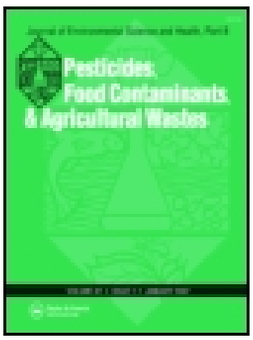

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ABSTRACT

Polymer poly(3-hydroxybutyrate) [P(3HB)] has been used as a matrix in slow-release formulations of the herbicide metribuzin (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 from the polymer matrix has been 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.

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KEYWORDS
Poly(3-hydroxybutyrate); metribuzin; embedding; slow-release formulations; controlled release

Introduction

The rapid development of chemistry and intensive farming has facilitated the production and use of a vast variety of chemicals intended for protecting crops from pests, weeds, and pathogens. Their application, however, creates certain risks. For instance, only small percentages of pesticides applied to crops reach their targets, while the major part of these substances kill useful organisms, accumulate in biological objects, unbalance natural ecosystems, and pollute soil, water, and air. The amounts of pesticides used in various areas are enormous, and they are growing continuously. The accumulation of pesticides in the biosphere via their buildup and concentration in trophic chains of biota in agroecosystems and natural ecosystems is one of the world's most pressing environmental issues. Therefore, alternative means and methods of crop protection, which would be more effective and harmless to humans and the whole environment, need to be developed.[1] A new direction, aimed at reducing the risk of the uncontrolled distribution and accumulation of pesticides in the environment, is the development of a new-generation environmentally friendly formulations with a targeted and controlled release of the active ingredient owing to the use of special coatings and/or matrices produced from biodegradable materials.[2]

Pesticides are mostly used in agriculture for controlling arthropods, nematodes, fungal and bacterial diseases of animals and plants, and weeds. One of the modern systemic herbicides that are widely used and extensively studied is metribuzin. Metribuzin (MET) is an asymmetric triazine herbicide, which has very high biological efficacy in different climate zones. Metribuzin is used as a pre-emergence and post-emergence herbicide to control such weeds as *Phalaris minor*, *Cynodon dactylon*, *Chenopodium album*, *Cyperus spp*., etc. This herbicide readily dissolves in water, and it is weakly sorbed in soil, contaminating groundwater. The loss of activity of metribuzin is mainly due to its being washed out of the lower soil layers. Owing to these limitations, metribuzin makes a good test pesticide for experiments with controlled pesticide delivery systems. The first studies describing the preparation of slow-release metribuzin formulations appeared in the late 1980s to early 1990s. They reported metribuzin encapsulation in polymer matrices based on 2-methyl-4-chlorophenoxyacetic acid and pentachlorophenol, a malic anhydride/methyl methacrylate copolymer, and chitin, and metribuzin binding with β-cyclodextrin.[3–7] Slow-release metribuzin (MET) formulations with the following natural and synthetic materials have been described so far: sepiolite;[8] alginate, bentonite, anthracite;[9] polyvinylchloride, carboxymethyl cellulose;[10] acrylamide/bentonite;[11] methacrylic acid combined with ethylene glycol and dimethacrylate;[12] phosphatidylcholine;[13] kraft lignin;[14] ethyl cellulose and lignin/polyethylene glycol blends.[15,16] Some authors reported the preparation of metribuzin chemically bound with polymer-clay composites through free radical polymerization with *N*-diacryloyl and methyl methacrylate and formulations of metribuzin embedded in polymeric systems of natural origin (chitin, dextran, cellulose, starch).[17,18] Granular metribuzin formulations were prepared by using the gelling sodium alginate and bentonite, anthracite, and activated carbon.[19]

It is important that materials used as matrices for pesticides should both enable slow-release and targeted and effective delivery and be readily processable and reasonably priced. The cost of the product is one of the major factors determining whether a given line of research should be developed and whether the novel agrochemical formulations will be used in practical agriculture.

The analysis of the literature suggests lively interest in synthesis and investigation of polymers based on derivatives of

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carbonic acids. In addition to polylactides and polyglycolides, special attention is given to such biodegradable polyesters as polyhydroxyalkanoates (PHAs)—microbial polyesters that have many useful properties. PHAs are promising materials for various applications.[20,21] PHAs are thermoplastic and have good physical and mechanical properties, like synthetic polyolefins such as polypropylene and polyethylene, but, in addition to this, they are also biocompatible and biodegradable. These polymers are degraded in biological media (soil, rivers, lakes, seas) by natural microflora. In contrast to polylactides and polyglycolides, PHAs do not undergo rapid chemical hydrolysis; they decompose via truly biological degradation, and, thus, it takes months for them to be fully degraded in biological media, which is very important for construction of long-term formulations. The ability of PHAs to be degraded in soil is a basis for constructing pre-emergence herbicide formulations, which can be buried in soil together with seeds of cultivated plants and thus prevent the growth of undesirable plants. The advancement of PHA biosynthesis processes has enabled the scale-up of the industrial production of these polymers.[22] This is the way to reduce their factory cost and widen the range of their applications. So far, very few studies have reported the use of PHAs to construct environmentally safe pesticide formulations. The first studies addressing the use of P(3HB) and P(3HB/3HV) copolymers as matrices for embedding pesticides Ronilan, Sumilex and α-hexachlorocyclohexane, lindane, were reported by Savenkova et al.,[23] and Voinova et al.,[24,25] respectively. Some authors reported encapsulation of pesticides ametrine, atrazine and malathion in microspheres prepared from P(3HB) and P(3HB/3HV).[26–29] In a more recent study, Prudnikova and co-authors described embedding of herbicide Zellek Super in P(3HB/3HV) granules and films to prepare slow-release formulations.[30]

The purpose of this study was to construct slow-release herbicide (metribuzin) formulations by using a natural degradable polymer, poly(3-hydroxybutyrate).

Materials and methods

Materials

Chemically pure metribuzin [$C_8H_{14}N_4OS$] (99.7% pure) was used (State Standard Sample 7713-99—the state standard accepted in Russia (Blok-1, Moscow). Metribuzin has a systemic effect against many undesirable plants in vegetable and grain crop fields, and both of them have a foliar action and can penetrate into plants through their roots; this herbicide inhibits plant photosynthesis. The structural formula of metribuzin is shown in Figure 1. It has the following main physicochemical properties: colourless crystals; molecular weight 214.3 g mol^{-1}; melting point 126.2°C; solubility at 20°C (g L^{-1}) in water: 1.2, in chloroform: 850, and in acetone: 820. Log K_{ow} 1.60. pK_a 7.1.

