition of soil microflora at the end of the experiment (83 d) revealed certain changes in the proportions of the major taxa in soil samples with slow-release herbicide formulations compared to the control soil. *Bacillus* species, constituting up to 28.7%, prevailed in both groups of soil samples. The proportion of *Streptomyces* increased to 21-24.5% on average and *Pseudomonas* to 5.6-12.3%, while *Stenotrophomonas rhizophila* decreased to 8-9.7%. The type of the filler in formulations did not substantially influence the taxonomic composition of the dominant species in microbial communities. Representatives of *Bacillus*, *Pseudomonas*, and *Streptomyces* were previously identified as P(3HB)-degrading microorganisms (Volova et al., 2017). Thus, application of slow-release herbicide formulations did not decrease soil capacity for microbial degradation of complex organic compounds, including pollutants.

3.4. Monitoring concentrations of herbicides released from the experimental formulations in soil

Measurements of residual concentrations of herbicides in the experimental formulations and monitoring of their concentrations in soil provided data for estimating their accumulation in soil (Fig. 7). The herbicide concentrations in soil were measured at definite time points of the experiment, without taking into account transformation and degradation of the formulations induced by biogenic and abiogenic processes occurring in soil.

Herbicide release kinetics was influenced by various factors such as kinetics of degradation of formulations in soil, shape of the specimens, composition of the matrix, solubility of the herbicides, and stability of the herbicides in soil. Soil is a complex and multifactor system, in which various biogenic and abiogenic processes influence concentrations of the chemicals applied to the soil. First, the chemicals are transformed by soil microflora in processes of co-metabolism and enzymatic oxidation. Furthermore, they are dissolved and degraded due to natural abiogenic processes occurring in the soil.

Herbicide concentrations in soil at different time points of the experiment are shown in Figure 7; kinetic constants of herbicide release rates are given in Table 4. Herbicides released from the granules reached their peak concentrations in soil after 20 days of incubation while the herbicides released from most of the pellets reached their highest concentrations in soil much later, at Days 48-50. The solubility of the rapidly dissolving metribuzin was 1.2 mg/L, whereas the solubilities of the poorly soluble tribenuron-methyl and fenoxaprop-P-ethyl were 2 mg/L and 0.7 µg/L, respectively. Moreover, the herbicides tested in this study had different lifetimes in soil. The half-life of metribuzin was found to vary between 44 d²⁸ and 172 d,²⁹ depending on the soil type and environmental conditions. The half-life of tribenuron-methyl reported in different studies varied between three days³⁰ and less than three weeks.³¹ For fenoxaprop-P-ethyl, this parameter varied between 0.5 d³² and several days,^{33,34} its longest half-life measured at 16.61 d.³⁵ Therefore, the contents of these herbicides in soil were determined not only by their release kinetics but also by their lifetimes in soil.

Figure 7 – Concentrations of herbicides released from the formulations with the active ingredient embedded in the P(3HB)/natural material matrix in soil at different time points: I - metribuzin, II – tribenuron-methyl, III - fenoxaprop-P-ethyl

Regardless of the type of the filler, metribuzin released from the pellets reached its peak concentrations in soil after 6-7 weeks of incubation: 60 to 80 mg/g soil, i.e. 50-80% of the loaded active ingredient, and remained steady for a long time, declining insignificantly by the end of the experiment. Residual metribuzin in the formulations was no more than 5-8% of its starting amount, i.e. the loaded herbicide was almost completely released to soil. Concentrations of the metribuzin released from the granules varied in a similar way, but the peak concentration was reached sooner: after three weeks for the specimens containing peat and after four weeks for the granules containing clay and wood flour. Metribuzin release from the pellets corresponded to the Fickian diffusion mechanism and from the granules to the non-Fickian transport, regardless of the filler type, suggesting considerable structural differences between the carriers (Table 4). Thus, metribuzin was steadily and gradually released from both pellets and granules and, hence, was present in the soil over extended time periods.

The literature data on metribuzin, an extensively studied herbicide, suggest that by loading this herbicide into matrices made from different materials, one can change its release kinetics considerably, i.e. construct controlled herbicide delivery systems. A study of acrylamide-bentonite (aluminum silicate clay mineral) composites as a matrix for metribuzin encapsulation suggested that metribuzin release kinetics was influenced by the amounts of metribuzin and bentonite loaded into

the polymer matrix.²⁵ The authors of that study showed that 50% of the encapsulated metribuzin was released after 25 to 51 days. Metribuzin release to soil from the granules prepared from a composite of alginate and bentonite or anthracite (metribuzin/alginate/bentonite or metribuzin/alginate/anthracite) was about 80% for the first 6 days at 25°C.³⁶ Other authors studied metribuzin release to the buffer solution from natural polymers (chitin, dextran, cellulose and starch) and synthetic acrylic polymers linked with metribuzin by the amide bond. Metribuzin was generally released in 40 days, and the process was influenced by the hydrophilicity of the polymer and types of bonding with the polymer.

Slow-release formulations of the metribuzin herbicide (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 study of the kinetics of MET release to water⁴⁵ showed that passive release of the herbicide to water, mainly due to leaching and dissolution, occurred in the first 12-14 days; then the curves plateaued. Theoretical analysis showed that the kinetic constant, K , was the highest for microspheres, 0.081 h^{-1} , and the lowest for films and 3D constructs (0.01 h^{-1}). 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. The kinetics of polymer degradation and MET release and accumulation in soil were studied in laboratory soil microecosystems with higher plants.³⁷ The study showed that MET release could be controlled by using different techniques of constructing formulations and by varying MET loading. MET accumulation in soil occurred gradually, as the polymer was 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/d over 60 days for microparticles, films, microgranules, and pellets, respectively.

