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INFLUENCE OF BIOPLASTICS´ BIODEGRADATION ON SOIL QUALITY
VLIV BIODEGRADACE BIOPLASTŮ NA KVALITU PŮDY

MASTER'S THESIS
DIPLOMOVÁ PRÁCE

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Abstract

Over the past decade, the society has brought attention to microplastics. They are produced by various industries and spread across the environment. For long time, they were considered inert, without further effect on plants and any other living organisms, however as recent studies found out, they might be a serious threat. Therefore, several researchers, including us, begun to focus on their transport and transformation in the environment. Most of the researchers, however, focus only on their presence in the marine and fresh waters and hence their behaviour in air and soil remains rather unclear. Furthermore, bioplastics has been brought to the media spotlight. They are presented as an ecological alternative to solve all the problems mentioned so far (and more). But what is often forgotten, their main advantage might also be their bottleneck. For that reason, this master's thesis focuses on negative effects associated with the presence of microplastics (specifically poly-(R)-3-hydroxybutyrate bioplastic) in soils as their common receptor. We combined respirometry, elemental analysis, thermogravimetry and enzymatic assays to investigate physico-chemical changes in soil induced by the presence of the bioplastic. Our results showed a negative effect on soil organic matter and water retention in the soil. In this sense, priming effect was widely investigated as acceleration and also retardation of soil organic matter decomposition took place. We registered different influence of selected concentrations of biopolymer on the soil and also the influence of soil properties on the course of degradation. Last but not lest, increased enzymatic activities clearly suggested impact of biopolymer presence on the microbial community. Such findings let us conclude, that biopolymer addition leads to long-term impact on a range of soil ecosystem services.

Abstrakt

V poslední době se pozornost polečnosti obrátila k mikroplastům. Jsou produkovány různými odvětvími a šíří se napříč prostředím. Po dlouhou dobu byly považovány za inertní, bez dalšího vlivu na rostliny a jiné živé organismy, avšak jak zjistily nedávné studie, mohly by představovat vážnou hrozbu. Několik vědců, včetně nás, se proto začalo soustředit na jejich transport a transformace v životním prostředí. Většina se však zaměřuje pouze na jejich přítomnost v mořských a sladkých vodách, a proto jejich chování ve vzduchu a půdě zůstává nejasné. Kromě toho byla pozornost soustředěna i na bioplasty. Jsou prezentována jako ekologická alternativa, která má vyřešit všechny dosud zmíněné problémy (a další). Avšak často se zapomíná, že jejich hlavní výhoda může být zároveň nevýhodou. Z tohoto důvodu se tato diplomová práce zaměřuje na negativní účinky spojené s přítomností mikroplastů (konkrétně bioplastu poly-(R)-3-hydroxybutyrátu) v půdě jako jejich běžný receptor. Kombinovali jsme respirometrii, elementární analýzu, termogravimetrii a enzymatické testy, abychom zkoumali fyzikálně-chemické změny v půdě vyvolané přítomností bioplastu. Naše výsledky ukázaly negativní vliv na půdní organickou hmotu a zadržování vody v půdě. V tomto smyslu byl zkoumán i tzv. "priming effect", jelikož docházelo k urychlení a také zpomalení rozkladu půdní organické hmoty. Zaznamenali jsme rozdílný vliv vybraných koncentrací biopolymeru na půdu a také vliv půdních vlastností na průběh degradace. V neposlední řadě zvýšení enzymatické aktivity jasně naznačovalo vliv přítomnosti biopolymeru na mikrobiální komunitu. Na základě takových zjištění jsme došli k závěru, že přidání biopolymeru vede k dlouhodobému dopadu na řadu funkcí půdního ekosystému.

Keywords

Bioplastics, Biopolymers, Degradation of Bioplastics, Biodegradation, Soil Quality Indicators, Priming Effect, Respirometry, Thermal Analysis, Thermogravimetry, Enzymatic assays, elemental analysis.

Klíčová slova

Bioplasty, biopolymery, degradace bioplastů, biodegradace, indikátory půdní kvality, priming effect, respirometrie, termická analýza, termogravimetrie, enzymatické testy, elementární analýza.

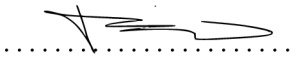
Reference

PALUCHOVÁ, Natálie. *Influence of bioplastics' biodegradation on soil quality*[online]. Brno, 2021. Available from: https://www.vutbr.cz/studenti/zav-prace/detail/131508. Master's thesis. Brno University of Technology, Faculty of Chemistry. 108. Supervisor prof. Ing. Jiří Kučerík, Ph.D.

Influence of bioplastics' biodegradation on soil quality

Declaration

I hereby declare that this Master's thesis was prepared as an original work under the supervision of prof. Ing. Jiří Kučerík, Ph.D. I have listed all the literary sources, publications and other sources, which were used during the preparation of this thesis.



Natálie Paluchová
July 17, 2021

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Contents

Theoretical part

Chapter 1

Introduction

The soil can be considered as a recycling facility. In this manner carbon is circulated to the atmosphere as carbon dioxide, nitrogen is made available as ammonium and nitrate, and the rest of the associated elements appear in forms required by higher plants [1]. However, during microbial decomposition of dead plants and organisms (and perhaps other external carbon-rich compounds), only part of the carbon is released and the rest remains in the form of soil organic matter. This matter is available to all living organisms and is being constantly reused. Addition of any kind of substrate to such an environment will cause imbalance in this system, that might be a serious threat to a soil quality and hence plants and living organisms. And that is even though the soil microorganisms have the ability to cope with such a situation. In this sense (but not only), the land has been drastically transformed since the beginning of agricultural production. At the beginning, these changes were not so challenging, however in recent decades with growing demand for agricultural production and problems with soil quality, science had to step in. In general, indicators of soil quality are used to assess its state. This assessment focuses on overall quality or specific soil property. However, the assessment of specific soil properties is often individual or not standardized (or there are several standards so it is difficult to compare in between results). Therefore, attempting to estimate the potential impact of external substrate (such as fertilizers or in the case of this work biopolymer) may be difficult. This substrate can affect various soil properties and therefore the choice of soil quality indicators should be carefully considered.

The following chapters are devoted to the study of the effect of biodegradable substrates (specifically biodegradable polymers and microplastics overall) on the environment and organisms in it. The reason for this focus is their increasing production and application across various industries without complex investigation. It is based on our previous research focused on the analysis of microplastics in soil [2]. This research suggested a possible negative impact of bioplastics on soil organic matter and is hence investigated closely in the following chapters of this work.

Chapter 2

Soil quality

Significant decline in agricultural soil quality has occurred worldwide due to reckless agricultural use [3]. This ignorance stood out of the slow reactivity of soils to land use. It is therefore essential to identify a set of sensitive soil attributes that reflect the capacity of soil to function [4]. These information are provided by sampling and following analysis or visual examination of soils to assess theirs status and use potential is highly dependent on the choice of soil attributes and interpretation of measurements. This is highly affected by complexity and site-specificity of soils, legacy effects of previous land use, and trade-offs between ecosystem services [4]. The evaluation is necessary not just to determine the current soil condition, but also for further land management.

Evaluation of soil is complicated due to its complexity. Soil, together with water and air is the main component of environmental quality [5, 4]. However, there is a main difference between defining the degree of pollution. Within water and air, the quality is defined by a direct impact on natural ecosystems, human health and or animal consumption and health [4, 6, 7]. Soil, on the other hand, is not limited to degree of pollution, but in general is defined as 'the capacity of a soil to function within ecosystem and land-use boundaries to sustain biological productivity, maintain environmental quality, and promote plant and animal health' [4, 8, 9]. Which implies that soil quality is more complex than the quality of air and water and therefore cannot be assessed in the same way.

There are several expressions that describe soil functionality. The suitability of soil for agricultural production is described by **soil fertility**, i.e. the ability of the soil to supply plants with essential nutrients and water in adequate amount and proportions needed for growth and reproduction [4]. Some authors extend this definition by the ability of fertile soil to support a diverse and active biotic community, exhibit a typical soil structure and place of undisturbed decomposition [4, 10]. In fertility assessment are however investigated only chemical and physical properties associated with provision of water and nutrients to crops [4]. **Soil capability** then describes the capacity of a soil to contribute to ecosystem services (its potential) [11]. **Soil quality** links these two definitions and also includes the interactions between humans and soil. This term is hence often interchanged with **soil health**, which originates from the observation that soil quality influences the health of animals and humans via the quality of crops [4, 12]. Meanwhile soil quality focuses on the capacity to meet defined human needs (e.g. growth), soil health focuses more on the soil's capacity to sustain plant growth and maintain its functions [4, 13].

2.1 Soil properties

Soil properties differ significantly for different soil types. This causes problems in soil quality assessment. Soil cannot be evaluated without knowledge of the origin and specifically selected soil properties that are sufficiently important for the assessment of the soil. In other words, the selection of specific properties as soil indicators must be adapted to the soil type and to the specific environment.

Soil consists of four main components: minerals, soil organic matter, water, and air. These components influence soil physical properties including texture, structure, porosity and the fraction of pore space in a soil. Physical properties in turn affect air and water movement, and thus the soil's ability to function [14, 15]. These are supported by biological attributes (biota: flora, fauna and microorganisms) of soil, that allow the proper function, development, structure and productivity of soil. Chemical interactions on the other hand mostly occur on charged colloidal surfaces, affecting the nutrient cycle. Details on the physical, biological and chemical properties in relation to the assessment of soil quality, their contribution and determination will be discussed later in the Chapter 2.2.

2.1.1 The taxonomic soil classification system

Understanding the soil origin and its classification based on the soil taxonomic classification system, is the basis of soil quality assessment. Taxonomic soil classification system categorizes soils based on distinguishing characteristics as well as criteria that dictate choices in use. The soil in the same taxonomic class have similar properties and forms as a result of similar pedogenic processes. The primary objective of taxonomic soil classification system is to establish hierarchies of classes that permit to understand the relationship among soils and between soils and the factors responsible for their character [16]. Second, it provides a means of communication for the discipline of soil science [16].

The soil resources are diverse. For instance in Europe (see Figure on page 95), northern soils have higher organic matter content than southern ones and young soil predominate in central Europe. The Mediterranean Basin mostly consist of poorly developed soils or soil with accumulations of calcium carbonate [17]. This is given by both natural background and anthropogenic activity (management) of given area. But despite this general division of European soils, significant differences can be found even within small distances (e.g. meters, kilometers).

There are several taxonomic soil classification systems. Countries are commonly using their own, but if there is a lack of their own classification system, World Reference Base for Soil Resources (WRB), International Union of Soil Sciences (IUSS) or soil taxonomy by Food and Agriculture Organization of the United Nations (FAO) are commonly used. Based on the Czech Taxonomic Soil Classification System, soils can be sorted into 15 main categories (see Table 1). These categories then include subcategories, that divide soils more specifically according to its further characteristic. This classification is based on diagnostic horizons, diagnostic features and soil properties [18].

Table 1: General soil classification based on translated* Czech Taxonomic Soil Classification System [18, 19, 20, 21, 22].

Reference class	**Soil specification**	**Location**
Leptosols	Shallow soil over hard rock Gravelly and/or stony Do not hold water	Hard rock areas Areas where soil formation kept pace with erosion
Regosols	Weakly developed soils Unconsolidated sediments (sands/gravels)	Eroding lands in arid/semi-arid and mountain regions
Fluvisols	River sediments, lacustrine and marine deposits Rich in humus content	Areas of alluvial plains, river fans, valleys and tidal marshes
Vertisols	High content of expansive clay minerals Heavy texture Unstable behaviour (shrinking and swelling)	Areas that are seasonally humid or subject to droughts, floods or drainage
Andosols	Weekly developed soils Rich in vitreous materials	Mainly in volcanic areas
Chernosols	High content of humus, phosphorus, ammonia and phosphoric acid High moisture storage capacity	Tallgrass steppe and prairie (mostly Eurasian steppe, Canadian Prairies, Great Plains)
Luvisols	Soils with eluvial horizons from which clay has been leached and illuvial horizons in which clay has been deposited	Areas with temperate climates in general Forested areas with of subhumid to humid climate
Cambisols	Aggregate structure High content of weather able minerals Absence of a layer of accumulated clay, humus, soluble salts, iron and aluminum oxides	Temperate and boreal regions Regions with high rates of erosion
Podzols	Rich on humidified organic matter combined with aluminum and iron Relatively porous	Cold, humid areas Coniferous or boreal forests, eucalyptus forests and heathlands Under-ashed soils
Stagnosols	Wet and mottled soils Possible concretions and/or bleaching Oxygen deficiency	Flat to gently sloping land Cool temperature to subtropical regions Humid to perfumed regions

Reference class	Soil specification	Location
Gleysols	Saturated with water (periodically or permanently) Depleted of oxygen	Subhumid and humid areas Areas of shallow depressions or level areas
Sodisols	Hard when dry; Compact, swelling to sticky material when wet More than 15 % of sodium	Semiarid and subhumid regions
Salisols	'Salt marsh' Rich on soluble salts Usually absence of layering, limited leaching	Adrid to subhumid, poorly drained areas Warm to hot climate
Organosols	Rich on organic matter Do not have permafrost near the surface	Peatlands Areas saturated with water
Anthrosols	'Anthropogenic soil' Formed or heavily modified by human activity (irrigation, addition of organic waste, wet-field cultivation etc.)	Long term agricultural areas

* For the most accurate translation into Czech, following taxonomic classes were used: International Union of Soil Sciences soil classification and The Canadian system of soil classification.

2.2 Soil quality indicators

Soil quality indicators are attributes of the soil which may be measured to assess quality with respect to a given function [3]. Given complexity of soil and large number of soil properties that may be determined, it is important to select the right indicator (see the most common ones in Figure 1). The challenge in selection of indicators is that they are interrelated (each describes different processes in soil) and that there is no single indicator or suite of indicators that would reflect soil function [16]. Moreover, the selection of representative indicators highly depends on land use, item function, reliability of measurement, spatial and temporal variability, sensitivity to changes in soil management, comparability in monitoring systems and skills required for the use and interpretation [3, 23]. Principally, good soil indicator is sensitive to management changes, but stable in response to non-management changes (such as weather) and simultaneously reflects some of the functioning of the system [16]. The most commonly used soil quality indicators therefore are soil organic carbon, soil pH, available phosphorus, indicators of water storage, and bulk density [24].

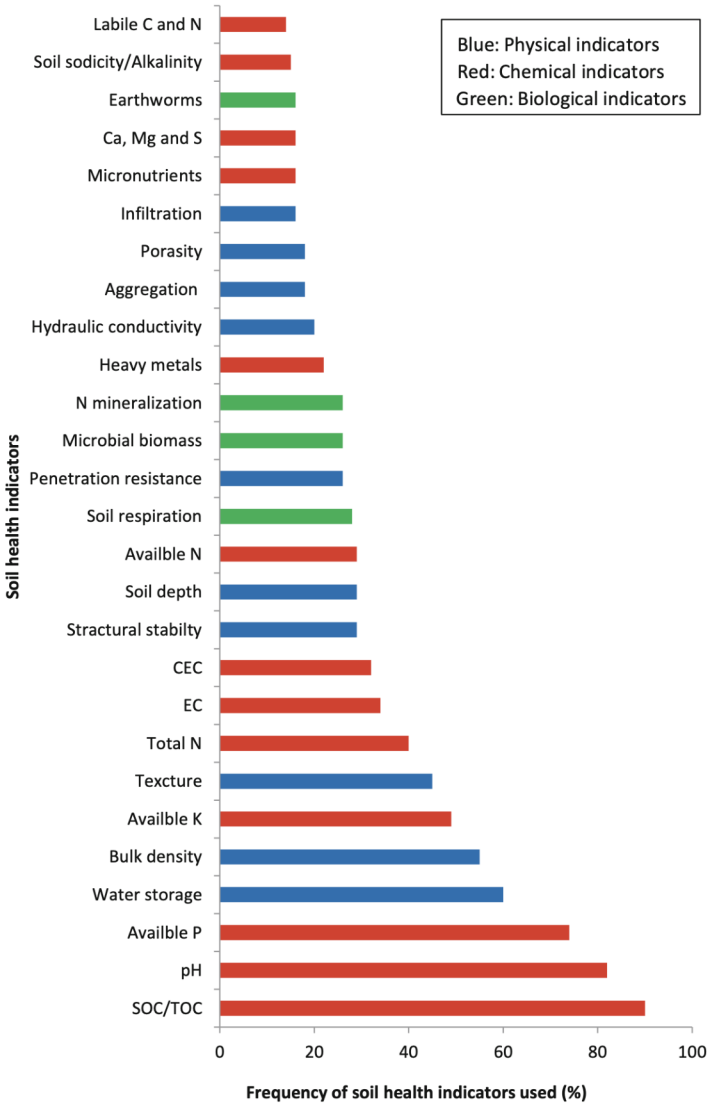

Figure 1: Frequency of different indicators used for soil quality assessment [4, 24].