Polymer poly(3-hydroxybutyrate) – P(3HB) – was used as a degradable polymer 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. Inoculum was prepared by resuspending the museum culture maintained on agar medium. Museum culture was grown in 1-L to 2-L glass flasks half-filled

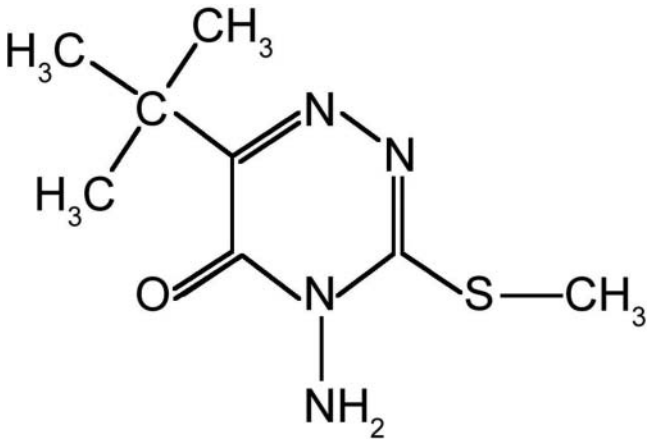

Figure 1. The structural formula of metribuzin.

with saline liquid medium. Then, the inoculum was transferred to the fermenter. Cells were batch-cultured in a BioFlo/CelliGen115 (New Brunswick, NJ, USA) automated the fermentation system of total volume 12 L and working volumes 3–6 L, under strictly aseptic conditions following the previously developed technology.[31,32] Cells were grown on Schlegel's mineral medium:[33] $Na_2HPO_4{\cdot}H_2O$ – 9.1; KH_2PO_4 – 1.5; $MgSO_4{\cdot}H_2O$ – 0.2; $Fe_3C_6H_5O_7{\cdot}7H_2O$ – 0.025; NH_4Cl – 1.0 (g L^{-1}); the medium was supplemented with glucose in amounts corresponding to cell concentration in the medium. Due to the use of urea, no pH adjustment was needed. A solution of iron citrate (5 g L^{-1}), which was used as a source of iron, was added to reach a concentration of 5 mL L^{-1}. Hoagland's trace element solution was used: 3 mL of standard solution per 1 L of the medium. The standard solution contained H_3BO_3 – 0.288; $CoCl_2{\cdot}6H_2O$ – 0.030; $CuSO_4{\cdot}5H_2O$ – 0.08; $MnCl_2{\cdot}4H_2O$ – 0.008; $ZnSO_4{\cdot}7H_2O$ – 0.176; $NaMoO_4{\cdot}2H_2O$ – 0.050; $NiCl_2$ – 0.008 (g L^{-1}). The air was pumped through the culture medium using a system of microbiological filters, with an air pump. When cell concentration reached 2.0–2.5 g L^{-1}, nitrogen (60 mg g^{-1} biomass) and glucose solution were fed to the culture medium in two separate flows. Substrate feed rates were regulated with a peristaltic pump. Nitrogen concentration in the culture medium was maintained at trace levels, and glucose concentration did not exceed 10 g L^{-1}. Nitrogen supply was stopped after 20–25 h, and the second phase was started; it lasted for 20–55 h, until P(3HB) reached (83 ± 2)%. 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. P(3HB) had the following physicochemical parameters: weight average molecular weight (M_w) 920 kDa; polydispersity (D) 2.52; degree of crystallinity 74%; melting point and thermal decomposition temperature 179.1 and 284.3°C, respectively.

Metribuzin analysis

Detection of metribuzin was performed by using gas chromatography. Measurements were done on the gas chromatograph equipped with a mass spectrometer (7890/5975C, Agilent Technologies, Santa Clara, CA, 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^{-1}; sample introduction temperature 220°C; initial temperature of chromatography – 150°C; temperature rise to 310°C at 10°C per 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. We used State Standard Sample 7713-99—the state standard accepted in Russia: 99.7% pure. The calibration curve was prepared by using a wide range of concentrations of metribuzin in acetone (0.1–4.2 μg μL^{-1}). The range of linear detection was obtained for a wide variety of concentrations: between 0.1 μg/μL and 4.2 μg μL^{-1}. The standard error of the method was no more than 3%.

Methods of preparation of P(3HB)/MET mixtures

Various phase states of P(3HB) (powder, solution, emulsion) were used to prepare polymer/metribuzin mixtures. Each polymer matrix was loaded with 25% (w/w) metribuzin.

The powder was prepared by grinding the polymer in a ZM 200 ultra-centrifugal mill (Retsch, Germany). The fractional composition of the powder was determined by using an AS 200 control analytical sieve shaker (Retsch, Germany). The fraction of the particles of sizes below 0.50 mm constituted 65%, and the fraction of the particles between 0.80 and 1.00 mm was 45%. P(3HB) and MET powder samples were weighed on the analytical balance and then homogenized with a laboratory stirrer for 2 min.

P(3HB) solutions of different concentrations were prepared by adding a polymer sample to chloroform. The polymer sample was dissolved in chloroform, and a solution of metribuzin in chloroform was added to the polymer solution. The polymer/metribuzin solution was mixed for 2–3 h (until completely dissolved) by using an MR Hei-Standart magnetic stirrer (Heidolph, Germany) and heated to 35–40°C under reflux condenser or was left to stay at room temperature for 3–4 h.

Polymer emulsion was prepared as follows. The oil phase, represented by a 1% P(3HB) solution, and metribuzin (25% of the mixed solution) in chloroform were combined with the aqueous phase—polyvinyl alcohol (PVA) solution (Sigma, St. Louis, MO, USA, M_w 30 kDa)—and mixed for 24 h, until complete solvent evaporation took place.

Preparation of slow-release metribuzin formulations

The P(3HB)/MET mixtures (solutions, powders, and emulsions,) were used to construct metribuzin-loaded films, granules, pellets, and microparticles. In every formulation, the content of metribuzin was 25% of the polymer matrix (w/w).

Polymer/metribuzin solutions in chloroform were used to prepare metribuzin formulations in the form of films and granules.

Films were prepared as follows: the P(3HB)/MET solution was cast in glass or Teflon-coated metal moulds, and then solvent evaporation occurred. We used 2 and 4% (w/v) polymer solutions in chloroform. Viscosity of the solutions was measured by using a HAAKE Höppler Falling Ball Viscometer C (Thermo Scientific, Germany). The following procedure was used to embed the active ingredient into the polymer matrix (25% w/w). The homogeneous polymer/metribuzin solution was filtered and poured into the degreased mould 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 the analytical balance. The film thickness was measured with a digital micrometer (LEGIONER EDM-25-0.001, Germany). Squares of 25 mm^2 in area (5 mm × 5 mm) were then cut from the film.

Metribuzin-loaded polymer granules were prepared as follows: the polymer was precipitated from the solution into the sedimentation tank filled with the reagent in which P(3HB) did not dissolve (hexane), and, thus, crystallization occurred and polymer granules were formed. We used P(3HB) solutions in chloroform of three different concentrations: 8, 10, and 12% (w/v). A solution of metribuzin was added to the polymer solution; 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 the sedimentation tank that contained hexane, where the polymer was crystallized and granules formed. We varied polymer concentration in the solution, needle diameter, the rate at which the solution was fed to the sedimentation tank, and the thickness of the layer of the precipitating agent.

Pellets were prepared from the powdered polymer/ metribuzin mixture ground in a ZM 200 ultra-centrifugal 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). The powdered P(3HB) and metribuzin were mixed mechanically. Samples of the two powders of different fraction compositions (0.10–1.0 mm) were weighed on the analytical balance and then homogenized with a laboratory stirrer for 2 min. The mixture was used to cold press pellets, 13 mm in diameter, by using a Carver Auto Pellet 3887 press (Carver, Wabash, IN, USA) under different pressing forces: 6,000, 14,000, and 24,000 F.

Microparticles were prepared by the emulsion technique. To produce large particles (10 μm diameter and more), we tested different production conditions. We varied the concentration of the polymer solution (between 1 and 4%); agitation speed (300, 750, 1,000, 6,000, and 16,000 rpm), and the type of the surfactant (polyvinyl alcohol, PVA, M_w 30–50 and 150 kDa; sodium dodecyl sulphate (SDS), and polyoxyethylene-20-sorbitan-monooleate (Tween ® 80). To vary the conditions and speed of agitation of the emulsion, we used a Silent Crusher M high-speed homogenizer (Heidolph, Germany: between 6,000 and 16,000 rpm) and an MR Hei-Standard magnetic stirrer (Heidolph, Germany: between 300 and 1000 rpm). 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®, The Netherlands).