Slow-release formulations of the other two herbicides (tribenuron-methyl and fenoxaprop-P-ethyl) were first prepared and described in the present study.

Tribenuron-methyl release kinetics and the time when it reached its maximum concentration in soil were determined by the shape of the specimen as well. The highest concentration of this herbicide in the soil was reached after three weeks for the granules and after 7 weeks for the pellets. Herbicide release from the specimens containing wood flour and peat corresponded to Case II (relaxation) transport ($n=0.89$). For pellets, the highest herbicide concentrations in soil (about 6 $\mu\text{g/g}$) were found for the specimens containing clay or peat as the filler (Fig. 7). Yet, pesticide concentration in soil was below 10% of the loaded amount, about 6-7 $\mu\text{g/g}$ soil or less, throughout the experiment.

This can be attributed to the more rapid decomposition of tribenuron-methyl in soil compared to metribuzin, which is supported by the literature data on the half-life of this herbicide – between three days and three weeks.^{30,31}

Herbicide release from the specimens containing wood flour and peat corresponded to Case II (relaxation) transport ($n=0.89$).

Table 4 – The constant of the rate of herbicide release from polymer/filler formulations with different compositions to soil

Herbicide formulations	Kinetic constant, k (h ⁻¹)	Diffusional exponent, n	Approximation accuracy, R ²
Pellets			
P(3HB)/wood flour/metribuzin	0.060	0.35	0.96
P(3HB)/peat/metribuzin	0.001	0.89	0.98
P(3HB)/clay/metribuzin	0.031	0.44	0.91
P(3HB)/wood flour/tribenuron	0.002	0.83	0.97
P(3HB)/peat/tribenuron	0.039	0.42	0.90
P(3HB)/clay/tribenuron	0.040	0.43	0.92
P(3HB)/wood flour/fenoxaprop-P-ethyl	0.029	0.45	0.98
P(3HB)/peat/fenoxaprop-P-ethyl	0.039	0.42	0.94
P(3HB)/clay/fenoxaprop-P-ethyl	0.025	0.45	0.93
Granules			
P(3HB)/wood flour/metribuzin	0.011	0.69	0.94
P(3HB)/peat/metribuzin	0.010	0.66	0.94
P(3HB)/clay/metribuzin	0.010	0.65	0.99
P(3HB)/wood flour/tribenuron	0.005	0.89	0.99
P(3HB)/peat/tribenuron	0.007	0.89	0.98
P(3HB)/clay/tribenuron	0.009	0.70	0.94
P(3HB)/wood flour/fenoxaprop-P-ethyl	0.052	0.37	0.97
P(3HB)/peat/fenoxaprop-P-ethyl	0.033	0.42	0.98
P(3HB)/clay/fenoxaprop-P-ethyl	0.112	0.30	0.97

For the specimens containing fenoxaprop-P-ethyl, an herbicide with much lower water solubility (0.7 µg/L), herbicide release corresponded to the Fickian diffusion mechanism, regardless of the filler type and the shape of the specimen. The highest fenoxaprop-P-ethyl concentrations in

soil in the experiment with the pellets were reached at different time points: after three to seven weeks of incubation. The absolute values of the concentrations differed considerably as well: about 2 mg/g soil in 7 weeks when released from the pellets made from P(3HB)/peat and about 0.5 mg/g soil in three weeks when released from the pellets made from P(3HB)/clay and P(3HB)/wood flour. The highest concentration of the herbicide released from the granules containing peat was about 1.8 mg/g soil, and it was reached after three weeks of incubation in soil. When, however, fenoxaprop-P-ethyl was released from the specimens containing clay or wood flour, the highest concentration of the herbicide in soil was reached much later (in 7 weeks). These differences were evident in the kinetic constants, which varied for granules between 0.33 and 0.112 at a high level of approximation accuracy (0.97-0.98). The curves describing fenoxaprop-P-ethyl concentration in soil declined more or less dramatically after peaking as fenoxaprop-P-ethyl broke down quickly (the $T_{0.5}$ of this compound is usually no more than three days). The amount of fenoxaprop-P-ethyl released to soil was considerably lower: throughout the experiment, concentrations of the active ingredient in soil were no more than 2.5 $\mu\text{g/g}$ soil, or 2.5% of the amount loaded into the matrix (Fig. 7). Yet, the loading of this extremely unstable herbicide into the matrix enabled its presence in soil during the experiment (83 days).

4. Conclusion

Herbicide formulations enabling slow controlled release of the active ingredients were fabricated. The degree of solubility of the herbicide considerably affected its release rate and degradation of the formulations in soil: the rapidly dissolving metribuzin and tribenuron-methyl were released at faster rates than the less readily soluble fenoxaprop-P-ethyl. Loading of the herbicides into the polymer matrix composed of the slowly degraded P(3HB) and available natural materials enabled both sustained function of the formulations in soil (lasting between one and a half and three or more months) and stable activity of the otherwise rapidly inactivated herbicides such as tribenuron-methyl and fenoxaprop-P-ethyl.

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