These indicators are represented by either biological, chemical or physical properties (see Figure 1). **Physical indicators/attributes** are concerned with a physical arrangement of solid particles and pores [23]. Namely soil texture, dry bulk density, porosity, aggregate strength and stability, soil crusting, soil compaction and top soil depth. The **chemical indicator** may be pH, salinity, organic matter content or cation exchange capacity. The selection of a specific chemical indicator depends upon the function under consideration [23]. **Biological indicators** are very dynamic and sensitive to changes in soil conditions and therefore are referred for short-term evaluations [23]. Biological indicator may be a population of microoganisms, enzyme activities, fatty acid profiles or respiration rate. More empirical indicators are visible attributes, that include evidence of erosion in the form of rills and exposure of subsoil, surface ponding of water, surface run-off and poor plant growth [23]. Following sections describes the most important and used soil indicators for soil quality assessment.

2.2.1 Soil texture, structure and aggregation

Many important soil processes take place in soil pores, influenced by soil texture and structure. They influence porosity by determining the size, number and interconnection of pores [14].

Soil texture describes particle size distribution and influences the physical and chemical characteristics of the soil [25]. It has an impact on many properties such as movement and retention of air and water with subsequent effects on plant water use and growth [14]. Soil texture based on the classification system of International Union of Soil Sciences is shown in the following Table 2:

Table 2: IUSS classification of soil separates according to the particle size [26].

Soil separate	Clay	Silt	Fine sand	Coarse sand	Gravel
Size [mm]	<0002	0.002	0.02	0.2	2.0

The texture is generally estimated by the 'feel method', Bouyoucos hydrometer method or International pipette method. The field method determines the textural class of soil by its feel performed by expert. The hydrometer method is based on the decrease of the suspension at a given depth as a homogenous dispersed suspension settles [27]. The rate of decrease in density is related to the sizes of particles and based on this particles can be particles distinguished using Stokes's law (see Equation 1) particles can be distinguished [27]. The pipette method is more time consuming and cannot be used for large number of samples. It is done by using sieves to separate out coarse sand from the finer particles, the silt and clay contents are then determined by measuring the rate of settling of these two separates from the suspension in water, which requires Stokes's law as well [28]:

$$\nu = \frac{2gr^2(\rho_s - \rho_w)}{9\eta} \qquad (\text{cm} \times \text{s}^{-1}) \quad (1)$$

where ν represents settling velocity, g gravitation acceleration, ρ_s density of particle, ρ_w density of fluid and η fluid viscosity.

Soil structure is the relative arrangement of the soil particles (primary and secondary aggregate particle), that provide stability of the soil [25]. Based on the structure is soil

classified either as very coarse (>10 mm), coarse (5–10 mm), medium (2–5 mm), fine (1–2 mm) or very fine (<1 mm) [25]. Coarse (sandy) soils have many large macro pores and fine (clayey) soils are tightly arranged with small micro pores (see Figure 2) [14].

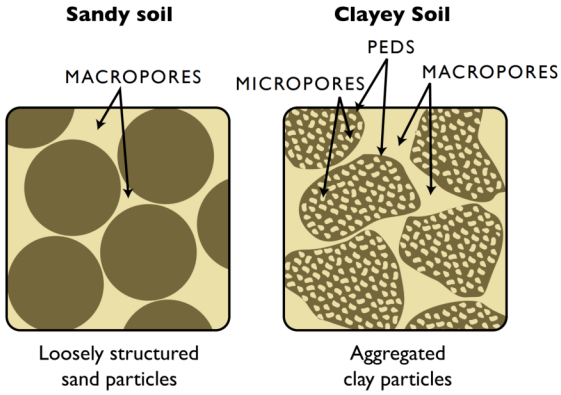

Figure 2: Generalized porosity in coarse and fine soils [14].

Soil aggregation reflects the arrangement of the primary particles into structural units, aggregates using cementation or binding together the floccules with different forces [25, 29]. It influences the amount and size of pores in the soil. Soils with an optimum level of aggregation allow more rapid water and air penetration, and thus provide better soil quality [29]. In general, sands have fewer aggregates and lower aggregate stability than loam, clay loam, or clay soils [30]. Soil aggregation is influenced by tillage intensity and residue management [29, 31]. Depending on the aggregate stability and the ease of separation is the structure characterized as either poorly, weakly, moderately, well or highly developed [25]. **Aggregate stability** reflects the level of biological activity, organic matter content and nutrient cycling in the soil [24, 30]. It is an important soil health indicator since it is maintaining ecosystem functions, such as organic carbon (C) accumulation, infiltration capacity, movement and storage of water, and root and microbial community activity) and it can also be used to measure soil resistance to erosion and management changes [24, 32, 33]. Aggregation is assessed either by aggregate size classes (the proportion of aggregates in different size ranges), stability (the percentage of the aggregates in a specified size class that remain intact after tests) or distribution of stable aggregates (the proportion of stable aggregates in different size ranges) [30]. There are several ways to measure aggregate stability (dry vs. wet, sieving vs. agitation, etc.), the appropriate technique is selected based on soil type and climate [24, 30].

2.2.2 The porosity, Water Holding Capacity and Bulk Density

The texture and structure of soil is closely linked to the **porosity** (the air or water-filled spaces between particles). If the pores are saturated, the water within macropores drains freely from the soil via gravity (see Figure 3). In case that the water is drained, it is held in micropores via attractive capillary forces or surface tension between water and solids [14]. The capillary water doesn't flow freely, because it is retained in the soil and is only removed by plant uptake or evaporation [14]. The amount of capillary water available to plants can be expressed by water holding capacity (WHC). If the water isn't available to the plants, it adheres to soil with help of hygroscopic forces.

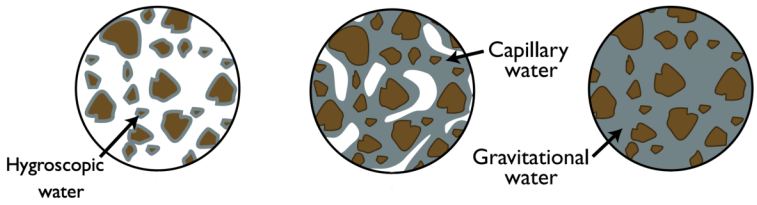

Figure 3: Simplified representation of water retention in pores (adapted from McCauley, Jones, Olson-Rutz, 2005 [14]; and Brady, Weil, 2016 [15]).

Water Holding Capacity (WHC) represents the amount of water that can be held in soil against the pull of gravity [30, 25]. The importance of a WHC lies in its ability to retain water, because soils with higher water holding capacity retain water better, thus support plant growth and development and reduce leaching losses of nutrients [24]. WHC is highly dependent on the texture, amount of organic matter and structure and percent of sand, silt and clay [30]. The WHC is determined by measuring the amount of water (expressed in percentage), that was held in the soil after the excess gravitational water was drained. The stage of field water holding capacity is attained in the field after 48–72 h of saturation which is considered as the upper limit of plant-available soil moisture [25]. The same period is therefore advised for laboratory measurement as well.

The rate at which water enters the soil surface and moves through soil depth is called **infiltration** [24, 30]. Infiltration rate changes with soil use, management, and time [24, 34, 35]. It is therefore (together with aeration) significantly affected by a soil compactness, which is determined by the measurement of **Bulk Density (BD)** [24]. BD represents the weight of dry soil per unit of volume expressed in $g \cdot cm^{-3}$. In a range of BD 1.3 to 1.7 $g \cdot cm^{-3}$ the restriction of root growth and decrease of plant yield may occur [36, 37, 38]. Bulk density is determined by recording the fresh weight of the sample in the field and dry weight of the sample in the laboratory [24].

2.2.3 Cation Exchange Capacity, pH and Electrical Conductivity

Cation-Exchange Capacity (CEC), or nutrient retention capacity, measures the amount of positively charged nutrients or toxic compounds, such as cations calcium (Ca), magnesium (Mg), potassium (K), aluminium (Al) and manganese (Mn), that the soil could hold onto electrochemically and release for plant use (see Figure 4) [24, 30]. In other words, it determines the amount of ions, that the system is able to bind as well as soil adsorption. The particles in the soil with charged surface are called colloids. The ions are either adsorbed and held to the colloid surface or exchanged with other ions and released to the soil solution depending on the charge, size and concentration of ions in the soil [14]. The soil's ability to adsorb and exchange ions is its exchange capacity and because soils are overall negatively charged, it is further described as 'Cation Exchange Capacity'. CEC is usually measured by ammonium acetate method at pH 7 or the barium chloride-triethanolamine method at pH 8.2 [24, 39, 40]. The CEC is dependent on soil texture, type of clay minerals, the amount of organic matter, and the pH [30].

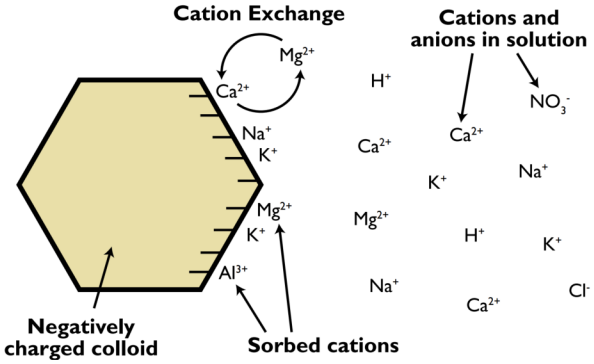

Figure 4: Simplified representation of cation exchange capacity (adapted from McCauley, Jones, Olson-Rutz, 2005 [14]; and Brady, Weil, 2016 [15]).

Soil pH is an indication of chemical properties and reactions that take place in soil [24, 30]. It affects solubility of several compounds, relative bonding of ions to exchange sites, and the various microorganisms [24]. Soil pH depends on parent material, climate, and is strongly affected by the application of ammonium fertilizers, liming, and animal manure [30, 29]. The soil pH (a negative logarithm of the active hydrogen ion concentration in gmol/L in the soil-water suspension) is measured by a pH meter after stirring the suspension with a glass rod for 25 minutes [25].

Electrical Conductivity (EC) measures concentration of ions (salt) in solution [30]. It is generally used as an indicator of salinity, crop performance, nutrient cycling (particularly nitrate), biological activity and, along with pH, indicates soil structural decline especially in salisols/sodisols [24, 41, 42]. Electrical conductivity can be determined using a conductivity meter in 1:2 (soil:water ratio) [24, 43].

2.2.4 Soil Organic Matter/Carbon

Soil Organic Matter (SOM) is a part of soil, which includes carbon-based materials left behind by plants and animals and which has been produced by microorganisms [30]. Because it is difficult to measure SOM directly, the measurement of **Soil Organic Carbon (SOC)** is preferred. SOC correlates positively with crop yields and it affects functional processes in soil like the storage of nutrients (mainly N), water holding capacity, stability of aggregates and microbial activity [24, 44]. It is assumed that SOM contains about 58% of organic carbon [25]. In case of SOM measurement, loss of weight on ignition is used [25]. Both SOM and SOC can be determined by volumetric (using potassium dichromate or potassium permanganate as an oxidizing agent) and colorimetric methods [45, 15, 46].

2.2.5 Available nutrients, micronutrients and heavy metals

The most important available nutrients that meet requirements of soil indicators are nitrogen (N), phosphorus (P), potassium (K), sulphur (S) and micronutrients copper (Cu), manganese (Mn), boron (B), molybdenum (Mo), zinc (Zn) and iron (Fe) [24, 25]. These nutrients are considered extractable nutrients and their measurement provides indication of a soil's capacity to support plant growth and conversely, critical or threshold values for environmental hazard assessment [47, 48].

Nitrogen (N) is linked to cycling of other available nutrients, especially soil organic C [24]. There are four main forms in which is N indicated: total nitrogen,

mineralizable nitrogen, inorganic N-NO_3^- and NH_4^+ [25]. **Total nitrogen** is a sum of all forms of inorganic and organic N. The determination of total N is based on the available form of N present in a sample. Organic N is usually converted into simple inorganic ammoniacal form (sulfuric acid is used as a reducing agent), which is distilled using Kjeldahl method and estimated using standard acid (salicylic acid or alternatively Devarda's alloy) [25]. **Mineralizable nitrogen** is measured as an index of plant-available N content, using potassium permanganate (oxidizing and hydrolyzing it to ammonia). Ammonia is then condensed and absorbed in boric acid and is titrated against standard acid [25]. Inorganic N in soil is present as **N-NO_3^- and NH_4^+**. They reflect effects of many practices including crop rotation, fertilization strategies, and use of animal manure and at the same time provide information about potential for leaching and contamination of ground and surface water and for release of nitrous oxides (NO_x) emissions [29, 49, 50]. There are various methods for N-NO_3^- and NH_4^+ determination, such as the methods of extraction (extraction solution: 2Ml KCl), methods using specific ion electrodes, colorimetric techniques, micro-diffusion, steam distillation and flow injection analysis [25, 24].

Available **phosphorus (P)** is the most commonly used indicator of all available nutrients. It is important because of its role in supporting plant growth, but also as a monitoring tool in order to prevent environmental hazard if surface runoff occurs [29, 51]. Available P is influenced by fertilizers, animal manure application and maintaining a near neutral pH of soil [29]. Based on pH of a soil a method for available P determination is used. Bray's method is commonly used in case of acid soils and Olsen's in case of neutral and alkali soils [25]. In these cases a specific reagents (Olsen's: 0.5M $NaHCO_3$, pH 8.5 or Brax's: 0.03N NH_4F and 0.025N HCl) are used to extract available phosphorus [24, 25, 52, 53]. And because these solutions tend to fade frequently, making it challenging for further analysis, ascorbic acid method has been presented and became preferred over these former methods [24, 54]. Regardless of the method used, this blue-coloured solution is then measured spectrophotometrically and evaluated base on Beer's Law 2:

$$A = 2 - log_{10}(\%T) \qquad (-) \quad (2)$$

where A represents absorbance and $\%T$ transmittance in percentage [25].

Potassium (K^+) is a regulator of metabolic activities, that is highly mobile in the soil. Its deficiency causes plants' sensitivity to drought, frost and a high salinity [55]. Potassium is usually extracted with 1N ammonium acetate solution (pH = 7) and estimated by flame photometer [25].

Sulphur (S) is a constituent of proteins and its deficiency therefore causes inhibition of protein synthesis [56]. It is also a structural constituent of coenzymes and secondary plant products and acts as a functional group involved in metabolic reactions [56]. The soil available S occurs as adsorbed SO_4^{2-} which may be extracted using $CaCl_2$ solution to produce phosphate ions and estimated turbidimetrically with UV/VIS spectrophotometer [24, 25, 57].

The last macronutrients are exchangeable cations **calcium (Ca^{2+}) and magnesium (Mg^{2+})**. Calcium plays an important role in nutrient transport and plant membrane strength and its deficiency cause stunted roots and poor plant standability. Magnesium is essential for enzyme production, chlorophyll structure and photosynthesis and its shortage cause chlorosis. Ca^{2+} and Mg^{2+} are extracted with the same ammonium acetate solution as in the case of potassium [25]. They are then determined either by atomic absorption spectroscopy (AAS) or the Ethylenediaminetetraacetic acid (EDTA) titration method [25].

Micronutrients, as macronutrients, are investigated to find out whether the soil can supply crops with adequate micronutrients for its optimum production or whether crops are grown on nutrient deficient soils [25]. The most commonly studied micronutrients are Zn, Cu, Fe, Mn, B and Mo. They can be extracted either with neutral ammonium acetate or chelating agents EDTA and Diethylenetriaminepentaacetic acid (DTPA) and then determined colorimetrically or using AAS [24, 25].

Heavy metals refer to a group of metals and metalloids, with an atomic mass greater than 20 and specific gravity greater than 5, possessing biological toxicity [58]. The contamination by heavy metals has been increased due to industrialization and intensified agriculture. They both cause accumulation of heavy metals, resulting in soil/water degradation, consequently food contamination, effect on organisms health and ecosystem malfunction [59]. Therefore, heavy metals are considered as an important soil health/quality indicator. The most commonly assessed heavy metals are cadmium (Cd), mercury (Hg), copper (Cu), arsenic (As), lead (Pb), chromium (Cr), nickel (Ni), and zinc (Zn). Heavy metals as an indicator for soil quality assessment are usually determined by AAS [25]. In case of arsenic and mercury determination is AAS equipped with a hydride generator in addition [25].