A physicochemical study

Initial substances in the form of powders (metribuzin and poly-3-hydroxybutyrate); powdered P(3HB)/MET mixture, and

metribuzin 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, Switzerland). Samples of films, powders, granules, and pellets (4.0 ± 0.2 mg) were placed in aluminium crucibles and heated at 5°C per min. The melting point (T_{melt}) and thermal decomposition temperature (T_{degr}) were determined from exothermic peaks on thermograms, using the StarE software. 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 operating at 40 kV × 40 μA. Fourier transform infrared spectroscopy (FTIR) was conducted as follows. Infrared spectra of the films and powders were taken in the 500–4500 cm^{-1} range, using an INFRALUM FT-02 FTIR spectrometer (Lumex, Russia).

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).

Granules were examined to determine their size, morphology, and active ingredient encapsulation efficiency (EE).

Parameters of microparticles of sizes under 10 μm (size distribution and surface charge (ξ-potential) were investigated with a Zetasizer Nano ZS particle analyzer (Malvern, UK), employing dynamic light scattering, electrophoresis, and laser Doppler anemometry. The surface charge of microparticles was characterized by the value of ξ-potential, which was measured with a Zetasizer Nano ZS microparticle analyzer, using Henry's formula. Microparticles of sizes between 10 μm and 100 μm were measured with a Flow Cam system for quantitative and qualitative particle analysis (Fluid Imaging, Scarborough, ME, USA). Triplicate measurements of each sample were performed.

The EE of metribuzin in microparticles and granules was calculated using Eq. (1)

$$EE = (M_{enc}/M_{init}) \times 100\% \quad (1)$$

where M_{enc} is the mass of the encapsulated metribuzin in the polymer matrix (mg) and M_{init} is the mass of the initial amount of metribuzin (mg).

A study of release kinetics of metribuzin from polymer matrices

Release kinetics of metribuzin from the polymeric matrices was studied *in vitro* in laboratory systems: the specimens were sterilized and placed into 500-mL sterile conical flasks filled with sterile distilled water (100 mL). The number of granules, large microparticles (of average size of 54 μm), films, or pellets placed in a flask was determined in such a way that the samples in each flask contained equal total amounts of the active ingredient (50 mg). The flasks were incubated at 25°C in an Innova 44 (New Brunswick, NJ, USA) temperature controlled incubator shaker at 150 rpm. Samples (2 mL) for analysis were collected periodically, under aseptic conditions, and an aliquot of water was added to the flask to maintain a constant volume of liquid in it. Metribuzin was extracted with chloroform three times to determine its concentration. The chloroform extracts were passed through sodium sulphate. Chloroform was removed in a rotary vacuum evaporator (Büchi, Switzerland). After chloroform was removed, we added 200 μL of acetone.

The amount of metribuzin released (RA) was determined as percentage of the metribuzin encapsulated in the polymer matrix, using Eq. (2)

$$RA = r/EA * 100\%, \quad (2)$$

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. (3)):

$$M_t/M_\infty = Kt^n. \quad (3)$$

Here, 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, $0.45 \leq n$ 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.[34,35]

To analyse the stability of the formulations incubated in water for long periods of time, we used electron microscopy and monitored the temperature properties and degree of crystallinity of the samples.

Statistical analysis

Statistical analysis of results was performed using the standard software package of Microsoft Excel, STATISTICA 8. Arithmetic means and standard deviations were tested using Student's t test. Results are given as $X \pm m$ (X is the arithmetic mean and m is the standard error).

Results

Characterization of P(3HB)MET mixtures

P(3HB)/MET solutions, emulsions, and powders were prepared from the preliminarily purified and thoroughly studied P(3HB) and crystalline metribuzin powder. The properties of the initial samples of the polymer, metribuzin and the polymer/metribuzin powdered mixture are given in Table 1. Figure 2 shows thermograms and X-ray images of initial P(3HB) and MET powders and P(3HB)/MET mixtures. Figures 4(A), 5(A), and 6 (A) show photographs and SEM images of the metribuzin

Table 1. Physicochemical properties of P(3HB), metribuzin, and the P(3HB)/metribuzin mixture.

Parameter	P(3HB)	Metribuzin	Initial powder
C_x, (%)	74	90	61
T_{melt} (°C)	178.1	126.2	126.5/168.9
H_{melt} (J g^{-1})	89.1	83.4	13.7/58.6
T_{degr} (°C)	284.3	274.5	280.1
H_{degr} (J g^{-1})	731.8	238.9	610.6

formulations (films, granules, pellets, and microparticles) prepared in this study. Table 2 lists their physicochemical properties.

For the powdered P(3HB) and metribuzin and for their mixture, thermograms were taken within a wide temperature range, including the polymer melting point and thermal decomposition temperature (Fig. 2(A)). The thermogram of the P(3HB)/MET mixture showed two peaks: one melting peak is at 126.5°C and the other at 168.9°C. The thermal decomposition temperature had one peak, at 280.1°C, as the thermal decomposition temperature of MET was in the same region. The enthalpies of melting of the mixture were 13.7 and 58.6 J g^{-1} and the enthalpy of its thermal decomposition was 610.6 J g^{-1}, and that was also lower than the enthalpies of the initial polymer, whose melting temperature was 179.1°C and thermal decomposition temperature