2.2.6 Soil Microbial Biomass and Respiration

Soil microbial biomass (microbial biomass carbon and nitrogen) plays an important role in nutrient cycling, plant nutrition, and functioning of different ecosystems as it is responsible for organic matter decomposition [24]. In general, it provides information about biological activity within soil [30]. It is highly influenced by management practices which makes it a sensitive indicator of soil quality and health [24, 60]. There are several physiological, biochemical, and chemical techniques that assess soil microbial biomass, such as chloroform fumigation incubation (CFI) [61], chloroform fumigation extraction (CFE) [62, 63], substrate-induced respiration (SIR) [64], and adenosine triphosphate (ATP) analysis [65, 66, 67].

First two methods are used to analyze microbial biomass carbon and nitrogen. In **chloroform fumigation incubation** method is moist soil fumigated with chloroform (chloroform is later removed by repeated evacuation) and then is reinoculated with unfumigated soil and incubated for 10 days (at constant 22 or 25 °C, at field capacity or 50% of its water holding capacity) [24, 61]. CO_2 evolved is measured during the incubation by a gas chromatography or by sorption in alkali followed by titrimetric, conductometric, or colorimetric determination [24, 61]. In addition, an unfumigated control sample is used. Finally, as the net C mineralized as CO_2 is only a proportion of total microbial biomass, the kC factor is used to calculate microbial biomass C based on the following equation 3 [61]:

$$\text{Biomass C} = \frac{(\text{C}_{\text{fumigated}} - \text{C}_{\text{control}})}{kC} \qquad (\mu\text{g} \times \text{g}_{\text{soil}}^{-1}) \quad (3)$$

where $C_{fumigated}$ represents CO_2 evolved from fumigated sample and $C_{control}$ from unfumigated one. The kC is estimated to 0.41 at 22 °C or 0.45 at 25 °C [61, 64]. In case of microbial biomass N calculation, mineral N (see subsection 2.2.5) from fumigated and unfimigated (control) samples is determined, and kN factor is used to correct incomplete mineralization of N (killed microorganisms) 4:

$$\text{Biomass N} = \frac{(\text{N}_{\text{fumigated}} - \text{N}_{\text{control}})}{kN} \qquad (\mu\text{g} \times \text{g}_{\text{soil}}^{-1}) \quad (4)$$

where kN value varies from 0.30 to 0.68 [24]. Soils with relatively low microbial biomass but high respiration activity often have low or even negative biomass estimates because of unequal amounts of non-microbial biomass C is mineralized [24, 68]. To overcome this issue a **chloroform fumigation extraction** is used. The measurement of evolved CO_2 here is replaced by direct extraction of C and N (extraction solution: 0.5 mol K_2SO_4/L) [62, 63]. Equations 3 and 4 are then used to determine soil microbial biomass. The kC and kN value is estimated to rage from 0.2 to 0.68 [24].

Third method, **substrate-induced respiration**, is used to measure microbial activity as a response to substrate addition (e.g. glucose) [64]. This response varies between soils [24]. SIR is using excess substrate (glucose) to increase biological activity in incubated soil (with constant temperature and moisture) to measure respiration rate (CO_2 evolved per hour) [24, 30]. The evolved CO_2 is captured in NaOH traps and then determined using for instance electrodes or titration method (with HCl, phenolphthalein and BaCl2) [69].

ATP analysis is used to estimate the amount of living microbial biomass. ATP is usually present also in a soil as part of dead microbial cells, however it is rapidly degraded and therefore doesn't interrupt the microbial biomass estimation [24]. ATP is extracted with acid reagents from moist, preincubated soil, and estimated by the luciferin–luciferase system [65, 66, 67].

2.2.7 Soil Enzymes

Biochemical reactions are related to the presence of enzymes, which play a key role in energy transfer through decomposition of soil organic matter and nutrient cycling [1, 24]. The importance of enzymes leans on two properties. First, they are catalysts, which means that they allow without undergoing permanent alteration cause chemical reactions to proceed at faster rates [70]. And second, their specificity (they combine with specific substrates with specific stereospecificity) for the types of chemical reactions in which they participate [70]. Due to the variety of soil types and their physio-chemical and biological properties, there is a difference in level of soil enzymatic activity [1, 24]. Furthermore, each enzyme has a different role in maintaining soil health and quality.

Enzymes are highly sensitive to any change in soil management practices and environmental conditions [24]. And therefore, they are used as sensors of soil microbial status, soil physio-chemical conditions, and the influence of soil treatments or climatic factors on soil fertility [24]. Enzymes in soil are not determined by direct analysis but indirectly through their ability to transform a given organic substrate into a known product [1]. The determination is possible due to the selectivity of the enzymes, which catalyzes the metabolic conversion of only one substrate or chemically similar substrates [71]. Enzymatic activity is therefore most often determined using artificial substrates and sometimes even an additional substrate (e.g. hydrogen peroxide), which is normally provided by the microorganism [71]. Certain enzymes are ubiquitous, such as urease, catalase, phosphatase, and peptide hydrolysases [1]. Other enzymes can be on the contrary produced only under special circumstances. Some of the frequently analyzed soil enzymes (for soil quality/health assessment) and their important functions are discussed in the following paragraphs. The detailed description of the determination of soil enzymes activity has been summarised for example by M. A. Tabatai, 1994 [70].

Urease regulates supply of a nitrogen to plants after urea fertilization and it is therefore used as an index of N transformation in soil and in management of soil fertilizers [72, 73, 74]. In other words, urease is an enzyme responsible for urea degradation (biological

turnover and bioavailability of nitrogen) forming ammonia and carbon dioxide and thus it is considered a proxy of nitrogen mineralization. This catalytic reaction 5 is described below:

$$\text{Urea} + H_2O \rightarrow CO_2 + 2\,NH_3 \tag{5}$$

Phosphatase is an enzyme that catalyses hydrolysis of ester-phosphate bonds [75]. Both acid or alkaline phosphatase convert organic P compounds into inorganic forms (hydrogen phosphate, dihydrogen phosphate), in order to make P directly available to microorganisms and plants [72, 76, 77] It cleaves the ester bond between the phosphate group and the organic residue of the organic phosphates, causing the phosphate to be removed from the proteins [75]. Phosphatases are the functional antagonists of protein kinases, that regulate the biological activity of proteins by phosphorylation of specific amino acids with ATP as the source of phosphate (inducing conformation change from an inactive to an active form of the protein) [78, 79]. All soil microorganisms produce extracellular phosphatases and therefore their activities in soil are generally greater than any other enzymatic activities [80]. Phosphatase indicates soil fertility, as it represents phosphorous cycles in soil associated with phosphorous nutrition and hence growth of plants [81]. An example of possible reaction 6 is presented below:

$$\text{Phosphate monoester} + H_2O \rightarrow \text{Alcohol} + \text{Phosphate} \tag{6}$$

Glucosidase is responsible for cellulose decomposition by hydrolysis of glucosides to glucose [82]. Its products are important energy sources for soil microorganisms as it is part of the carbon cycle for the growth and activity of soil microorganisms [82, 83]. Glucosidase mainly originates from fungi, and therefore may represent a shift of the microorganism community by decreased/increased glucosidase activities [84, 85]. Possible glucosidase reaction 7 is presented below:

$$\text{Glucoside} + H_2O \rightarrow \text{Alcohol} + \text{Glucose} \tag{7}$$

Arylsulfatase mobilizes inorganic sulphates for plant nutrition. Specifically, it catalyzes the hydrolysis of aromatic sulphate esters to phenols and sulphate [72, 86]. About 40-70% of the total S in soils is present in the form of ester sulphates an thus sulfatases play important role in S mineralization [87]. This process is described in the following reaction 8:

$$\text{Phenol sulphate} + H_2O \rightarrow \text{phenol} + \text{sulfate} \tag{8}$$

Beta-1,4-N-Acetyl-glucosaminidase (NAG) is one of three enzymes that catalyze hydrolysis of chitin, which is important for soil carbon and nitrogen cycling [88]. It participates in the processes of chitin conversion to amino sugars, that are major sources of mineralizable N in soils, since it is a major structural component in insects and fungal cell walls [88, 89]. In general, NAG is associated with microorganism N-acquiring activities and is correlated with fungal biomass [90, 83, 91].

Catalase is important oxidoreductase-enzyme, that protects cells from oxidative damage by reactive oxygen species, based on reaction 9 below [72, 92, 93]:

$$H_2O_2 \rightarrow O_2 + 2\,H_2O \tag{9}$$

Nitrogenase is an important contributor to a nitrogen fixation. It has the ability to convert atmospheric dinitrogen into ammonia using ferrodoxin, represented as Z in the

following reaction 10 [72, 94]:

$$8\ Z_{red} + 8\,H^+ + N_2 + 16\,ATP + 16\,H_2O \rightarrow 8\ Z_{ox} + H_2 + 2\,NH_3 + 16\,ADP + 16\ \text{phosphate} \quad (10)$$

Invertase is a hydrolyze-enzyme, that serves as an indicator of carbon transformation, responsible for the breakdown of plant litter in soil [72, 95]. This process is described in the reaction 11:

$$\text{Sucrose} + H_2O \rightarrow \text{glucose} + \text{fructose} \quad (11)$$

Cellulase is an enzymatic complex responsible for degradation of cellulose, that provides readily available C for soil microorganisms and thus is increasing soil microbiological activity, and soil fertility [72, 96]. This process is either based on endohydrolysis or hydrolysis of $(1 \rightarrow 4)$-β-D-glucosidic linkages in cellulose releasing cellobiose from the non-reducing ends of the chains [72, 96].

2.2.8 Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal fungi (AMF) live in a symbiotic relationship with terrestrial plants, by a formation of communities of infectious propagules (spores, hyphal, arbuscules and vesicles) that penetrate the root of a vascular plant increasing its resistance [24, 97]. The formation of such community is highly affected by agricultural management practice, soil type, concentration of nutrients and host species [98, 99, 100, 101, 102]. Therefore is AMF used as a potential indicator of a sustainability of a long-term farming systems [24]. Quantification of AMF biomass is mainly performed through microscopic methods [103, 104]. This method is based at first on spore extraction by wet sieving, followed by decantation and filtration of spores [24, 103]. Finally, the spores are observed and counted under the microscope. If the objective is an assessment of a root colonized by AMF, root staining followed by quantification with gridline intersect method (to estimate the colonized root length) is used [24, 104, 105]. Biochemical methods, such as analysis of signature fatty acids and glomalin are also applied [24].

2.2.9 Earthworms

The earthworms may also function as an indicator of soil quality, since their quantity is affected by many factors and activities, such as tillage, pH, chemical additives, and especially crop residues. They create tunnels, that help to increase air space and thus, improve infiltration rate and create habitats for invertebrates. Furthermore, they improve aggregate stability, promote soil mixing, increase the surface area of residue to help with decomposition, and enhance microbial activity in the casts [30]. However, the influence of worms on the soil quality varies from species to species and their presence may not be a guarantee of soil quality. In general, the presence of earthworms indicated either high productivity or good soil quality and thus it is essential to not consider earthworms as a single and direct indicator of a good soil quality (their absence doesn't necessarily indicate poor soil quality) [30].

Earthworms are sampled during cool and wet season (autumn, spring, winter) by the extraction of a soil bulk with a spade fork [24, 106]. Traditionally, they are hand sorted during an examination of a soil bulk [24]. Sometimes, in order to collect deep burrower species, an irritant suspension (mustard powder suspension) is poured into the soil, acting as an expellant of earthworms [24].

2.3 Soil quality assessment

Soil quality assessment focuses on evaluation of a sustainability of soil management practises, considering multiple soil uses (e.g., agricultural production, forest, rangeland, nature conservation, recreation, or urban development) [3]. It is based on monitoring of physical, chemical, and biological effects of management decisions that may affect soil and water resources [29]. The soil quality assessment is constantly evolving in terms of objectives, tools, methods, and even an overall approach (see Figure 5) and hence, there are still number of steps to be taken [4]. The following section of this chapter provides a brief overview of possible approaches to soil quality assessment.

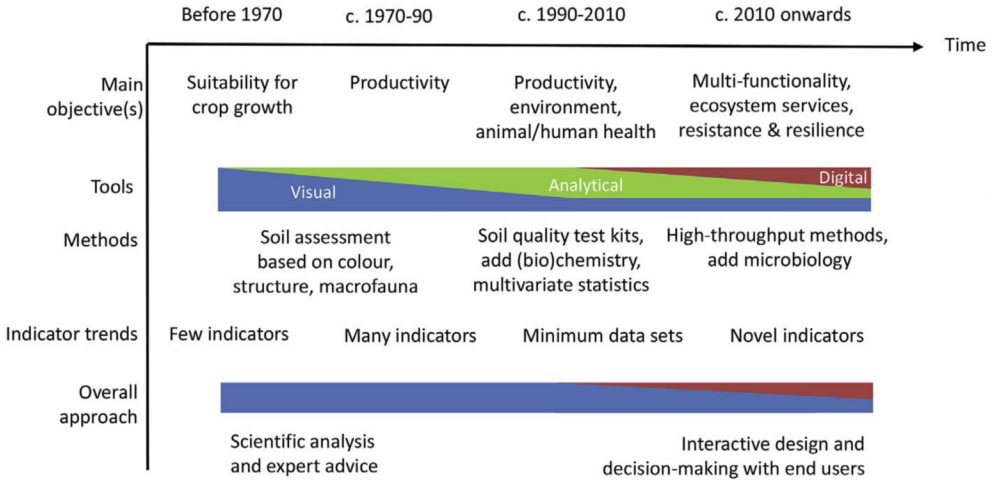

Figure 5: Main objectives, tools and approaches of soil quality assessment through history [4].

2.3.1 Analytical and visual approach to soil quality assessment

The analytical approach to soil quality assessment is the most common approach used at national level [4]. It consists of laboratory analysis of specific soil properties/indicators (more about indicators will be discussed later in section 2.2). **Visual approach** on the other hand, targets on farmers that evaluate soil directly in the field, i.e. they can evaluate soil based on qualitative indicators, deliver immediate results, and facilitate communication with scientists [4]. Visual soil assessment approach mostly utilizes indicators linked to a soil structure, sometimes in relation to productivity [4].

The simplest soil quality assessment uses benchmark sites to assess changes in soil quality over time, especially in relation to the soil threats erosion, compaction, organic matter loss, acidification and salinization [4, 107]. Another simple approach to assess soil quality is to establish soil pits or to use soil quality test kit, that allows to measure and evaluate important parameters, such as water infiltration, bulk density, soil respiration, water content, water holding capacity, water-filled pore space, temperature, pH, electrical conductivity, and soil nitrate [23, 108].

More complex method is The Soil Management Assessment Framework (SMAF), that clearly defines indicators (out of 81 potential ones) that are selected for evaluation using selection rules [4, 109, 110, 111]. The indicator values are then interpreted based on scoring curves [110]. The flexible and context-specific approach of the SMAF has also been an

inspiration for multivariate statistical methods (see Section 2.3.2) to select the most relevant indicators to arrive at a soil quality index geared to the specific conditions [4]. A similar approach as used in SMAF is the AgroEcosystem Performance Assessment Tool (AEPAT) and the Cornell Soil Health Test program [29]. AEPAT utilizes computer program to assess agronomic and environmental performance of soil and crop management practices [29, 112]. It is based on ranking of agroecosystem performance among management practices for functions and indicators included in the procedure [112]. In the latter case, the assessment is targeted at land users. The Cornell Soil Health test is offering them various testing packages and is supplying them with management advice together with the results (values are interpreted using scoring curves) [4, 29]. These two applications describe the soil status or conditions and reflects current or past management decisions, i.e. 'dynamic soil quality', rather than 'inherent soil quality' which reflects the basic soil forming factors of climate, parent material, time topography and vegetation on soil attributes, which are unresponsive to recent management [29]. In other words, dynamic characteristics change on human time scales—biological activity (structural features, and water and nutrient movements) and inherent characteristics are those that change over geologic time scales (texture, slope, mineralogy, and depth) [30].

There is a plethora of soil monitoring programs in Europe, hence there is a need to determine and monitor soil quality in a manner which allows comparison within and between countries [23]. Therefore, International Standardisation Organisation (ISO) established a Technical Committee (ISO/TC 190 Soil quality) that is focusing on a development of methodologies for monitoring the soil quality [113]. The Technical Committee is not concerned with the actual assessment of soil quality, but seeks to provide a set of proven, widely used standard methods which can be reliably used by those seeking to evaluate soil quality [23]. The ISO program on soil quality presents standard procedures covering many aspects of description, sampling, and analysis and evaluation procedures to assist in establishing indices of soil quality [23].