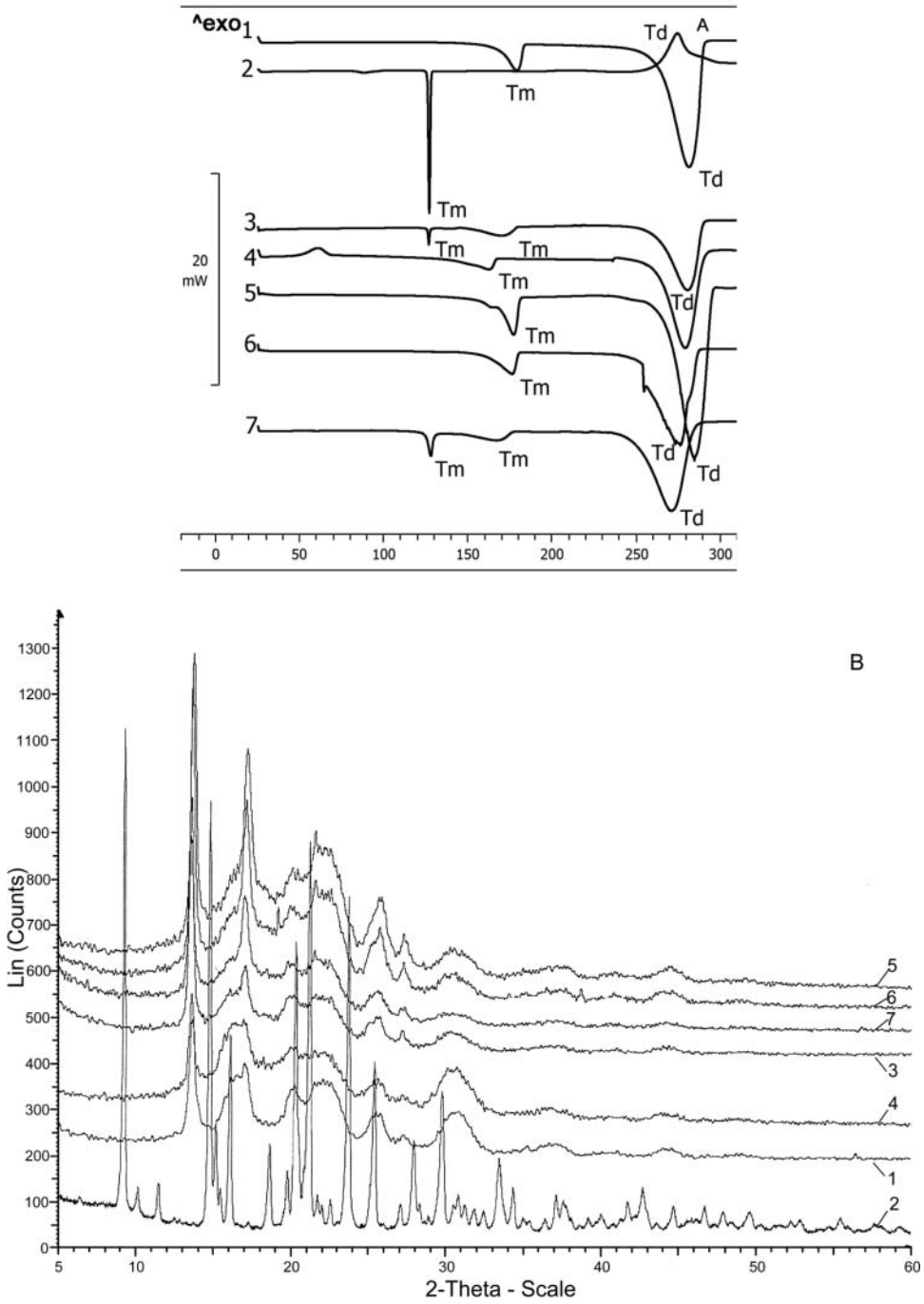

Figure 2. The DSC thermograms (A) and X-Ray images (B) of: (1) poly(3-hydroxybutyrate), (2) metribuzin, (3) P(3HB)/metribuzin mixture (powder) and P(3HB)/metribuzin formulations: (4) films, (5) microparticles, (6) granules, and (7) pellets.

284.3°C: 89.1 and 731.8 J g^{-1}, respectively. A similar result—the presence of two melting peaks (127.2 and 168.2°C)—was obtained for the pellets.

The presence of two melting peaks suggested that in the dry mixture, the MET part did not interact physically with P(3HB), causing the mixture to split into layers during heating. However, the decrease in the melting temperature of P(3HB) in the mixture compared with the initial polymer was indicative of the weak physical interaction. The formulations prepared from solutions (microparticles, films, and granules) did not show a metribuzin peak, suggesting that the system was a physical mixture of components. In all formulations, the temperatures of melting and thermal decomposition and enthalpy (T_{melt}, T_{degr}, enthalpy) were lower than those of the initial polymer (Table 2). The most significant decrease in the melting temperature was observed for films and pellets (161.1 and 168.2°C, respectively; Table 2). We assume that during the preparation of formulations, the size of crystallites may have changed. MET molecules may have occupied the free space in the polymer and prevented P(3HB) crystals from growing. This may have led to the formation of small crystals, whose melting temperature was lower. The temperature properties of the formulations may have been influenced not only by the size of the crystals but also by the processes used to produce them, as they were prepared in different ways. Films were crystallized when the solvent was evaporated; crystallization of granules occurred during their precipitation in hexane; pellets were produced by high-pressure processing. These processes affected the structure of the crystals.

By contrast, analysis of the number and shapes of endothermic peaks on the thermograms of films, granules, and microparticles showed the formation of a stable mixture of the polymer and metribuzin, which was not separated under heating, as there was only one peak of melting and thermal decomposition. The T_{melt} decrease suggested an increase in the viscosity of the melts and, thus, inhibition of polymer crystallization. The small metribuzin crystals had greater interfacial energy and, therefore, they began to melt before the polymer did, and this lowered the melting temperature of the mixture and made the structure of P(3HB) more amorphous. This conclusion was also supported by a decrease in the enthalpy of melting, which was indicative of a decrease in the degree of crystallinity of the initial polymer, and certain smearing of the peaks, which is typical of melting of amorphous regions.

X-ray structure analysis showed that the loading of the P(3HB)/MET decreased the degree of crystallinity of the polymer. The C_x of the P(3HB)/MET powders was 61% (Table 1). That was lower than the C_x values of P(3HB) (74%) and metribuzin (90%) (Fig. 2(B)). The C_x of metribuzin formulations (granules, microparticles, and pellets) was also lower than that of the initial polymer (62–64%); the C_x of the films was even lower (51%) (Fig. 2(B), Tables 1 and 2). That was indicative of changes in the crystallization process in P(3HB)/MET, i.e. an increase in the proportion of the amorphous region.

The results of FTIR of metribuzin, P(3HB), and P(3HB)/MET films and powders (Figs. 3(A)–(C)) suggested that the most informative range of the wavenumbers was that between 1450 and 1700 L cm^{-1}. The absorption peaks (bands) observed in the P(3HB)/MET films were those associated with the

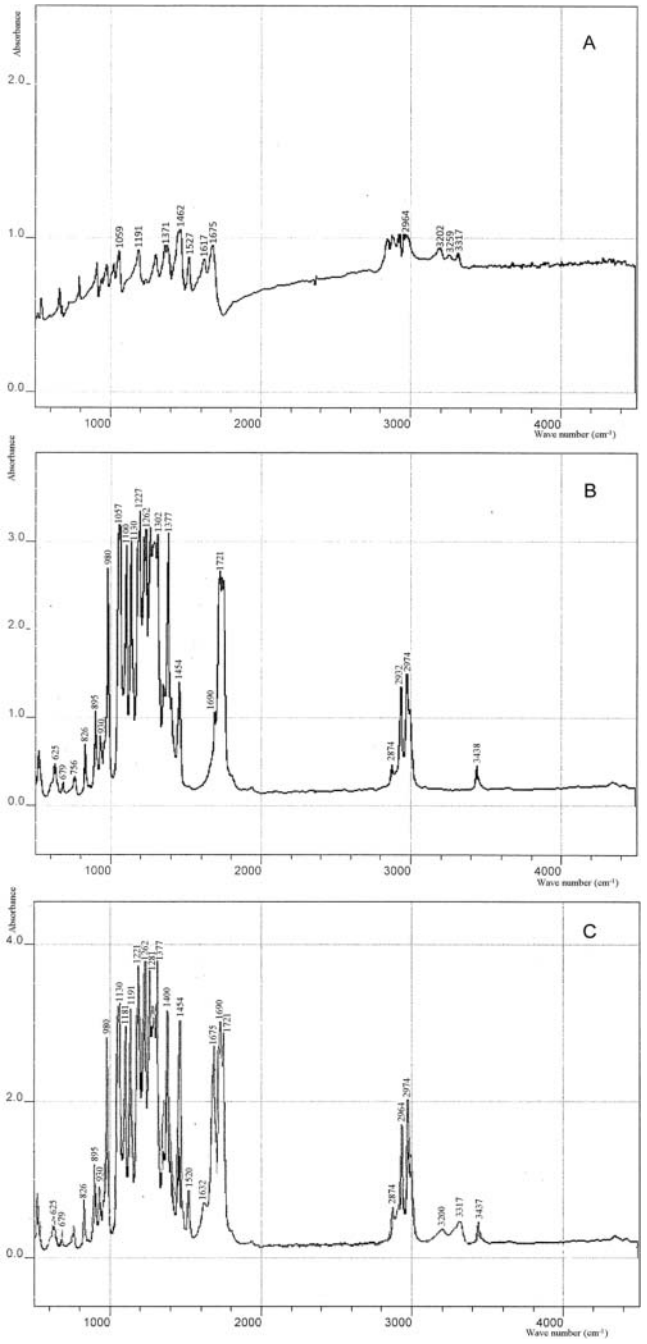

Figure 3. Infrared-spectra of metribuzin (A), poly(3-hydroxybutyrate) (B) and P(3HB)/metribuzin mixture (C).