2.3.2 Multiparametric indices and Soil Quality Index

Several methods of soil quality evaluation have been developed, such as soil card design and test kits, geostatistical methods or soil quality index methods [4, 114]. These methods assess soil quality based on specific indicators of soil quality (set of soil properties) [114, 115]. Currently, different individual parameter (e.g., total organic carbon, texture) or single indices (e.g. the metabolic quotient/ratio) are utilized despite the fact that the use of one individual indicator or indices integrating only two parameters has many limitations [114, 116, 117]. Furthermore, information about soil quality and degradation is completely missing [116]. Therefore, there is ongoing development of multiparametric indices, that integrate different parameters, among which the most important are the biological and chemical ones (e.g., pH, soil organic matter, bulk density, water-stable aggregates, electrical conductivity, microbial biomass carbon, respiration or enzyme activities) [116]. This multiparametric aggregation of soil quality indicators yields single Soil Quality Index (SQI). Whereas numerous attempts have been made to estimate single SQI for major soils across the World, there is no standard method established yet [26]. However, there are three widely used SQI methods that stand out: simple additive SQI, weighted additive SQI and statistically modeled SQI. They are established on either expert opinion (subjective), or using mathematical–statistics methods (objective) [116].

Simple Additive SQI method is a method where soil parameters are given threshold values (SQI_{max} for maximum, SQI_{min} for minimum) based primarily on the literature review and expert opinion [26]. The individual index values are then summed up in Equation 12 to obtain a total SQI :

$$\sum \text{SQI} = \sum \text{Individual soil parameter index values} \tag{12}$$

in which Simple additive SQI of individual soil is calculated by Equation 13:

$$\sum \text{Simple SQI} = \frac{\sum SQI - SQI_{min}}{SQI_{max} - SQI_{min}} \tag{13}$$

where $\sum SQI$ represents the sum of 'Individual soil parameter index values' taken from an Equation 12 [26, 118].

Weighted Additive SQI assigns to each soil parameter score ranging from 0 to 1 by applied linear equation based on criteria: 'more is better', 'less is better' and 'optimum' [5, 26]. Less clear parameters are assigned to 'more/less is better' criteria. For 'more is better' (e.g., cation exchange capacity, soil organic matter, available nitrogen/potassium, etc.), each observation is divided by the highest observed value of the entire data set (the highest observed value has a score of 1) [26, 118]. For 'less is better' (e.g., bulk density, percentage of $CaCO_3$, available calcium, electrical conductivity etc.), the lowest observed value is divided by each observation (the lowest observed value has a score of 1) [26, 118]. 'Optimum' properties are those with indicators that have a positive influence on soil quality up to a certain level beyond which their influence is harmful (e.g., pH, available phosphorus, percentage of sand, silt, clay, basal soil respiration etc.) [119]. In this mathematical algorithm functions 'optimum' scored up to a threshold value as 'more is better', and thereafter above the threshold values were scored as 'less is better' [5, 26, 120]. To each score are then assigned their weights depending on soil function for improving and maintaining soil quality [118, 119, 121]. Weighted SQI is then computed in Equation 14:

$$\sum \text{Weighted SQI} = \sum (W \cdot Sf) \tag{14}$$

where W represents assigned weight and Sf soil function. Overview of tables with threshold values, detail description of soil functions and assigned weights necessary for calculation of both Simple and Weighted Additive SQI can be then found (for instance) in publication of Atanu Mukherjee et Rattan Lal (2014) [26].

Statistically modelled SQI uses Principal Component Analysis (PCA) to extract information and reduce data, specifically to choose the most important indicator in a Minimum Data Set (MDS) [118]. The MDS consists of measurements, that are considered basic to assessing soil [16]. Other measurements can be included depending on local goals and soil conditions [16]. The main difference between the additive methods and statistically modelled SQI is that additive SQIs rely mainly on subjective expert opinion and literature review, while the PCA method is more objective of using a number of statistical tools (multiple correlation, factor and cluster analyses) [5, 8, 26]. The function of PCA is to reduce the dimensionality of the entire data set consisting of a large number of interrelated variables, which is achieved by transformation to a new set of variables, that are linear functions of those in the original data set, the principal components (PCs) [26, 122]. The non-correlated parameters are considered important and retained in the MDS and in case of correlated variables, only the ones with the highest eigenvalues (highest influence) are selected [5, 26, 118]. These new independent variables successively

maximize variance and at the same time minimize information loss [122]. Simplified, PCA helps to select the most appropriate indicators to represent and estimate SQI [26]. All selected parameters are transformed using linear scoring functions (see Weighted additive SQI) and a weighted additive approach is used to integrate them into indices for each soil [5, 26]. Thereafter, the weighted additive SQI is computed using Equation 15:

$$\sum \text{Statistically modelled SQI} = \sum Weight \cdot \text{Individual soil parameter score} \tag{15}$$

Chapter 3

Degradation of plastics in soil

3.1 The degradation process

Degradation is a process of reduction in complexity of organic compounds [123]. In other words, this process causes transformation of organic compounds, such as fragmentation, loss of mechanical properties or complete degradation. The term 'biodegradation' in a view of environmentally degradable plastics (see chapter 3.2) describes, that this process operates through the action of living organisms [124]. The responsible organisms are microbial communities of bacteria, fungi, algae, lichenaceae or protozoa groups [123, 125, 126]. For example, bacteria and fungi that play important role in a microbial degradation of polyhydroxyalkanoates are *Bacillus*, *Pseudomonas*, *Klebsiella*, *Actinomycetes*, *Nocardia*, *Streptomyces*, *Thermoactinomycetes*, *Micromonospora*, *Mycobacterium*, *Rhodococcus*, *Flavobacterium*, *Comamonas*, *Escherichia*, *Azotobacter*, *Alcaligenes*, *Sporotrichum*, *Talaromyces*, *Phanerochaete*, *Ganoderma*, *Thermoascus*, *Thielavia*, *Paecilomyces*, *Thermomyces*, *Geotrichum*, *Cladosporium*, *Phlebia*, *Trametes*, *Candida*, *Penicillium*, *Chaetomium* and *Aerobasidium* [127, 128, 129, 130]. If the process is completed successfully, the organic compound is either incorporated into microbial biomass or mineralized [125]. This initial process yields end products, called inorganic species (e.g., carbon dioxide, water, or methane) [131]. However to do so, certain rules must be met during mineralization process:

- there must be an organism producing specific enzymes;
- this organism must be present in the contaminated environment (not every organism has the specific predisposition to degrade specific organic compound);
- the organic pollutant must be available to an organism with suitable catabolic potential (the organisms with catabolic potential let the pollutant or the products of transformation/intermediates to penetrate across the membrane);
- in addition, if the natural environment is not optimal for the biodegradation by microorganisms (e.g. microorganisms do not grow fast enough), physiological conditions can be adjusted [123].

On the contrary, incomplete biodegradation produces intermediates, that might have different properties and therefore affect the system in other way compared to its original form.

3.1.1 Biotic degradation

Degradation is a complex process that has multiple steps. The process is initiated by abiotic and/or biotic factors. In a case of **biotic degradation**, the microorganism is attached to a polymer's surface, where it grows using polymer as a carbon source and therefore allows its decomposition [131]. These organisms then act by mechanical, chemical and/or enzymatic means causing deterioration and fragmentation [132]. Later, this process might produce low molecular weight fragments like oligomers, dimers and monomers, which are further being used as a carbon and energy source and finally mineralized. Microbial attack starts with

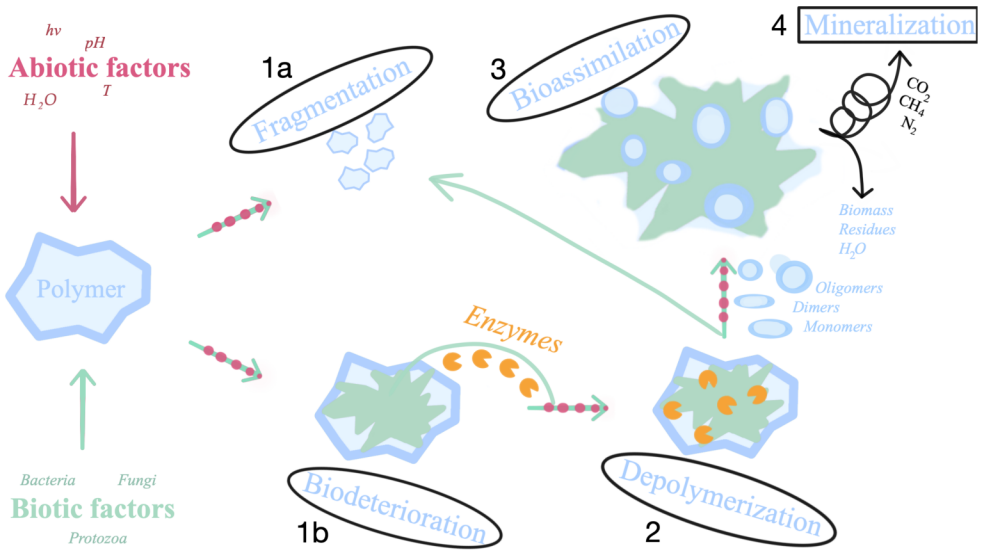

Figure 6: Schematic representation of the different steps involved in (bio)degradation.

biodeterioration, process of formation of biofilm on a polymer's surface or/and inside a given material [133]. Porous polymer is infiltrated by this slime matter, that changes the size and distribution of pores, moisture degrees and thermal transfers, in order to protect microorganism against unfavorable conditions (e.g., desiccation and UV radiation) [133]. During this process the size of pores expands and therefore causes cracks of the material. But just microbial presence is not enough to break down polymer's structure. Therefore microorganism, secretes extracellular enzymes (as catalysts) or by products (such as acids and peroxides) in order to cleave the main chain [131, 134, 124]. This is possible because (extracellular) biopolymers act as surfactants that allow exchange between the hydrophilic and hydrophobic phases, favouring the penetration rate of microbial species [133]. Biodeterioration then occurs because microorganism is using inorganic compounds (e.g., ammonia, nitrites, hydrogen sulphide, thiosulphates and elementary sulphur etc.) or organic substrates as energy and electron sources [133]. This microorganism releases active chemicals (mostly acids and peroxides) that either react with components of the material and increase the erosion of the surface [135] or it sequestrates cations present into the matrix (e.g., Ca^{2+}, Al^{3+}, Si^{4+}, $F_2{}^{e+}$, Mn^{2+} and Mg^{2+}) to form stable complexes [133]. In case of peroxides it is an oxidation of cations that causes catalytic degradation of hydrocarbons [133, 136, 137, 138, 139].

The role of enzymes during biodeterioration is to decrease the level of activation energy of polymers, in order to support chemical reactions and therefore to promote the **depolymerization** process [133]. This process is only possible if enzymes (present either

free in solution, bound to cells, colloids or particles) have access to the polymer and at the same time the polymer have chemical groups susceptible to attack [125]. The enzymes that support this reaction belong to the group of oxidoreductases and hydrolases [133]. In addition, some oxidation reactions produce free radicals conducing to chain reactions that also accelerate polymer transformation [133]. However, the microorganism does not have to be the only one initiating this reaction. Macroorganism also have the ability to directly consume and even digest polymers, causing mechanical, chemical or enzymatic ageing [124].

Even if macro- and microorganisms meets conditions specified in the paragraph above, this process does not have to lead to mineralization [125]. It is strongly dependent on the molecular weight of the fragments that are yield during depolymerization process. If the molecular weight of fragments is not low enough, the process does not proceed further. Otherwise, once are oligomers/dimers/monomers small enough to diffuse into the organisms, they undergo **bioassimilation**, a process of uptake by microbial cell [131, 134]. Here, are atoms from fragments of polymeric materials integrated, which gives the organism necessary sources of energy, electrons and elements (i.e. carbon, nitrogen, oxygen, phosphorus, sulphur and so forth) for the formation of the cell structure, growth and reproduction [133]. Inside cells, are molecules oxidised through catabolic pathways, producing adenosine triphosphate (ATP) and constitutive elements and primary and secondary metabolites [134]. These metabolites are further **mineralized** and formed products (carbon dioxide, methane, water, nitrogen etc.) are released into the environment [134]. Polymers are initially converted to biomass because, under natural conditions, mineralization is a very slow process [124, 140]. Therefore, attention should also be focused on complete biodegradation and not only mineralization, when we are assessing their presence in the environment [124].

Complete mineralization yields carbon dioxide and water or methane and carbon dioxide, depending on the degradation conditions (aerobic/anaerobic) [125]. Oxygen is essential under **aerobic conditions** (see equation 16), because degradation process is initiated by specific enzyme 'oxygenase'. The aerobic microorganism uses oxygen as an electron acceptor in order to break down biopolymers, producing by-products of carbon dioxide and water [131, 124].

$$C_{polymer} + O_2 \rightarrow CO_2 + H_2O + C_{residue} + C_{biomass} \tag{16}$$

Under **anaerobic conditions** bacteria must use more complex strategy (see equation 17). They use nitrate, sulphate, iron, manganese and carbon dioxide as electron acceptors to break down organic compounds [131]. The problem of anaerobic degradation is that the transformation is slow and organic products, that can only be broken down under aerobic conditions, are being accumulated, which can be a threat to the environment [123].

$$C_{polymer} \rightarrow CH_4 + CO_2 + H_2O + C_{residue} + C_{biomass} \tag{17}$$

The equations are written for polymers or intermediates that consist only of carbon, hydrogen, and oxygen. However, there might be other elements in the polymer structure, which would appear either in oxidised form under aerobic conditions or reduced form under anaerobic conditions [124].

3.1.2 Abiotic degradation

Nevertheless, biodegradation is not only affected by microbial activity, but it is also enhanced by **abiotic factors**. Abiotic degradation causes transformation and change in mechanical properties of polymer by climatic conditions or chemical reactions. Even though, the abiotic alike biotic factors affect structure of plastics, they must not be mistaken with biodegradation. For example physical deterioration or loss in physical integrity can result in fragmentation of the plastic but does not necessarily remove the plastic from the environment [125]. In fact, a reduction in particle size resulting from deterioration can cause wider distribution of plastic particles in the environment [125]. Low hydrophobic polymers are the only polymers that have a chance to fully degrade, while exposed to abiotic factors [124]. On the other hand, abiotic factors might increase surface area for microbial colonization or reduce molecular weight, which can start or promote biodegradation of polymers [125]. Therefore biotic and abiotic factors might be considered interdependent. However, if considered separately, the efficiency of biotic factor visibly exceeds the efficiency of the abiotic one [123]. Abiotic factors that can initiate transformation or changes of organic compounds are light (UV radiation), temperature, moisture and water uptake, other chemical conditions (such us exposure to specific chemicals, change of pH, type of chemical bond, co-polymer composition) and physical disintegration [141, 124].

Mechanical degradation takes place due to compression, tension and shear forces and can activate or accelerate biodegradation [133, 142]. In the field conditions, it can act in synergy with other abiotic parameters (temperature, UV radiation and chemicals) [133]. Mechanical degradation might be an issue for preparation of samples for test in the laboratory, because it can be initiated during homogenization with matrix. And it must be taken into consideration, because mechanical degradation is possible with poly-(R)-3-hydroxybutyrate, that is being tested in the experimental part of this thesis.

Second factor is **light degradation/radiation**. This process might lead to Norrish reactions (transformation of polymers by photoionisation and chain scission) and/or crosslinking reactions, or oxidative processes [143, 133]. These reactions were recently described during photodegradation of biopolymers poly(lactic acid), polycaprolactone, polybuytelne adipate terephtalate and again poly-(R)-3-hydroxybutyrate [133, 144, 145, 146].

Thermal degradation of thermoplastic polymers occurs at their melting temperature and may also influences organisation of the macromolecular framework, which is dependent on glass transition temperature [133]. The melting temperature is generally higher than the environmental conditions, for example the melting temperature of poly-(R)-3-hydroxybutyrate is 175 °C and therefore in the environment doesn't occur [133, 147]. Exceptions are for example polycaprolactone and some composites, whose melting point is around 60-65 °C [133]. This temperature can be reached during composting (more about composting will be later in this chapter). Above glass transition temperature (rubbery state), the polymers have disorganised chains (see Figure 7, which makes them more accessible to chemical and biological degradation [133, 148]. Therefore glass transition temperature of biodegradable polymers is typically within the temperature range which is common in the environment (e.g. 5 °C for poly-(R)-3-hydroxybutyrate) [133].