specific structural groups of metribuzin (Fig. 3(C)). FTIR spectra of the mixture showed an absorption band in the 1520 cm^{-1} region, which was the characteristic of pulsation vibrations of the carbon skeleton of four substituted pyridines. Enhanced intensity of absorption bands was observed in the 1400–1419 cm^{-1} region. They were characteristics of both P(3HB) and MET and were attributed to deformation vibrations of CH_3 groups, which were adjacent to C = O, and pendulum oscillations of CH3 groups in the 1130 cm^{-1} region. Similar intensity enhancement was observed in the 2874 cm^{-1} region for peaks of the bound OH groups. The absorption band in the 1632 cm^{-1} region was attributed to deformation vibrations of the NH_2 group of metribuzin, and the absorption band of the

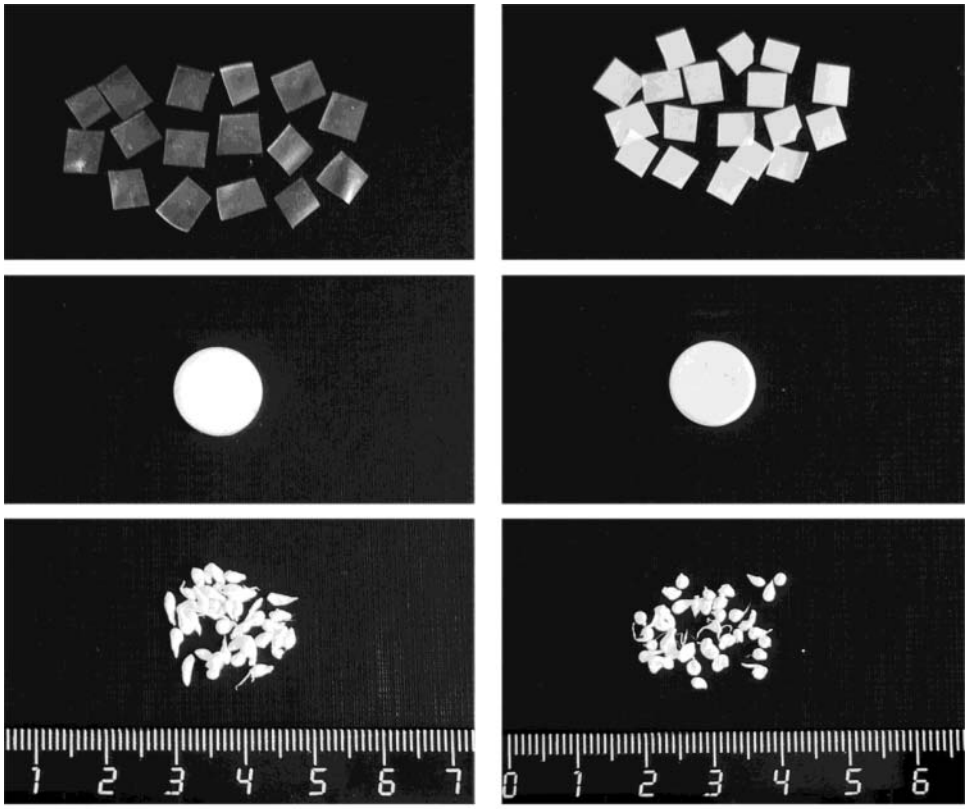

Figure 4. Photographs of the P(3HB)/metribuzin granules, films, and pellets: (A) initial and (B) after 49 days of incubation in water.

mixture was shifted relative to that of pure metribuzin (1617 cm^{-1}). Therefore, we assumed possible involvement of the NH_2 group in the formation of hydrogen bonds. The peaks in the 3200–3400 cm^{-1} region were attributed to stretching vibrations of the bound OH group. No groups that would form due to chemical interaction between P(3HB) and metribuzin were detected. Peaks of the existing groups became higher because of the overlap of the P(3HB) and metribuzin spectra, indicating that P(3HB)/metribuzin was a physical mixture of the polymer and the herbicide.

Thus, results of DSC, X-Ray, and FTIR suggested that there were no chemical bonds between the pesticide and the polymer and that the mixtures were physical mixtures of components. The decrease in the melting temperature and enthalpy (Table 1) leads us to conclude that the active ingredient of metribuzin plays the role of a filler of the polymer matrix.

Characterization of P(3HB)/MET formulations

Figures 4(A), 5(A), and 6(A) show photographs and SEM images of the P(3HB)/MET formulations (films, pellets, granules, and microparticles) prepared in this study. Table 2 lists their physicochemical properties.

Films

We studied the effect of polymer solution concentration on the quality of the films loaded with metribuzin. Solutions of polymer in chloroform of concentrations 4% or higher (the viscosity of the solution decreased from 236.91 to 87.30 cP as the temperature was increased from 5 to 60°C) did not enable complete dissolution and uniform distribution of metribuzin in the solution; the films prepared from these solutions exhibited nonuniform surfaces. The use of 2% solutions (the viscosity of the solution decreased from 26.79 to 12.82 cP as the temperature was increased from 5 to 60°C) resulted in complete dissolution and uniform distribution of metribuzin (Fig. 4(A)). The temperature of the solution influenced the shape and quality of the films, too: at room temperature, films were deformed during solvent evaporation. As the solution and the surface of the glass mould were heated to 30–35°C, flexible films of uniform thickness (0.045 ± 0.005 mm) were produced. P(3HB) films loaded with metribuzin had smooth dense structure with a few small pores (Fig. 5(A)). X-ray spectral analysis showed that metribuzin was uniformly distributed inside the polymer matrix and was present on the film surface as dispersed particles (smaller than 1 μm), which were also uniformly distributed on the surface. The EE of metribuzin was 100%. We observed a few 2–3 μm pores on the film surface (Fig. 5(A)).

Granules

By varying P(3HB) solution density, hose diameters, and needle size, we determined the parameters that enabled production of high-quality granules: 10% polymer concentration of the solution, needle size—20 G, and the thickness of the layer of the precipitating agent—200 mm. At polymer solution concentrations below 10% and with a smaller needle size, the granules