Another important parameter of abiotic degradation is **chemical degradation**, which is activated by atmospheric pollutants and agrochemicals, that can change properties of

polymers [133, 142]. Whether the polymer will undergo chemical degradation depends on its structure. Atmospheric oxygen (O_2, O_3) is a good example, by its ability to attack covalent bonds and the ability to support light degradation [133, 149]. Another chemical way by which polymers can undergo degradation is hydrolysis [133, 150, 151, 152]. Molecules can be spilt by H_2O, if they contain hydrolysable covalent bonds (esters, ethers, anhydrides, amides and so forth) and meet specific parameters (specific: water activity, temperature, pH) [133].

In general it is true, that degradation is more easily performed within disorganised molecular regions (amorphous domains), because well organised molecular frameworks (crystalline domains) do not prevent just diffusion of O_2 and H_2O, into its structure, but in general any kind of degradation [133]. In reality there are only semi-crystalline and amorphous polymers (see Figure 7). Meaning that they both consist of amorphous regions that can be degraded. This is however highly dependent on temperature and especially on time.

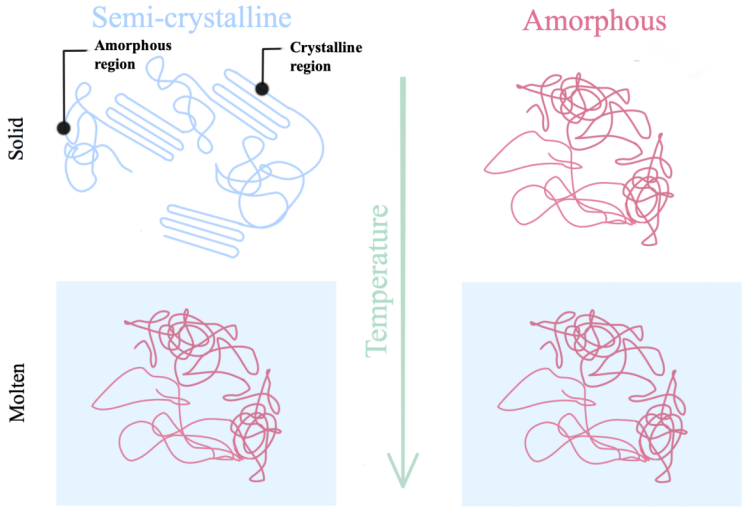

Figure 7: Structural difference between semi-crystalline and amorphous polymer chains.

3.1.3 Incomplete degradation

The degree of biodegradation depends on both the properties and the environment to which is organic compound, in this case biopolymer, exposed [153]. If the plastic material is not completely degradable or it takes long time to degrade, **fragmentation** may occur (see Figure 8). This process starts with embrittlement and cracking, that may later lead to fragmentation and thus production of microparticles. If the particle is made of polymer, it is called a 'microplastic'. **Microplastics and nanoplastics** are polymer particles in the sub-millimeter down to the sub-micrometer size. Precisely, according to ISO/TR 21960:2020, microplastics are defined as particles of 1 μm up to 1 mm range and nanoplastics are smaller than 1 μm (According to Organisation for Economic Co-operation and Development nanoparticles are up to 100 nm) [154]. The norm also specifies their origin and a shape: 'Typically, a microplastic object represents a particle intentionally added to end-user products such as cosmetic means, coatings, paint, etc. A microplastic object can also result as a fragment of the respective article. Microplastics may show various shapes' [154]. This means that microparticles can be divided in two

categories: secondary particles, that are result of degradation and fragmentation of larger plastic items and primary particles, that are directly produced in the form of microplastics [155]. Both, primary and secondary particles, may then undergo further fragmentation by chemical or physical processes [155]. Their environmental impact is described in chapter 3.4.

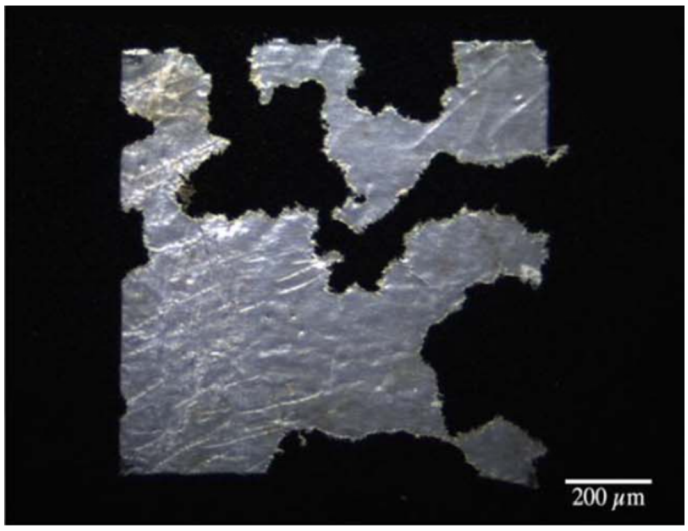

Figure 8: Fragment of biodegradable plastic sample after 7-day laboratory exposure to *O. gammarellus* [156].

3.2 Biodegradable plastics

Most conventional plastics, such as polyethylene, polypropylene, polystyrene and other, pose a threat to biota due to their high durability, their ability to accumulate and our inability to process them (especially in less developed countries). Therefore there has been a tendency to find an alternative, that would solve these problems. The first strategy are biodegradable plastics. Although, as it will be mentioned further in the text, their application should be chosen wisely. Biodegradable plastics are part of the **'Bioplastic'** family. Therefore is for further explanation essential to define bioplastics at the first place. The term 'plastic' refers to a group of synthetic polymers: thermoplastics and thermosets. Thermoplastic are inaccurately shortened to 'plastic'. The term refers to the property of a material able to deform without fracturing, meaning that thermoplastics are capable of being repeatedly moulded, or deformed plastically, when heated [157]. On the contrary thermosets, once they are formed, cannot be remoulded by melting [157]. In case that is the origin of polymer biological, the polymer is called **'biopolymer'** (polymer derived from biomass). There are diverse biopolymers, that can be acquired from various feedstocks (for instance, the poly-(R)-3-hydroxybutyrate's feedsotck is starch) [158]. Biopolymers are, along with synthetic polymers (fossil fuel derived), obtained from its matrices, processed and formed into plastic product. There are 4 major processes of biopolymer production. Firstly, polymer is directly extracted from biomass (e.g., starch, cellulose, casein, gluten). Secondly, polymers are synthesized from renewable monomers (e.g. polylactic acid) [159]. And finally, polymers can be obtained from microorganisms or genetically modified bacteria

(e.g. polyhydroxyalkanoates) [159]. Key point is that some of them have similar properties (see Table 3) to conventional plastics and therefore are used as an alternative.

Table 3: Biopolymer alternatives to conventional polymers.

Biopolymer	**Alternative conventional polymer**
Polyhydroxyalkanoates	High density polyethylene
Polylactic acid	Polyethylene terephthalate Polystyrene
Polytrimethylene terephthalate	Polytrimethylene terephthalate Nylon 6

Although there is the prefix 'bio-' in the word 'bioplastic', the prefix does not necessarily specify the origin, but it describes that plastic can be biologically degraded and/or have biological origin. This means that bioplastics can be both biopolymers and synthetic polymers. Specifically, bioplastics consists of either biodegradable plastics or bio-based plastics (see Figure 9). **Biodegradable plastics** can be degraded by various

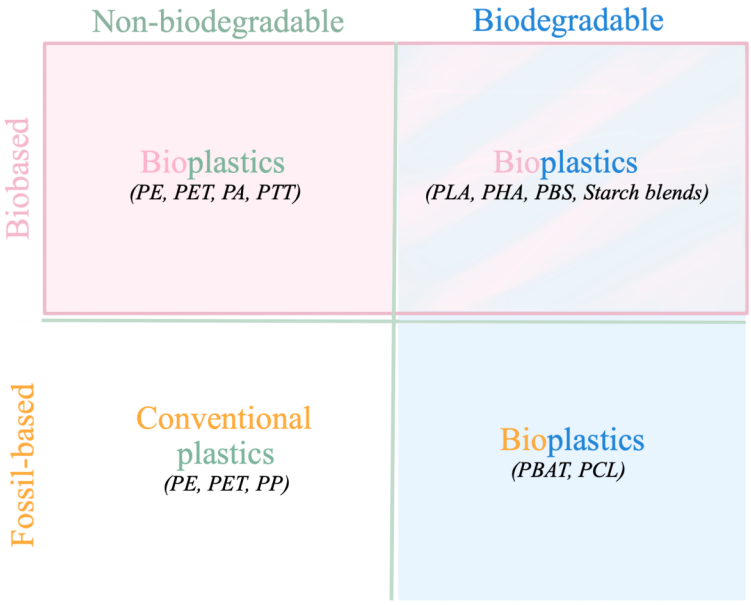

Figure 9: Classification of plastics *(PE = polyethylene, PET = polyethylene terephthalate, PA = polyamide, PTT = polytrimethylene terephthalate, PLA = polylactic acid, PHA = polyhydroxyalkanoates, PBS = polybutylene succinate, PP = polypropylen, PBAT = polybutylene adipate terephthalate, PCL = polycaprolactone).*

processes no matter if they are produced from fossil materials or are synthesized from biomass / renewable resources (see top and bottom part of the Figure 9) [141]. They break down upon interaction with UV, water, enzymes and gradual changes in pH [131]. The second case, **bio-based plastics**, are plastics that are made primarily from biomass or renewable resources, however it does not necessarily mean that they can be degraded (see left and right part of Figure 9) [141]. For example polyethylene can be produced from biomass, but cannot be degraded at the same time [160]. But plastics can be both

bio-based and capable to undergo degradation. Plastics such as starch, poly(lactide) and polyhydroxyalkanoates meet these conditions.

In the experimental part of this work **poly-(R)-3-hydroxybutyrate (P3HB)** as a representative of polyhydroxyalkanoates (PHA) has been chosen. It is a natural biodegradable polymer (see structure in Figure 10) produced by microorganism as a means to store carbon and energy [141]. In other words, excess nutrient (carbon, nitrogen

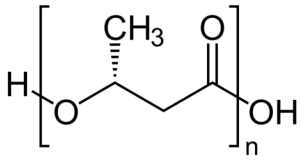

Figure 10: Structure of poly-(R)-3-hydroxybutyrate

and phosphorus) is biochemically assimilated and accumulated inside the intracellular cytoplasm in the form of granules (see Figure 11) with 0.2 - 0.5 mm diameter. These granules, that are made of PHAs (amongst which is P3HB predominantly widespread), are stored by microorganism in order to process it after stress exposure, for example for a cell division [130, 132]. The abundance of granules inside the dry mass of an organism is approximately 30 %, although under special circumstances, some microorganism (such as *Azotobacter* and *Alcaligenes sp.*) can accumulate up to 90 % [130]. The process of

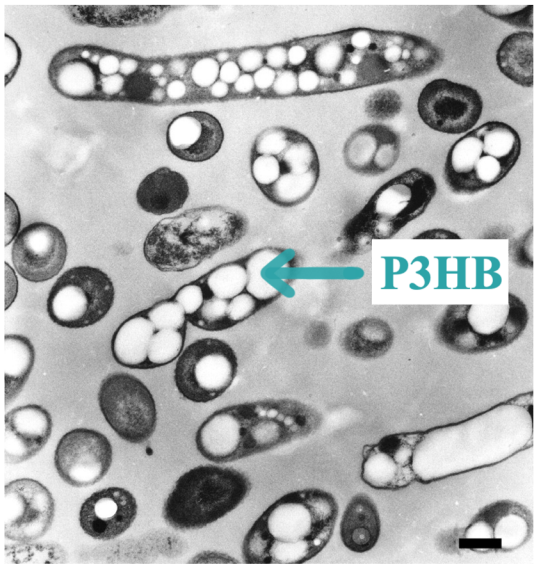

Figure 11: Image of P3HB granules in *W. eutropha* [161].

transformation of extra nutrients into granules is called polymerization (see Figure 12). It is carried out by a key enzyme called PHB synthase [162, 124]. Firstly, two acetyl-CoA molecules are coupled to form acetoacetyl-CoA in a condensation reaction catalysed by β-ketothiolase [163]. The product is reduced to (R)-3-hydroxybutyryl-CoA in a reaction catalysed by NADPH-dependent acetoacetyl-CoA reductase [163]. High concentration of NADPH and NADH inhibits the citrate synthase of the tricarboxylic acid cycle (TCA), which again ensures the availability of acetylCoA for the β-ketothiolase [163]. In the final step, (R)-3-hydroxybutyryl-CoA molecules are synthesized into P3HB [157]. This product

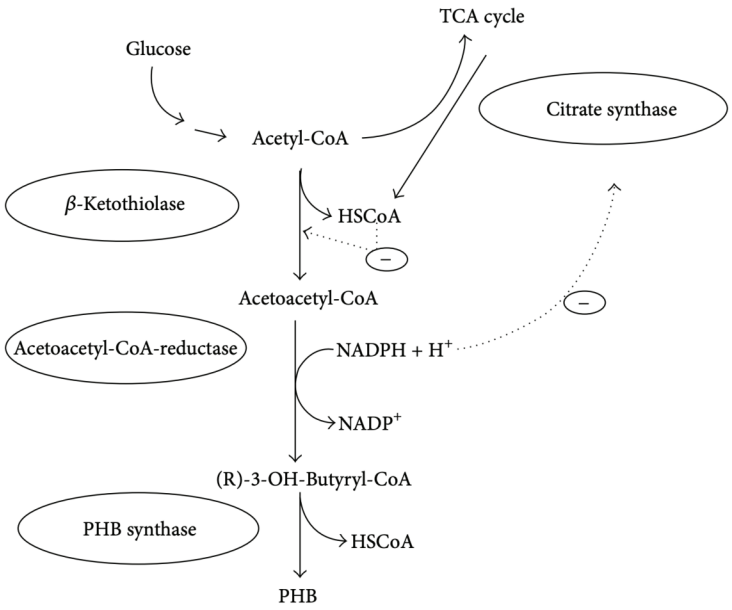

Figure 12: P3HB synthesis pathway of *R. eutropha* and regulatory circuits [163, 164].

is a semicrystalline (60–70% crystallinity) thermoplastic, which means that it becomes moldable at a certain elevated temperature and solidifies upon cooling [165, 166]. Its structure increases the density, strength, modulus of elasticity and hardness and decreases the ductility, impact toughness and transparency of the polymer [166]. These properties make the polymer similar to the polypropylene and polyethylene [124, 167]. The polymer lately gained plenty of attention, because it currently is the most common PHA found in nature, which makes it a renewable low-cost feedstock that can be easily synthesized at the same time [124, 141]. Plus the polymerization process is being operated under mild process conditions that allows minimal environmental impact [141].

3.2.1 Compostable plastics

There is a significant difference between degradation in nature and composting facilities. Industrially are therefore bioplastics referred to as biodegradable and compostable. The term **'biodegradable'** has become a marketing term that is misleading. It represents the ability of material to undergo biodegradation, which is in the most cases tested only under very specific conditions and does not represent a generic property of the material [168]. Organic material labelled as 'biodegradable' might not therefore undergo fast and effective biodegradation in the environment. The term **'compostable'** is more specific in the conditions in which the bioplastic degrades. It describes the ability of bioplastics to biodegraded at elevated temperatures in soil under specified conditions and time scales, usually only encountered in an industrial composter [157]. Yet, in natural environment may the degradation take much longer or does not have to happen at all. And because, typically the conditions in the composting facility must be controlled, the compost must respect certain conditions:

- suitable carbon to nitrogen ratio, between 20:1 and 40:1;
- required moisture content (depends on soil type and the porosity - optimized to 70 %);

- pH in the range from 6.5 - 8 (under pH 6 the organism dies and above 8.5 is Nitrogen transformed to ammonia);
- aeration of substrate (>10 %);
- regulation of temperature during composting (is given by the type of raw material and the phase - see Figure 13);
- sufficient content of organic substances [169, 170].