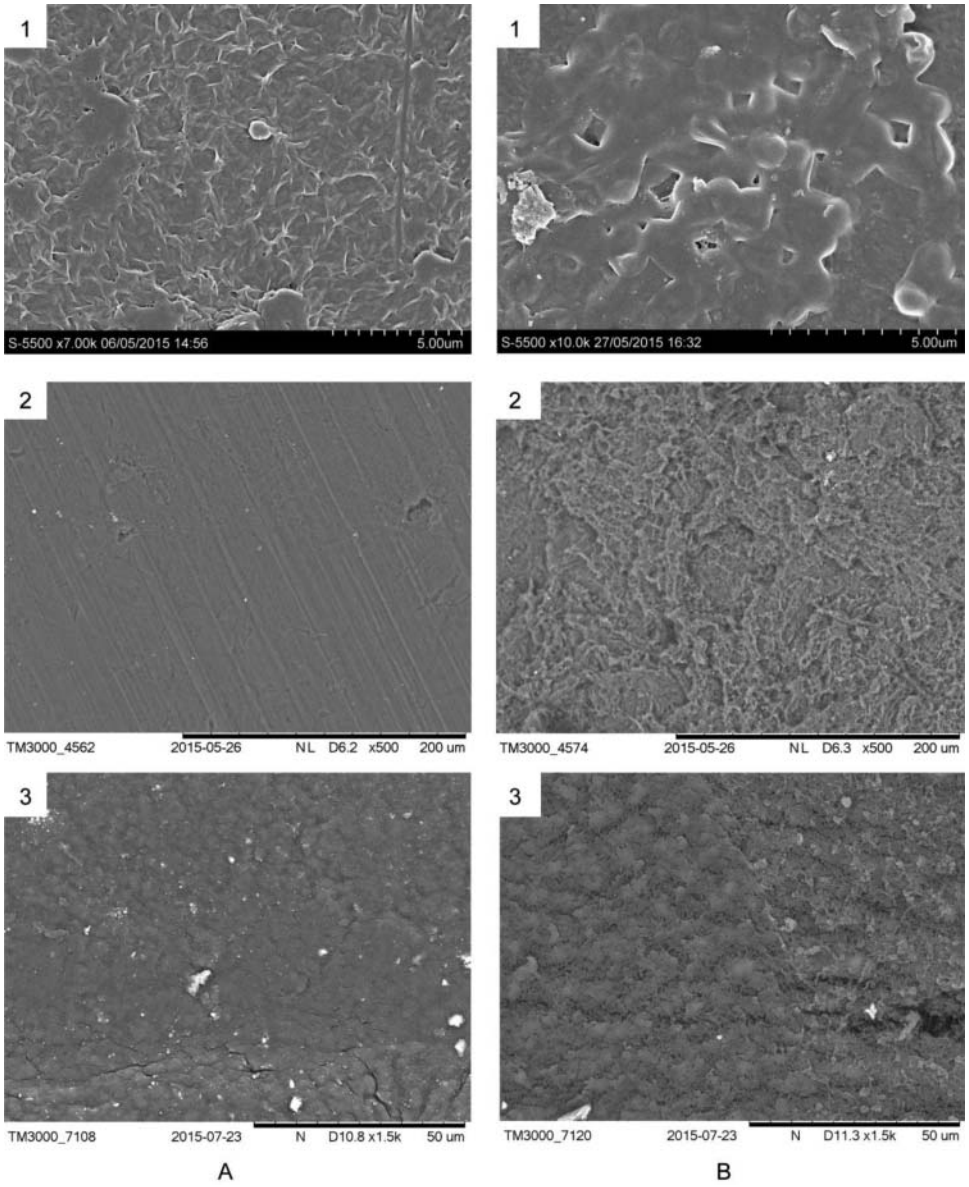

Figure 5. SEM images of P(3HB)/metribuzin granules, films, and pellets: (A) initial and (B) after 49 days of incubation in water. Bars: 1 – 5 μm; 2 - 200 μm; 3 – 50 μm.

were misshapen, and their formation was incomplete (some granules had voids or "tails", Fig. 4(A)). The use of solutions of higher concentrations was technologically infeasible. The size of metribuzin-loaded granules prepared from 10% polymer solutions was 2.5–3 mm. We developed a method for producing polymer granules with the maximum active ingredient EE (95–100%). The surface of the granules was slightly rough, with easily visible metribuzin crystals (Fig. 5(A)).

Pellets

The method of contact cold pressing was used to prepare pellets of diameter 13 mm and mass 200 ± 0.15 mg, with metribuzin constituting 25% of the pellet mass, from P(3HB)/MET powders (Fig. 4(A)). The amount of the applied force influenced the structure of the pellets: when the force below 6,000 F was applied, the resulting pellets had loose structure; at 24,000 F, partial surface sintering was observed. An optimal force for preparing high-quality pellets (with even surface and uniform distribution of chemicals) was 14,000 F. The surface of the pellets loaded with metribuzin was generally dense, with slight lesions, probably where metribuzin was located, as metribuzin particles were larger than the polymer particles (Fig. 5(A)). Spectral analysis of elemental composition proved that 100% of metribuzin was embedded in the polymer matrix and that its structure remained unchanged.

Microparticles

The study of the effect of microparticle preparation conditions on the size of microparticles showed that not all study parameters had favourable effects on this value. The main factors determining the size of microparticles were polymer emulsion concentration and agitation speed. As polymer concentration of the emulsion was increased from 1% to 2% and to 4% (surfactant PVA 30 kDa), the average diameter of the microparticles increased from 7.5 to 17 μm. Agitation speed influenced the particle size. At a high agitation speed, 6,000 and

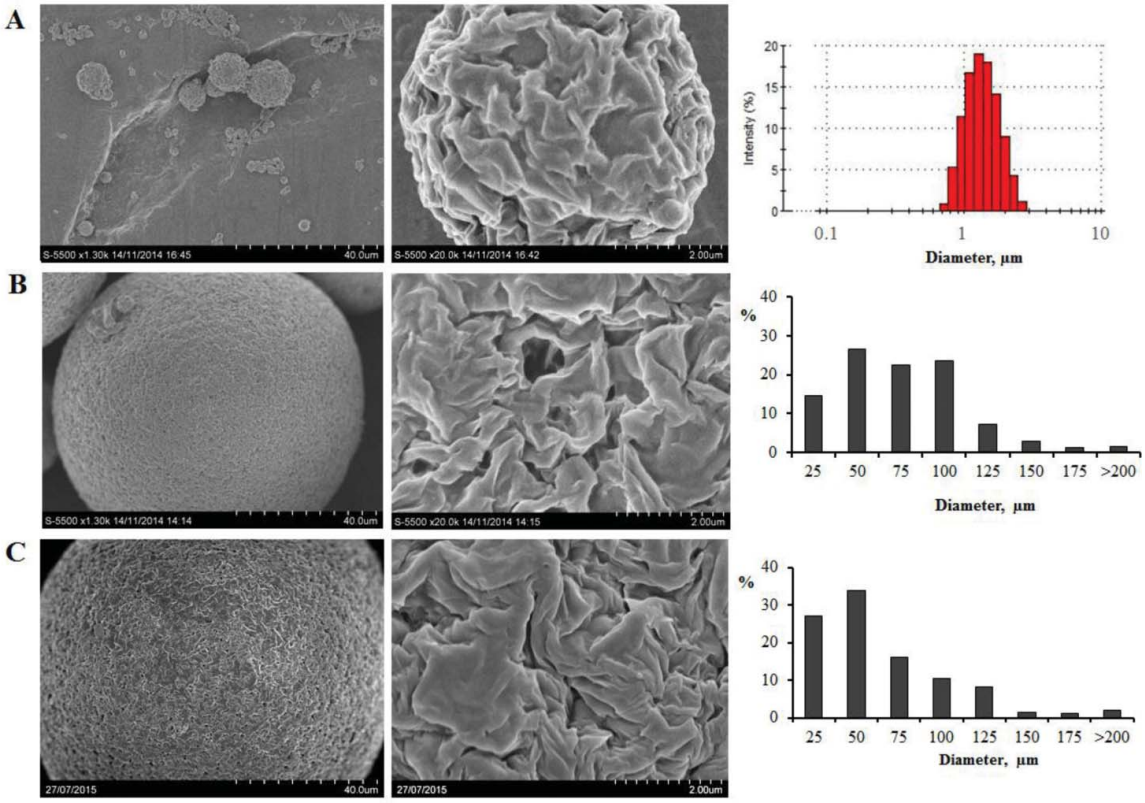

Figure 6. SEM images and size distribution of microparticles loaded with metribuzin at 25% of the polymer mass, prepared by using: (A) a high-speed homogenizer (6,000 rpm), (B) magnetic stirring (750 rpm), and (C) magnetic stirring (750 rpm) after incubation in distilled water for 49 days. Bar = 40 and 2 μm.