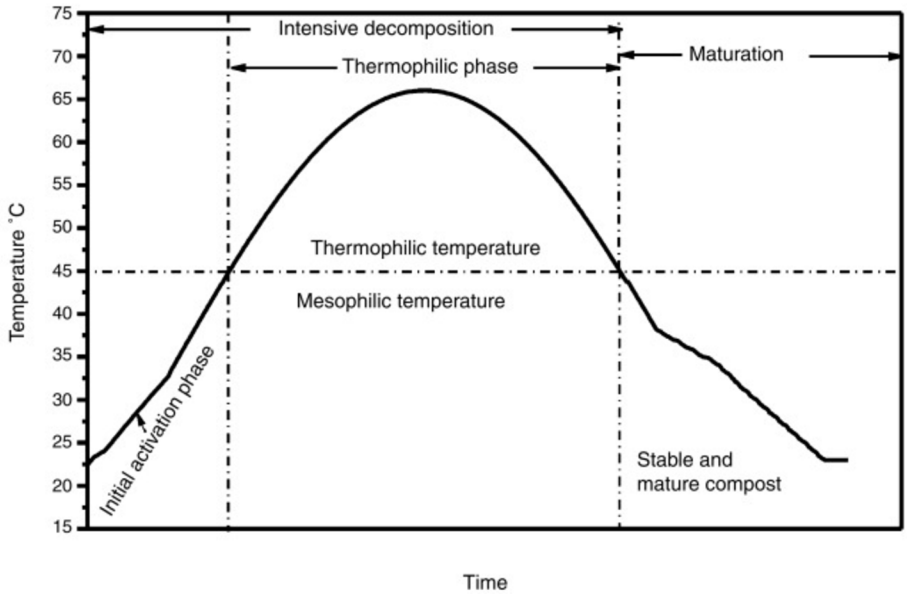

Figure 13: The Different Phases of the Composting Process [171]

These are difficult to meet under natural conditions. But not only that compostable plastics might not be environmentally decomposed but also must not be accepted by every composting facility (this varies across Europe). In fact, some composting facilities do not accept compostable plastics and therefore they are removed together with other non-compostable contaminants such us metal and glass [172]. Another concern is that compostable plastics appear as conventional ones and hence might be removed by sorting machines or by human operators in the facilities, where they are usually accepted. On the top of that, compostable plastics do not decompose at the same rate as other organic materials and therefore require higher temperature and longer time to break down. Also impurities must be taken into consideration, because they may stay in the compost and cause further contamination. Accordingly, composting facilities should either separate impurities after composting or before [173]. If they are separated before, no additional changes in the process must be applied. Otherwise the process must be adapted to new conditions and consequently, new methods, which allow better separation of conventional plastics, must be investigated [173].

First step of implementation of bioplastics on the market is labelling and standardisation. These should ensure, that product that goes on the market have certain properties and according to that can be handled. To give an example, there are multiple labels for industrially compostable products: the Seedling logo, OK Compost, and DIN-Gerprüft Industrial Compostable and Consorzio Italiano Compostatori (CIC) [174].

The issue is, that at the moment there is no comprehensive EU legislation specifically harmonising labelling for environmental and product marketing claims [174]. Standards as well as labelling are not harmonized neither. Currently, there are various institutions, including European Commission, national governments, ministries, and independent standardisation institutes have issued a multitude of standards, such as ISO (International Organization for Standardization), European Committee for Standardisation (CEN) and American Society for Testing and Material (ASTM), that try to serve as a basis for evaluating claims for bioplastic products [174]. Although, in the light of labelling and standardisation it would be much easier if the norms would be harmonized. For instance, standards on the EU-level through CEN, should apply equally across participants in the same market [174]. It would give clearer guidance and terminology for producers, consumers and final recipient of the waste. This goal has been successfully reached by plastic recycling, where there is legislation regarding minimum recycled content mandates and procurement policies, combined with recycled products labelling [168].

3.2.2 Oxo-biodegradable plastics

There is also an initiative to transform conventional non-biodegradable plastics (e.g., polypropylene, polyethylene, polyethylene terephthalate) into biodegradable. Regardless its meaning, these **'Oxo-degradable plastics'** are still being produced, despite the fact that the degradation process forms nothing more than microplastics [168].

To produce Oxo-degradable plastics additives are needed. These additives, so called prodegradants, are used to accelerate the oxidation process [175, 168]. This means that the oxo-degradable plastics become inherently more accessible to chemical reactions. The issue is, that when they are exposed to a sunlight and oxygen, they fragment into microplastics, which still take a long time to biodegrade, meaning that the threat to the environment continues [176, 168]. However, even truly biodegradable plastics containing additives, mustn't be biodegradable at the end, because additives may prevent or slow down degradation process [177]. Thus, the enrichment of polymers with additives doesn't necessarily mean the support of the degradation process, whether or not they are biodegradable.

3.3 Suitable application of bioplastics

Plastic pollution of the environment is nowadays a trendy topic. The negative effects, such us their capability to enter a food chain, disturb biological processes, cause physical damages to the organisms or the capability to change the habitat are abundantly presented. Bioplastics are therefore suggested as alternative. Prior research generally confirms that they are considered non-toxic. But recent studies have promoted that they might cause adverse effect indirectly. They enter organism by respiring atmospheric particles, drinking contaminated water or consuming aquatic organisms exposed to microplastics [178]. If they are not in their raw form, the serious toxic effect might appear. For instance, adsorption of elements/substance (e.g., metals, persistent organic pollutants) on the biopolymer surface, presence of additives (such as UV- and thermal-stabilizers) and pathogenic and opportunistic organisms (*Vibrio spp.*) or presence of residual monomers and intermediates (e.g., phthalates, Bisphenol A, styrene and other aromatics) may endanger organism [178, 140, 157, 179]. Another hazard might cause pathogens, that have been identified in the microbial communities colonizing microplastics [180]).

Plastic pollution is mostly associated with pollution of marine environment. Therefore, many scientists are trying to come with innovations, that would help to remove or eliminate this pollution. Some of these solutions are unfortunately not suitable and may cause more harm than good. As an example, the consensus 'bioplastics are the solution to ocean plastic pollution' can be discussed. In the marine environment UV radiation is the dominant weathering process (lack of biodegradation-supporting microorganisms) [157]. As already mentioned before, this abiotic factor is less efficient than the biotic one, meaning that the process of degradation might not lead to complete mineralization of bioplastics or it will be extremely slow. Application of bioplastics here might not be therefore solution (biodegradable and conventional plastics will both produce microplastics). These microparticles can then penetrate water organisms, where they might bioaccumulate and after that continue their path in a food chain. The bioavailability is influenced by particle density, abundance, color, and especially size (their bioaccumulation potential increases with decreasing size) [180, 178, 181]. Also higher temperatures, oxygen levels and mechanical abrasion both increase the rate of fragmentation in the oceans, so if plastic products become buried in sediments or covered in organic and inorganic films, the rate of fragmentation decreases rapidly [157].

It is a fact, that the application of bioplastics should be investigated before it is placed on the market. Unfortunately, bioplastic can be currently found in all possible industries. Biopolymers are used for products such as adhesives, resins, composites, plastic products, lubricants, coatings, solvents, inks, paints, and many other. The concern is, that in the most cases they are sold as a 'sustainable' solution to a plastic pollution, without further evaluation of impact on the environment. But for example, in 2019, the largest bioplastics application was in packaging production with more than 53 percent (1.14 million tonnes) of the total bioplastics market [182]. A throwaway non-recyclable bioplastic package is not sensible application, because a throwaway non-recyclable bioplastic package will probably end up on the dump or in the incinerator (see Section 3.2.1). Because of this 'sustainable' label (as they are generally viewed), the annual production rises each year and this trend is expected to continue. They represent about one percent of the 360 million tonnes of plastic produced annually and just polyhydroxyalkanoates together with bio-based polypropylene have the highest relative growth rates of all bioplastics [182]. This massive production is thus a reason, why there is a need of evaluation of specific biopolymer applications.

While using bioplastics, decisive is when we use it as alternative. In the first place, bioplastics shouldn't be used in order to 'disappear' in the environment. There is a wide range of bioplastics and their biodegradability varies considerably under different environmental conditions. For example thermoplastic PLA, that is commonly used as a material to produce bottles and packaging, only breaks down in a high-temperature industrial composting facility [183]. In other words, it cannot be degrade in an average household compost bin and nor in the nature [183]. And even that are bioplastics biodegradable in the 'environment' they may not be biodegradable everywhere, because in general, the environmental properties varies significantly within small distances (see Figure 7 in Appendices). Significant factor is their size. Smaller particles will biodegrade faster and more effectively than bigger one and at the same time one must take into consideration the fact, that many composting facilities do not accept biodegradable products (see Section 3.2.1). Therefore, it is essential to distinguish where to use recyclable material and where biodegradable/compostable one in case we cannot reuse the product for some reason. Accordingly before manufacture, producers and manufacturers must determine suitable material and format to use for specific application, the carbon

footprint, the current recycling infrastructure as well as the public understanding of plastics and bioplastics. In a light of these facts are in this chapter described promising application in biopolymer industry.

Biodegradable polymers are currently tested in many promising applications: mulch films, tunnel films, string, nets, clips, planting/flower pots, plant containers, controlled release of pesticides, herbicides, fertiliser, and pipelines for mulch [124]. Some of these applications are described in detail in following paragraphs.

Firstly, bioplastics can be used for delivery of specific substances at the right time to the right location. **Targeted drug delivery** systems are designed to deliver drugs at the proper dosage for the required amount of time to a specific site of the body where it is needed. It is used in order to prevent any adverse effect drugs, such us chemotherapeutic drugs and fragile biotechnological molecules (e.g., peptides and proteins), may have on other organs or tissues [163]. To exemplify Lee and others described drug delivery to tumor (breast cancer cells) by hybrid polyhydroxybutyrate (P3HB) nanoparticles (see Figure 14) [184]. In this case, P3HB nanoparticles are loaded with model drug and are

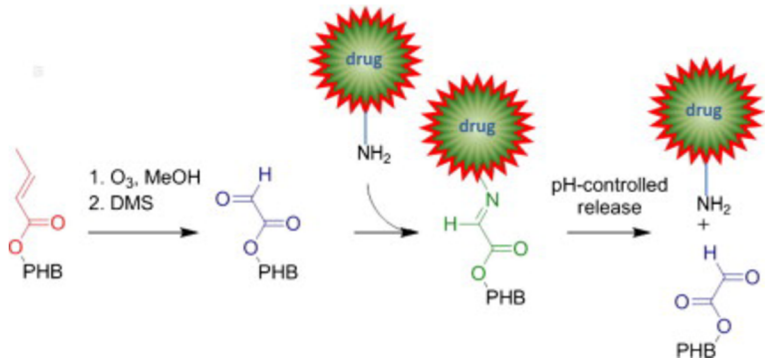

Figure 14: Representation of the targeted drug delivery system [185].

functionalized with tumor-specific ligand, fused with PHA synthase. This specific enzymatic modification shows P3HB nanoparticles' affinity to a breast cancer cells and therefore its capability to be delivered drug to this exact place [184]. This system can also be used in agriculture and biomedical application etc. Another promising application of biopolymers is their application as degradable carriers for **pesticides delivery**. Commonly are pesticides applied in a form of powder, suspensions, or emulsions, that often do not ensure targeted delivery of agents [186]. As a consequence, numerous insects and weeds remain uncontrolled and the pesticides accumulate in the biosphere. Besides these consequences, some of the pesticides exhibit mutagenic and carcinogenic effects, and thus can be ingested and pose a threat for human health [186]. Therefore, biopolymers are presented as carriers to promote delivery of pesticides in a regulated way [187]. Polyhydroxyalkanoates, specifically, have ideal properties for this application, because they are not prone to rapid chemical hydrolysis and are degraded as a result of true biological degradation in the environment [186].

Another example of agricultural application are **polymer-coated controlled-release fertilizers (PC-CRFs)**. They minimize nutrient losses and cater the specific plant nutrients demand at different plant growth stages to optimize the yield under specific soil conditions [188]. Uncoated mineral fertilizers have low efficiency of plant nutrients uptake due to losses on farmland by runoff, leaching and volatilization and can accumulate in soil, water and foodstuffs (because to prevent these issues the bigger

amount of fertilizers is used) [188, 189]. One of the approaches to overcome these problems is to coat nutrients (see Figure 15) [188]. According to this strategy, the nutrient is coated and then released into soil along with biopolymer degradation [189]. Depending on the geometry of the carrier and the amount of nitrogen loaded in it, can the

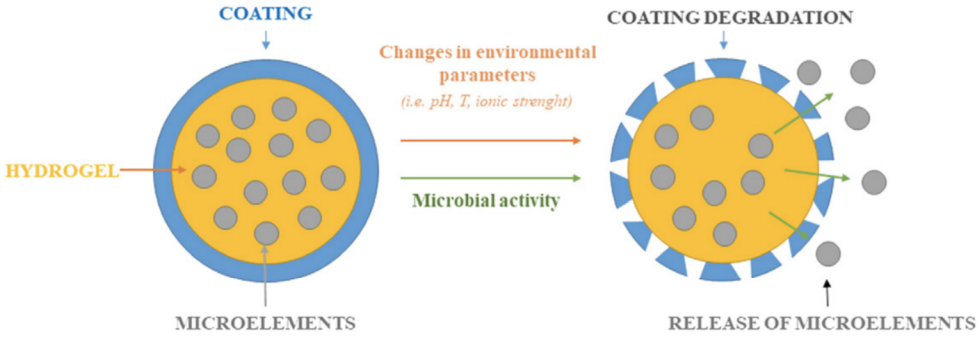

Figure 15: Representation of a nutrient release mechanism from the coated capsule [190].

nitrogen release last up to 30 days or longer and this process may be controlled by varying the fabrication technique employed [189]. In conclusion, the use of PC-CRFs can decrease the amount of chemicals in the environment and prevent their adverse effects on the biosphere. This system of controlled release can be applied to herbicides and pesticides as well [124]. And as in the previous case, coating is a promising technology in drug delivery. Newer studies reversed this method and instead of coating of a given material, the material is loaded (sort of encapsulation method) into prepared microcontainers (see Figure 16) [191]. The advantage of this method is, that it allows controlled release even for liquid samples in the desired location or/and in desired time.

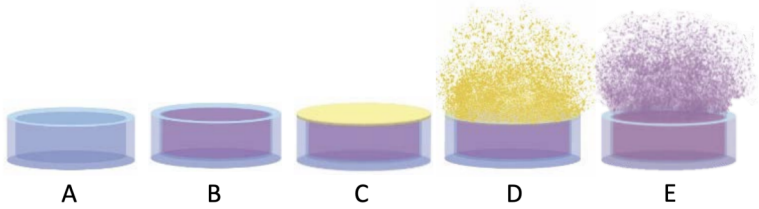

Figure 16: Example of loading principle of microcontainers [191]. *(A = fabrication of a microcontainer, B = loading of sample, C = closure with a lid, D = lid dissolution, E = sample release).*

Mulch films are usually made of polyethylene, which tend to fragment under environmental weathering conditions. Foils then produce macro/micro particles, which makes the plastic difficult to remove from the field [192]. However, their application is irreplaceable since they offer several benefits, including the suppression of weed growth, conservation of soil moisture, promotion of efficient nutrient uptake, and improved soil microclimate for plant growth [193, 194, 195, 124]. As a solution, biodegradable plastics were introduced. The benefit of biodegradable mulch films is, that the fragments are degraded and incorporated into the soil (see Figure 17). Some of biodegradable mulch films can even provide similar yield and quality benefits as a conventional ones [124]. But the risks of the application should be thought out. Much attention has been drawn to the ability of soil-biodegradable plastics to incorporate into the soil at the end of the crop

cycle, however little research has been conducted about the effect of the incorporated particles on the soil [195]. Also, biodegradable mulch films are often enriched by additives, which as already mentioned before, might have a negative impact on soil biota. Hence, further adverse effect of biopolymers on soil system should be studied.

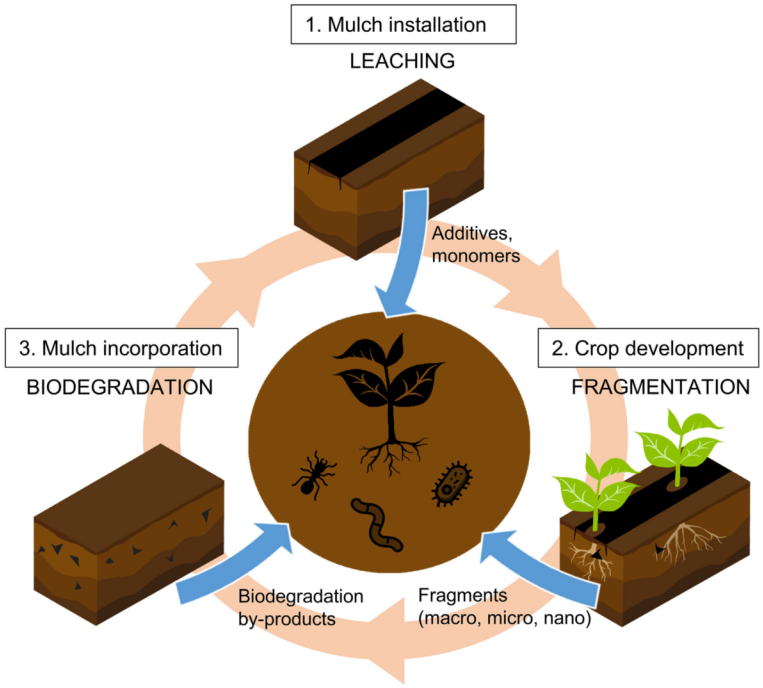

Figure 17: Representation of agricultural cycle of biodegradable mulch films [196].