16,000 rpm (PVA 30 kDa, polymer concentration 1%), the size of the resulting particles was 2.1 and 1.2 μm, respectively. At lower agitation speeds (300, 750, 1000 rpm) (PVA 30 kDa, polymer concentration 1%), when the magnetic stirrer was used, the size of the particles was 17.3, 11.4, and 4.25 μm, respectively. Thus, as the agitation speed was increased, the size of microparticles decreased. When PVA 30 kDa was replaced by PVA 150 kDa (polymer concentration 1%), the size of the particles increased from 1.2 to 1.5 μm. The use of SDS increased the size of the particles to 7.0 μm. The effect of Tween 80 and its concentration in the emulsion was studied at 16,000 rpm. At 1% Tween 80 in the emulsion, the size of the resulting particles was 3.0 μm; at 4%, it was somewhat greater—3.9 μm. The quality and the yield of microparticles were influenced by the type of the surfactant used: microparticles prepared from emulsions containing PVA and SDS had regular spherical shapes, and their yield was 77.1 and 75.5%, respectively. The use of Tween 80 produced some misshapen particles, and their yield was no more than 60%. Variations in the density of polymer emulsion did not have a significant effect on the particle yield. A similar result was obtained when we varied the speed and type of agitation (particle yield varied between 68 and 73%).

The loading of particles with metribuzin was studied at 750 and 6,000 rpm. The average diameter of the metribuzin-loaded particles prepared at the high agitation speed (6,000 rpm) was 1.83 μm, but at 750 rpm it was much greater (54 μm). The surface of microparticles was wrinkled, regardless of their size (Figs. 6(A) and (B)). The study of the ξ-potential as an indicator of the stability of metribuzin-loaded particles, showed that at 750 rpm, ξ-potential was 26 mV and at 6000 rpm – 30 mV. Furthermore, the increase in the surfactant (PVA)

Table 2. Physicochemical properties of the P(3HB)/metribuzin formulations after incubation in sterile water.

	Films		Granules		Pellets		Microparticles	
Parameter	Initial	After 50 days in H_2O	Initial	After 50 days in H_2O	Initial	After 50 days in H_2O	Initial	After 50 days in H_2O
C_x (%)	51	60	64	62	63	66	64	66
T_{melt}(°C)	161.1	176.9	173.5	177.5	127.6/168.2	126.7/175.3	176.8	177.1
H_{melt} (J g^{-1})	42.3	79.5	83.4	87.0	16.6/51.1	3.8/74.0	88.8	38.5
T_{degr} (°C)	278.6	283.4	275.6	250.4	270.6	276.1	283.5	278.6
H_{degr} (J g^{-1})	546.9	759.5	660.1	774.7	495.2	672.2	736.6	300.0

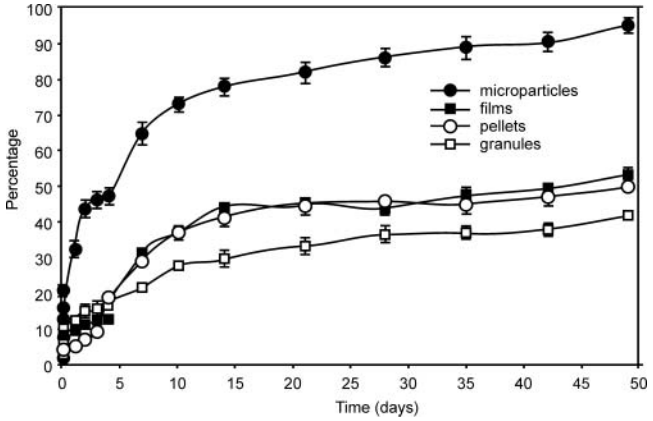

Figure 7. The release profile of metribuzin from: (1) microparticles, (2) films, (3) pellets, and (4) granules (% of the initial amount).

concentration from 1 to 3% had almost no effect on the ξ-potential. An important parameter is EE of the substances in the polymer matrix of microparticles. By varying the agitation speed (750 and 6,000 rpm) and the type of agitation, we prepared particles with similar values of metribuzin EE: 18% and 16.9%, respectively.

Thus, the best conditions for preparing metribuzin-loaded microparticles were as follows: P(3HB) concentration 1%, PVA 30 kDa concentration 1%, and agitation speed 750 rpm, on a magnetic stirrer. The size of the resulting particles was 54 μm, the yield of the particles was 71.6%, the ξ-potential was 26 mV, and metribuzin EE was 18%.

Release kinetics of metribuzin from P(3HB)/MET microparticles, granules, films, and pellets

Figure 7 shows metribuzin release from P(3HB)/MET formulations: films, pellets, granules, and microspheres; Figs. 4(B), 5(B) and 6(B) show photographs and SEM images of the formulations after incubation in water. Table 1 gives physicochemical properties of the formulations before and after incubation in water. As poly-3-hydroxybutyrate is neither dissolved nor hydrolysed in water, metribuzin was passively released from the polymer matrix, diffusing through the pores and microcracks of the polymer matrix. The differences that can be clearly seen in the graph are determined by the construction of the carrier. Metribuzin was released at the highest rate from microspheres.

The reason for this was that microparticles were the smallest metribuzin carriers and, thus, had the largest total particle/water interface area. In the first 3 days, the rate of metribuzin release from microparticles was 7.7 mg d^{-1}; in the following 11 days, it dropped to 1.5 mg d^{-1}. Then, between day 14 and the end of the experiment, the rate of metribuzin release from microparticles was very low (0.24 mg d^{-1}), the herbicide release reaching 95% by the end of the experiment (49 days). Metribuzin release rates from films and pellets were much lower. For 14 days, they were about 1.5–1.6 mg d^{-1}, and, then, both curves exhibited plateaus, i.e. no more metribuzin diffused through the pores of the polymer matrix. The total percentage of the encapsulated agent released from the polymer matrices of films and pellets in the experiment reached about 50–53%. Metribuzin encapsulated in granules showed similar release behaviour: in the first 10–14 days, the herbicide was released at a rate of 1.1 mg d^{-1}. Then, the curve exhibited a plateau, and the percentage of metribuzin released from the granules was the lowest: 42%.

Table 3. The constants derived from fitting the equation $M_t/M_\infty = Kt^n$ to release of metribuzin into water from P(3HB)/MET formulations.

Formulations	K (h^{-1})	n	R^2
Films	0.010	0.555	0.97
Granules	0.024	0.404	0.96
Pellets	0.010	0.512	0.97
Microparticles	0.081	0.405	0.98

R^2: validity coefficient of approximation.

Table 3 lists the main kinetic parameters of metribuzin release from different formulations into water. Exponent n varied between 0.40 and 0.55 for all formulations. For granules and microparticles, n was 0.4, suggesting diffusion of the herbicide through the polymer layers in accordance with Fick's law. For pellets and films, the exponent was equal to 0.51 and 0.55, respectively, indicating abnormal metribuzin release behaviour, which did not follow Fick's law. The highest value of kinetic constant K was shown by microspheres (0.081 h^{-1}) and the lowest by films and pellets (0.01 h^{-1}). The reason was that microparticles had the largest surface area for diffusion to occur. The lowest percentage of metribuzin was released from the pellets. Taking into account that metribuzin is a water-soluble substance, we assume that pellet production by compression blocked the pores of the polymer matrix, preventing water penetration and hindering herbicide diffusion. This is supported by the presence of the metribuzin melting peak in the diagram of melting of the pellet after incubation in water.

All specimens incubated in water were rather stable and retained their initial shapes (Fig. 4(B)). Only the initially transparent films had become cloudy. Slight changes in the surface structure were captured by electron microscopy (Fig. 5(B)). The films became more porous, and the pores were of larger sizes (reaching 2–3 μm). Lesions developed in some regions of the pellets, most likely where metribuzin was dissolved and released. The morphology of microparticles changed very little (Fig. 6(C)). The ξ-potential and size of the particles did not change, reaching 25.7 mV and 55.1 μm, respectively. The physicochemical properties, such as temperature parameters and crystallinity, of all P(3HB)/MET formulations did not change considerably (Table 2).