Polyhydroxyalkanoates (specifically poly-(R)-3-hydroxybutyrate) are also applied in cosmetics as a **replacement of microplastic particles** (e.g., peeling, decorative cosmetics) **and chemical UV filters**. Conventional particles often enter water environment (marine/fresh) and are not effectively removed if they are removed at all. Therefore, biodegradable particles replace the conventional plastics in cosmetic products and serve as more sustainable alternative.

3.4 Effect of soil exposure to microplastics

Despite the fact that there is a general opinion of bioplastics behaving differently in the environment than conventional plastics, recent studies claim that several of them are actually more prone to disintegration (formation of fragments) than degradation [197]. Behaviour of biodegradable plastics is often similar to a behaviour of polyolefins and hence must be evaluated alike. There is also a difference in particle size, which means that microplastics in the environment can behave differently than nanoplastics (see Figure 18). Larger particles affect mostly soil biogeochemistry (e.g. mulch films) and may have a negative effect on terrestrial and continental organisms (e.g. birds) when ingested [198, 199, 200, 201]. Smaller particles then have the ability to reduce a growth of macrofauna (especially earthworms), have a lethal toxic impact to plants and fungi and nanoparticles are generally cytotoxic [202, 203, 204, 205, 206, 207]. Soil type, along with other soil and weather factors, also plays a key role in microplastic behavior. All these

facts mentioned in this paragraph need to be taken into consideration when assessing the impact of microplastics on soil and terrestrial ecosystems in general. Possible routes of entry and impact of microplastics on terrestrial ecosystem with a deeper focus on the soil is discussed in this chapter.

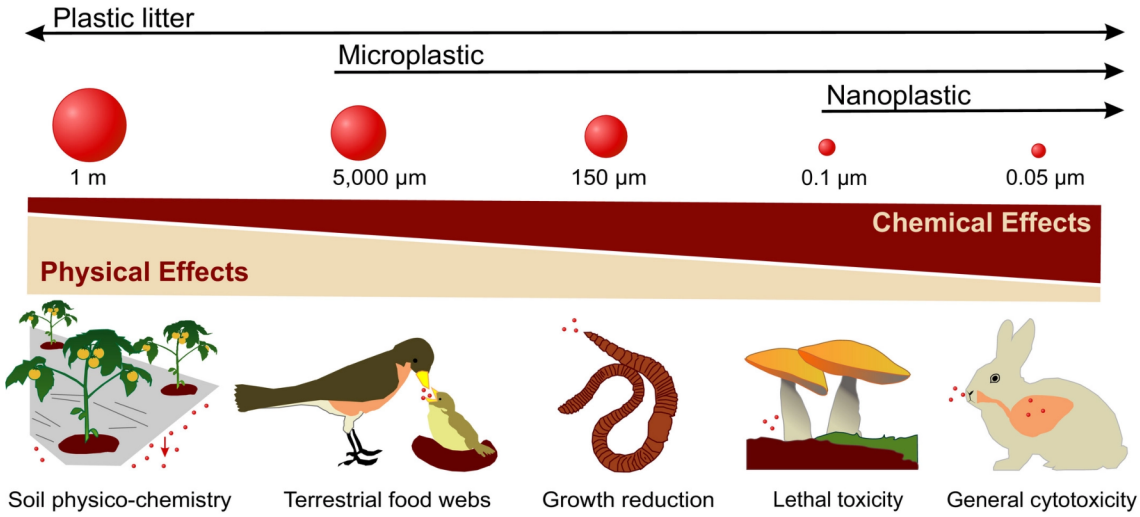

Figure 18: Distribution of physical and chemical effects of plastics on the environment based on particle size [208].

Polymers are applied into the soil intentionally or unintentionally by two main routes: agriculture and littering [124]. The **sources of microplastics** in agriculture are mostly sewage treatment plants, agricultural mulch films (and similar products) or compost, that are often applied to soils [124, 208]. Untreated sewage is rich in fibers from clothing and microplastic beads from personal care products [208, 209]. But even treated sewage might contain significant amounts of microplastics [208]. In Europe, it has been estimated that approximately 63,000-43,000 tons of microplastics per year is applied to farmlands via sewage [210]. Accordingly, compost is also added to the agricultural soil as a fertiliser. It contains residues of compostable plastics because the compostability criteria focuses on a disintegration of the compostable material rather than complete degradation [124]. For this reason, compost shouldn't contain particles larger than 2 mm in dimension, but it could still contain smaller particles due to incomplete degradation in the composting phase [124]. This strategy 'blindly' relies on the process of mineralization in the environment, which according to a theory should remove the remaining smaller particles. Admittedly, the complete degradation may occur, however it is not always guaranteed, especially if additives are present. Another application of plastics is in the form of mulch foils. As already mentioned, they tend to fragment under environmental weathering conditions and therefore have an impact on the environment. Their market is expected to reach an annual volume of 7.5 million tons by 2021 (in Europe about 700,000 tons a year), making it a significant source of microplastics for terrestrial environment [124, 211]. But there are other sources of microplastics with similar effect such us tunnel films, strings, nets, clips, planting/flower pots, plant containers and pipelines for mulch, which penetrate the environment [124]. Specifically for biodegradable plastics then controlled release of pesticides, herbicides and fertilisers. Littering of either biodegradable or non-biodegradable plastics is another way to expose the soil to plastics. Biodegradability of a material is sometimes used as a justification

of littering. On the contrary, biodegradability of plastics should not encourage nor excuse littering. Furthermore, microplastics enter soil system also by air transfer (and following deposition) from landfills, urban and industrial centers, that are often responsible for an accidental loss of particles, improper handling of waste and generation of contaminated soils and aerosols [208, 210].

A little is known about the exposure and effects of microplastics on terrestrial environment. Therefore, the risk assessments for ecological and human health is marked by a high degree of uncertainty [212]. While large particles of plastics have low lethal toxicity, smaller ones cause toxicity and act as a long-term environmental stressor and exert selective pressure on terrestrial organisms [208]. Additionally, plastic fragmentation causes changes in physical and (bio)chemical properties that increase their potential interaction with organisms causing direct and indirect toxicity [208]. The chemical toxicity to organisms associated with exposure to microplastics arises from sorbed persistent organic pollutants (POPs), from the chemical additives, residual monomers in the plastics, from the intermediates produced during the degradation of plastics or colonization of microplastics by pathogens (the negative effects will be discussed later) [178]. Humans or other animals are exposed to microplastics by respiration of atmospheric particles or consumption of plants or animals (mostly aquatic life, such us shellfish) [178]. Microplastics from agriculture are often ingested by continental animals, especially birds. Some of the ingested microplastics are considerably smaller than the usual food of those birds, which suggests microplastic ingestion to be either accidental or via trophic transfer [208, 201]. To exemplify, the trophic transfer may be described by a measurement of microplastic's concentration in the soil, earthworms and chickens. Lwanga et al., 2017 reported concentrations of 0.9 particles per 1 g of the soil, 14 particles per 1 g of the earthworm casts, and 129 particles per 1 g of the chicken feces [213]. Earthowrms and other 'plastic feeders' (for instance collembola) ingest regularly shaped microparticles over irregularly shaped ones with preference of aged particles, due to the presence of microorganisms [214, 215, 216]. The mentioned example confirms both, bioaccumulation and biomagnification of microplastics in the organism and that only specific particles are capable to bioaccumulate and biomagnify when transported from soil to plastic feeders. The most common translocation of particles is therefore via the intestinal lymphatic cells, which may cause decrease of the body weight and affect the composition of gut microorganisms involved in organic matter decomposition and N reactions [208, 214, 217, 218, 219]. Indeed, microplastics smaller than 0.5 μm may also accumulate in yeasts and filamentous fungi and hence potentially disturb detrital food web, that is considered amongst the longest food chains on Earth [208, 220, 221, 222]. Furthermore, poorly soluble biopersistent microplastics smaller than 1 μm may interact with biological membranes, organelles, and molecules [208]. Nanoplastics can also be internalized by cells which increases their cytotoxicity targets [208, 223]. This can incite inflammation, changes in membrane permeability (externally adsorbed nanoparticles to cells disturb membrane processes), oxidative stress, changes in gene expression, and biochemical responses, etc [206, 208, 220, 224, 207, 225]. There are evidence that plastics cross highly selective membranes such as the brain-blood barrier and the human placenta [208, 226, 227]. Despite that, microorganisms are not helpless. They might adopt different self-protecting mechanisms, such as changes in the structure of cellular membrane, the secretion of neutralizing molecules, and the barriers imposed by the bacterial cell walls and any biofilm matrix [214, 228, 229, 230, 231]. However, further effects of micro-/nanoparticles on cells and tissues, the uptake and toxicity mechanisms have remained rather unclear.

As already mentioned in previous chapter, microplastics are utilized as carriers. However, they might become carriers of compounds, that are dangerous for the environment. In this context **microplastics**' surfaces might be **enriched with pathogenic and opportunistic organisms**, especially if they come from sewage treatment plants [179, 232]. They are often released to the continental waters, where they integrate seston with a microbiome distinct and potentially dangerous from the ones on natural particles causing recently observed disease emergence [208, 179]. Furthermore, despite the fact that polymers themselves are inert against metals, they are important intermediary for metal transportation into the organism and through the environment [233, 234, 235]. In other words, microplastics are capable of **heavy metal adsorption** due to their specific surface areas [233]. The adsorption is affected by the type of microplastic, their physical properties, the size of pores and surface area [233]. Metals bound to or included in the mineral matrix, behave inertly in the ecosystem, but their mobilization or external application (mostly together with compost) cause serious harm to plants and animals and allows them to accumulate in the food chain [124]. **Extracellular enzymes** are another compounds that may be adsorbed on microplastics [214]. The **adsorption** may cause an increase in a half-life of the enzyme due to the protection against proteolysis and decrease in a thermal denaturation [214, 236]. For instance, there are reports of a decrease in extracellular enzymes activity of soil after 28 days of incubation for polystyrene nanoparticles [214, 237].

Another negative effect of microplastics on the environment is **leaching** from chlorinated microplastics. It may cause serious changes in soil. For instance, polyvinyl chloride (PVC) releases nonvolatile organolorides, which cause geochemical changes in soil structure. This has been observed near roads in Australia, where presence of microplastics was approximately 7% [208, 238]. In addition, chlorinated microplastics might persist in the environment for more than 100 years due to its high durability (low light input and oxygen conditions) and therefore interact with soil fauna by changing biophysical environment and thereby the condition and functionality of the soil [208, 202, 239, 218]. Over and above that, microplastics can be transported within soil in both horizontal and vertical directions via earthworms [208, 240]. Transportation and following release of microplastics attached to the earthworms may cause structural changes in their burrows, which is directly linked to soil aggregation and functioning (this effect will be described later in the text) [202]. Leaching of plastic additives, plasticizers, and components of the polymer matrix is another serious problem. Leached compounds, in particular phthalates and bisphenol A, may cause endocrine disruption (compounds with estrogenic activity) in vertebrates and some invertebrate species [208, 241]. Possible demasculinizing effects by endocrine disruptors may then have an impact on whole population [242]. This is likely to happen, since plastic additives are amongst the most common anthropogenic substances in the environment [208]. And what is more, leaching is supported by particle size since is polymer physically bound to additives [208]. In other words, with decreasing particle size, the surface exponentially increases and hence promotes leaching.

Soil contamination with microplastics can potentially lead to direct and indirect **affect on cultivated plants** as a consequence of their root uptake or effects on soil chemical-physical and biological characteristics [214]. There are evidences of microplastic contamination in plants, for instance lettuce [243]. The uptake of microplastics by plants depends on plant species, on plastics properties, and on environmental ageing affecting surface chemistry and behavior [214, 244]. To emphasize, negatively charged microplastics show much higher root uptake compared to positively charged ones, possibly due to a

higher binding affinity with radical mucilage and size increasing through the hetero-aggregation induction by root's exudates [214, 245]. The plastics in roots might induce risk such as the interruption of the nutrient transport, the excessive production of reactive oxygen species, and the reduction of the plant disease resistance [214, 245, 246]. They can further pilgrim to stem and leaves via the vascular system [214]. Except root uptake, microplastics might enter food chain also by their adhesion to the surfaces of plants [245].

Speaking of indirect effects which has not been mentioned yet, microplasics' nature affect soil through physico-chemical **changes of soil texture and structure**, which is consequential for water cycling and ecosystem functioning in terrestrial systems and diverse plant–soil feedback [208, 247, 248]. In these circumstances, microplastics drive changes in the hydrologic properties of soils, which may influence soil microbial biodiversity and have an impact on key symbiotic associations in terrestrial ecosystems, such as mycorrhizal and N-fixing associations [208, 249, 250]. Such impact arises concerns for the soil microbiome because the mechanism of biodiversity loss and extinction in those ecosystems is not fully understood [208, 251, 252]. In soils are present hydrophobic and amphiphilic compounds (secreted by fungi), that regulate species communication and ecosystem processes [208]. For example, hydrophobins (cysteine polypeptides) play important role in soil hydrophobicity and soil aggregate stability, with direct potential consequences for soil erosion and biogeochemical cycles [208, 253]. This balance is likely to be disturbed by microplastics due to their hydrophobic surface, that tends to interact with hydrophobic compounds [254, 255, 256]. That is to say, that hydrophobicity reduces surface wettability and thus the accessibility of organic matter to soil microorganisms, restricts their living conditions and enhances the aggregate stability [214]. Furthermore, there are possible effects of microplastics on soil pedological processes due to a prolonged time of residence in the soil and their high reactivity [214]. Base on this theory, the non-degraded microplastics become part of the soil's geological cycle [214].

Along with above mentioned, **microplastics interact with surface-reactive soil particles**, such as clay minerals and soil organic matter [214]. This interaction affects microplastics' mobility [214, 257, 258, 259]. In addition, the interactions of microplastics with soil reactive components, and extracellular biological molecules affects soil functionality, which has an impact on the soil fertility and consequently yield and quality of crops [214]. Such reactive components are for example dissolved fractions of dissolved organic matter (DOM), that might potentially cause formation of (toxic) organic complexes [214, 260]. This formation is caused by an interaction of microplastics with DOM (via $\pi - \pi$ conjugation), carboxyl groups and C=O bonds [214, 260]. Nanoplastics may even accelerate the kinetic assembly rate of DOM by weak electrostatic interactions and hydrophobic interactions thus forming the particulate organic matter [214, 260].