Discussion

This study presents results of constructing slow-release formulations of herbicide metribuzin embedded in the degradable polymer matrix. As metribuzin exhibits rather high physiological activity towards various weeds and is convenient to use, it has attracted the attention of researchers as a model for developing slow-release herbicide formulations.

There are quite a few studies that report the use of different natural (chitin, dextran, cellulose, starch, bentonite, magnesium silicate of the talc group) and synthetic materials (polyethylene glycol, acrylamide, methacrylic acid combined with ethylene

glycol and dimethacrylate) as matrices for embedding metribuzin.[8–19] Metribuzin formulations are usually prepared as granules and microparticles.

In this study, for the first time, the matrix was made of biodegradable polymer of 3-hydroxybutyric acid—poly-3-hydroxybutyrate [P(3HB)], the most widespread and thoroughly studied representative of polyhydroxyalkanoates (PHAs)—microbial polymers of hydroxy derived alkanoic acids. Metribuzin was loaded into degradable P(3HB) matrices shaped as microparticles, granules, films, and pellets, which were prepared from the P(3HB)/metribuzin powders, solutions, and emulsions. The preparation of metribuzin-loaded films and granules involved the use of chloroform and hexane, and surfactants were used to prepare microparticles. The use of these substances may be a limitation for the preparation of the formulations on an industrial scale, and the process may need to be optimized.

Examination of the specimens by FTIR, X-Ray, and DSC techniques did not show any chemical bonding between metribuzin and polymer, which indicated that P(3HB)/MET was a physical mixture of the two components. The decrease in the temperature and enthalpy of melting of the mixtures suggested that metribuzin was the filler of the polymer matrix.

In a laboratory model system (sterile water), we showed the stability of all P(3HB)/MET formulations and studied metribuzin release kinetics from the polymer matrix. The analysis of the literature indicates that the rates of metribuzin release reported by different authors vary between a few hours and dozens of days, depending on the form of the polymeric matrix and conditions of the medium.

Some of the authors observed fast release of metribuzin. Sepiolite-based granular formulations loaded at 28.6 and 16.7% of metribuzin showed a fast release of metribuzin (50–80% of the encapsulated herbicide) in the first hours.[8] Reported a study of metribuzin release from the granules prepared from the gelling sodium alginate combined with bentonite, acid-treated bentonite, anthracite, and activated carbon loaded at 12% of metribuzin.[19] Only 20% of the initially encapsulated MET was released from the alginate/activated carbon granules after 70 h, while the release of the herbicide from other formulations reached 100% after 40 h. Metribuzin release from molecularly imprinted polymer (MIP) prepared by in situ polymerization using methacrylic acid as the functional monomer and ethylene glycol dimethacrylate as cross-linker was studied in soil.[12] During 45 days, metribuzin concentration in soil gradually decreased from 0.81 to 0.05 mg kg^{-1}. Kumar et al.[10] studied release kinetics of metribuzin loaded in polyvinyl chloride, carboxy methyl cellulose, and carboxy methyl cellulose-kaolinite composite in water in comparison with the commercial formulation (75DF). After three days, metribuzin was completely released from the commercial formulation, while its release rate from the experimental formulations was slower. Fernandez-Perez et al.[15] studied metribuzin release from lignin-polyethylene glycol granules for 500 h in a glass reactor filled with silicone oil. Metribuzin release from the granules reached 90% over 48 h, while free metribuzin was dissolved in oil in 30 min. Metribuzin loaded into lignin-based granules was released into water in 13 days; the increase in the size of the granules from 0.5 to 2–3 mm slowed down the process.

Metribuzin formulations that show slower release have also been described. A study of acrylamide–bentonite (aluminium 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: at higher loads of bentonite, the release of the active agent became slower, and a decrease in metribuzin content speeded up its release in water.[11] The authors of that study showed that 50% of the encapsulated metribuzin was released after 25–51 days, while it took only 14 days for metribuzin to be completely released from the commercial formulation, 75DF. Flores Céspedes et al.[9] studied metribuzin release from alginate-based formulations, with bentonite and anthracite used as modifying agents, in soil for 60 days, at a temperature of 25°C in a thermostat incubator. About 80% metribuzin was released from the metribuzin–alginate–bentonite or metribuzin–alginate–anthracite granules in the first 6 days, i.e. the release of metribuzin was much slower from these formulations than from the commercial formulation (80% metribuzin was released in 2 days). Rehab et al.[17] studied metribuzin release into the buffer solution for 3–4 months at different pH values (5, 7, and 9) from the poly(*N*,*N*-diacryloyl)/MET and *N*,*N*-diacryloyl-methyl methacrylate/MET systems. The copolymers contained metribuzin via an imide linkage and were prepared by the free-radical polymerization of metribuzin monomer with N, N-diacryloyl or methyl methacrylate (MMA). During the first 5–10 days, at pH 7, 0.5–2.0 mg of metribuzin was released, depending on the proportions of components in the formulation. The release rate was increased as pH of the medium was increased, as at high pH values, the degrees of ionization and swelling of the copolymers were increased. At the same time, the amount of metribuzin hydrolysed in different buffer solutions after 4 months ranged from 0.15 to 0.45 mg L^{-1}. The *N*-diacryloyl-methyl methacrylate/MET system showed a higher release rate—up to 4.5 mg for the first 3–5 days. Metribuzin release kinetics was influenced not only by pH of the medium but also by the hydrophilic properties of the polymer matrix. McCormick [18] studied metribuzin release in buffered solutions from natural polymers (chitin, dextran, cellulose, and starch) and synthetic acrylic polymers with amide bonds to metribuzin. In most cases, metribuzin was released after 40 days, but this process was influenced by the origin of the polymer and the type of the bond between the polymer and metribuzin. Dextran and polyvinyl alcohol formulation showed the lowest levels of release (4.7%). Metribuzin release from cellulose and chitin formulation reached 7–10%. Synthetic acrylic polymers with amide bonds to metribuzin released at higher rates (metribuzin release reached 14–16%) as hydrolysis proceeded apparently from neighboring group assistance and increasing hydrophilicity.

Analysis of the literature shows that release kinetics of metribuzin can be varied by loading it into different materials, suggesting the possibility of constructing controlled-release herbicide formulations.

In this study, the influence of the type and shape of the formulation on metribuzin release kinetics was investigated in laboratory systems with sterile water. Metribuzin diffused from the polymer matrices and dissolved in water in the first 12–14 days; then, the curves reached plateaus. However, metribuzin

release from microparticles lasted longer. Gradual release of metribuzin from the particles was observed throughout the experiment (for up to 49 days). Theoretical analysis showed that the highest values of kinetic constant K were characteristic of microspheres (0.081 h^{-1}) and the lowest for films and pellets (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.

Conclusions

In the present study, for the first time, the natural polymer poly-3-hydroxybutyrate was tested as a degradable matrix for embedding metribuzin in order to prolong its release into the surrounding medium. The biodegradable polymer poly(3-hydroxybutyrate) was used as a basis for constructing controlled release formulations of metribuzin: microparticles, granules, pellets, and films. Analysis of the polymer/metribuzin mixtures by DSC, X-ray, and FTIR methods showed that metribuzin and polymer formed a stable physical mixture. Metribuzin release from the constructed formulations into water occurred gradually, and release rates depended on the geometry of the forms.

Funding

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