Interactions of **microplastics** with organic matter can **affect nutrient availability** to biota in soil for example, by decreasing the dissolved organic N and dissolved organic P forms [214, 261]. In case of soil intoxication by biodegradable plastics, the increased microbial activity might potentially increase requirements for nutrients (importantly disbalance C/N ratio) and possibly cause priming effect. **Priming effect** refers to an increase in soil organic matter decomposition rate after input of fresh organic matter [262]. It results from an increased microbial activity due to a higher availability of energy, which in the end intensifies mineralization of soil organic matter. Accelerated CO_2 evolution in response to the activation of microbial metabolism and higher microbial biomass turnover are not measured directly, but by a measurement of CO_2 efflux or

nitrogen mineralization rates [263]. However, experiments should aim to identify priming effects not only based on CO_2 evolution, but also by identification of mechanisms of these effects [264]. Information about CO_2 efflux should be therefore enriched with information about microbial biomass and community structure (microbial diversity - different species could be activated by added substrate) and enzymatic activity (degradation of soil organic matter involves enzyme activity) [263, 264]. Adding a substrate to soil (see Figure 19) may cause either acceleration (positive priming effect) or retardation of soil organic matter decomposition (negative priming effect) [263]. The intensity of both processes is

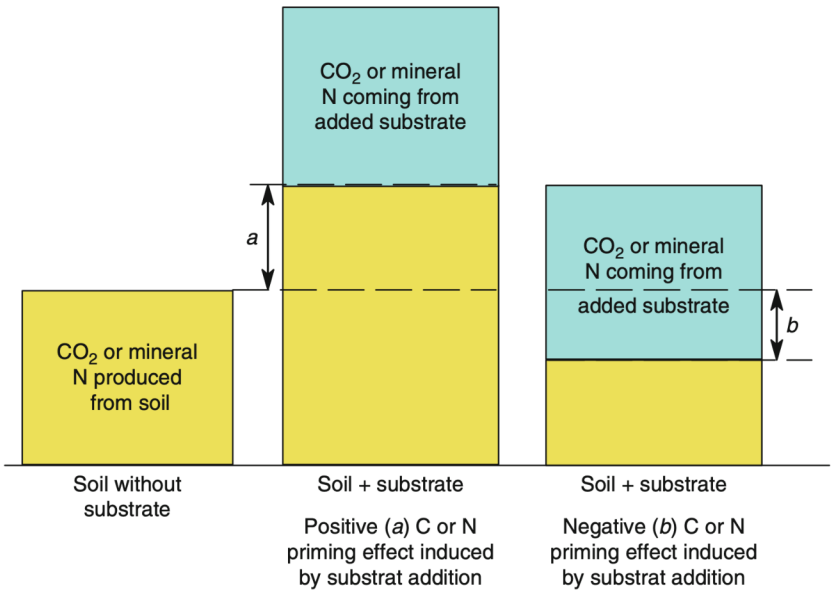

Figure 19: Schema of positive (a) and negative (b) priming effect [263].

not only affected by biotic factors, but also by physicochemical factors such as temperature, soil moisture, and pH, which act indirectly [263]. In a matter of mechanisms, there are two main components of priming effect: real and apparent priming. They are both governed by microbial activity [263]. Real priming corresponds to the change in CO_2 release from soil organic matter [265]. It is caused due to lack of nutrients (mostly nitrogen) or due to co-metabolism of soil organic matter. In the first case, the nutrients are supplemented by an accelerated soil organic matter decomposition [264]. In the second case, specific soil microorganisms use the energy of most available compounds to synthesize enzymes hydrolyzing the low available compounds and hence can be soil organic matter co-metabolized by microorganisms [264]. Apparent priming corresponds to a change in the CO_2 evolved from microbial biomass turnover after the input of substrate [265]. To summarize, real priming effect is caused by soil organic matter decomposition and apparent priming effect by changes in microbial biomass turnover without effects on soil organic matter decomposition [264]. The priming effect has been proven for substances such us glucose, fructose, alanine, celulose, plant residues, manure or slurry [263, 266, 267, 268]. The priming effect has also been observed in a soil exposed to a thermoplastic starch (TPS)-blend (PLA-TPS blend) as a substrate [269, 270]. However, the authors suggests that it could have been caused by degradable glycerol plasticizer [270]. Based on previous work and research, the experimental part of this work further

investigates the possibility of the priming effect caused by biopolymer poly-(R)-3-hydroxybutyrate [2].

Aim of the Work

Polyhydroxybutyrate is considered as a carbon neutral plastic given to its biologic origin [271]. This theory is however only valid if it doesn't disrupt the balance of the environment in which its degradation occurs. On top of that, it is believed that they do not enhance nitrous oxide and methane emissions which might offset these benefits [272]. In this sense, most of the research focuses on marine and fresh water environment only. However, recent studies suggest that soil may be adversely affected by the presence of P3HB, although comprehensive evidence is lacking. This work is therefore aiming to investigate P3HB degradation in soils and its subsequent effect on properties of various soil types. In addition, it will closely focus on 'Priming Effect' investigation as it was discovered in our previous research, that did not provide sufficient information to confirm our theory [2].

To meet this goals, several methods must be implemented. **Respirometry** is the first method used as an effective method of CO_2 release monitoring. It is used to investigate P3HB's degradation in soils; its rate of reaction and amount of carbon transformed. To investigate whether the carbon dioxide is released from P3HB or from P3HB and soil organic matter simultaneously, the **elemental analysis** is used. Third method, **thermogravimetry**, is used to investigate whether the degradation was complete and to investigate additional impact of P3HB on soil system (soil properties). **Enzymatic assays** are the final methods used for the investigation of the activity of microbial community and again effects on soil properties.

Experimental part

Chapter 4

P3HB degradation in selected soils

4.1 Specification and sample preparation

To investigate biodegradation of P3HB in soils, three soils (topsoils, sampling depth about 10 – 15 cm) were selected. According to the Czech Taxonomic Soil Classification System first two soils are classified as Chernosols and the third one belongs to the group of Cambisols. General overview of soil specification according to the Czech Taxonomic Soil Classification System was described in the Table 1. Soil analysis was performed at the Mendel University in Brno. The overall evaluation of individual parameters was based on a book 'Kritéria pro hodnocení produkčních a ekologických vlastností půd' (translated as : Criterias for evaluation of soil's production and ecological properties)[273].

First Chernosol soil was sampled in Šaratice and was classified as 'černozem černická' (*chernozems*). It was relatively rich on humus 3.73 % (usually 2.0 – 4.5 %), which signifies a predominance of bound humic components over free humic components and absence of free fulvic acids. The humus quality 0.89 (ratio of humic and fulvic acids) was classified as low (usually greater than 2 for chernozems). The pH_{KCl} 7.09 (refers to the acidity of a soil solution, together with reserve acidity in the colloids) is classified as neutral. The soil had a high clay content/fine texture (46 % of particles is smaller than 0.002 mm and 85 % is smaller than 0.01 mm) and therefore was classified as a heavy soil. The lower bulk density (1.51 $g \cdot cm^3$), higher cation exchange capacity (298 $mmol_+ \cdot kg^{-1}$) and relatively high porosity (46 %) were typical for soils with a higher content of organic matter. The soil airiness (6.62 %) was low as expected for heavy soils. The total nitrogen (0.32 %) was considered moderate [274].

The second Chernosol was sampled in Sloveč and was classified as 'černice pelická' (*phaeozems*). It had a high humus content 3.89 % (usually higher than chernozems), however its quality (0.59), was very low (high-quality humus is indicated by ratio greater than 1) [275]. It's pH_{KCl} was about 7.18, which as well as in the previous case is considered as neutral. The soil contained high clay content/fine texture (48 % of particles is smaller than 0.002 mm and 62 % is smaller than 0.01 mm) and hence indicated a heavy soil. The bulk density 1.48 $g \cdot cm^3$, cation exchange capacity 278 $mmol_+ \cdot kg^{-1}$ and porosity 44 % were typical for soils with a higher content of organic matter. The soil airiness was low (3.34 %) as expected in case of heavy soils. The total nitrogen was considered moderate (0.38 %) as in the previous case [274].

The third contrast soil was sampled in the site of Postoupky (nearby Kroměříž) and it was classified as 'kambizem luvická' (*cambisols*). As a contrast cambisol soil, it contained low humus content 1.43 % (usually 1 – 6 % for cambisols) of a low quality 0.56. This lower

humus content is adequate in case of cambisols, because it was sampled at an altitude of approximately 100 m above sea level (below 600 m is expected humus content less than 3 %). The pH_{KCl} 4.86 corresponds to moderate acidic soils. The low clay content/coarse or medium texture (22 % of particles is smaller than 0.002 mm and 30 % is smaller than 0.01 mm) and therefore indicated a light soil (sandy). The low cation exchange capacity (90 $mmol_+ \cdot kg^{-1}$), high bulk density high (1.70 $g \cdot cm^3$) and low soil porosity (11.65 %) is typical for mineral soils. The soil airiness (11.65 %) was high as expected for sandy soils. The total nitrogen corresponded to 0.21 %.

The soil was air dried and sifted (diameter: 2 mm). Right after, using an analytical laboratory balance, 5 g of each soil was mixed in a Petri dish with powdered P3HB of a selected quantity of approximately 0.5, 1 and 3 %. The P3HB was obtained from 'Duslo a.s.'. To identify it's particle size, photographs were taken via FEI's electron microscope QUANTA 250. Based on the results, the particle size was about $0.6 - 1.3\,\mu$m (see Figure 20). This size indicates that the P3HB powder included both micro and nano particles of this polymers. Except contaminated samples, 5 g of each soil was prepared and used as a

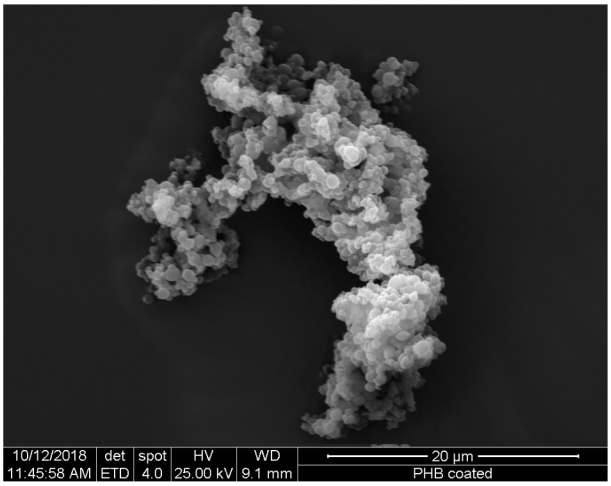

Figure 20: The photo of powdered P3HB taken with an electron microscope.

blank sample. To compare degradation of P3HB with other frequently used contaminant, two types of samples containing cellulose were prepared (serving as control samples). First type consisted of 5g of soil and 1 % of cellulose. The second one included 1 % of cellulose and 1 % of P3HB. The mixture of samples and contaminants was then properly homogenized, with respect to the preservation of both the soil's and contaminants' structure. To reduce possible fluctuations caused by sensors, pure P3HB was separately prepared as well. Because each soil had a different structure and was therefore able to hold different amount of water, it was necessary to add a certain amount of water to each soil. To do this, the field water holding capacity pF 1.8 (corresponding to coarse narrow pores) was measured. The field water capacity allowed to achieve similar conditions for each soil. The data obtained was recalculated because 76 % of the water in the sample was required (to simulate environmental conditions). In this context, indicated amount of water was added to the samples using a micropipette: the *pheozem* was enriched with approximately 1.09 mL of water, the *chernozem* with 1.22 mL and the *cambisol* with 0.97 mL. The samples were once again homogenized and placed in a respirometer.

4.2 Device construction, materials and methods

The respirometer Respicon VI (Nordgren Innovations, Sweden) was used to collect data of respiration. This equipment runs on a fully-automated system for the measurement of soil respiration, that can be used to record evolved carbon dioxide (CO_2) from up to 96 samples. The samples are incubated in a closable, well-sealed container (with extra tape around the lid and around the rubber stopper), consisting of a bottom part for the sample vessel and an upper part for a smaller container with 0.6 M potassium hydroxide (KOH) (see Figure 21). Here, potassium hydroxide served as an intermediary between the electrode

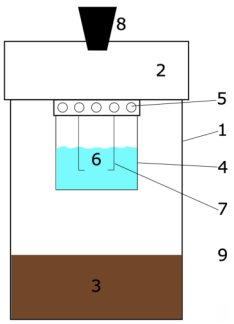

Figure 21: Interpretation of the vessel of the respirometer (experimental vessel (1), screw cap (2), soil (3), conductivity cell (4) with holes for CO_2 flow (5), KOH solution (6), platinum electrodes (7) and rubber stopper (8) [276]).

and the released carbon dioxide. The CO_2 evolved during degradation was dissolved in the KOH absorbent (aqueous solution) located above the sample and its amount was hence determined using electrodes placed in the absorbent solution based on the electrochemical potential change. In a principle, as potassium hydroxide reacts with carbon dioxide to form aqueous potassium carbonate solution the electrode potential changes (see Reaction 18).

$$CO_2 + KOH \rightarrow K_2CO_3 + H_2O \tag{18}$$

In addition, the temperature of the vessels was regulated by their placement to two temperature-controlled water baths (see Figure 22). Electrodes connected to the multiplexer send a signal via a conductometer to a computer. In the computer, the signal is processed by the Versatile control software, that records the progress of the analysis and also allows data editing using selected functions. In the experiment, the evolved carbon dioxide was recorded in mg (in total), but it was also possible to display it per hour or to display conductivity change in mS.

4.3 Procedure

The experiment was inspired by the standard 'ČSN EN ISO 17556:2012 Plastics - Determination of complete aerobic biodegradability of plastic materials in soil by measuring oxygen consumption in a respirometer or by measuring the amount of carbon dioxide released' [277], however the standard was later updated to 'ISO 17556:2019' [278].

The samples were incubated for 9 months at a maintained temperature of (20±0.01) °C without access to light. The respirometer read the values per hour. During this period, potassium hydroxide was carefully and regularly replaced on software's command. At the

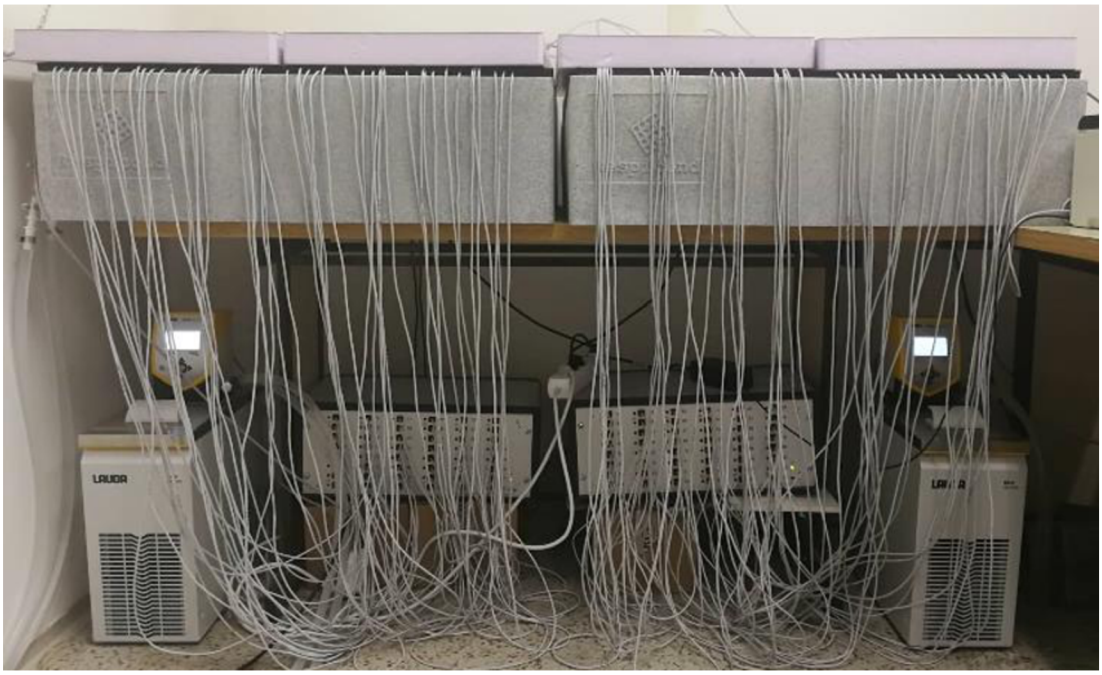

Figure 22: Photograph of the Respicon VI respirometer with two water baths (two gray containers with 4 white lids made of polystyrene) connected by 96 cables to two multiplexers (2 central devices) with cooling devices on the sides.

end of the experiment, part of the samples was frozen and sent to elemental and enzyme analysis. The second part was further used for thermal analysis.

4.4 Data processing

The results were obtained in mg as accumulated CO_2. To compare results within each other, several adjustments had to be made.

First, data was adjusted as a consequence of technical problems, which had occurred during these 9 months of measurements. This was done by basic mathematical operations, that shouldn't affect the accuracy of the results. However, to discard any suspicious anomalies, further experiments were performed (will be described in the following chapters). Second, since the concentrations of P3HB and cellulose in the soil were not precisely 0.5 %, 1 % or 3 %, mathematical operation 'direct proportion' was applied to recalculate the data. This way, the results of recalculated CO_2 evolved represented degradation of precise concentration (0.5 %, 1 %, 3 %) of these two contaminants and hence, allowed the comparison within results (including theoretical values) without unnecessary inaccuracies.

To find whether the contaminants had been fully degraded or the priming effect had cured, the theoretical amount (mass) of CO_2 accumulated was calculated. Before all else, the degradation of P3HB and cellulose into the CO_2 was taken into consideration. To do so, the carbon content in molecules of P3HB and cellulose was calculated (Equation 19) :

$$Carbon\,content\,(\%) = \frac{M_{\mathrm{carbon}}\,(\mathrm{kg}\times\mathrm{mol}^{-1})\times X\,(-)}{M_{\mathrm{molecule}}\,(\mathrm{kg}\times\mathrm{mol}^{-1})}\times 100\,(\%) \tag{19}$$

where M represents a molar mass and X represents number of carbons in the molecule (either of P3HB or cellulose). The results represent percentage of carbon in either P3HB or cellulose. To continue with, the carbon content was used to calculate the amount (mass) of carbon in P3HB in mg. To do so, the theoretical amount of P3HB and cellulose added to the soil had to be calculated based on the Equation 20 :

$$m_{\text{contaminant}}\,(\text{mg}) = \frac{c\,(\%) \times m_{\text{soil}}\,(\text{mg})}{(100\ -\ c)\,(\%)} \tag{20}$$

where m represents the mass and c represents the concentration of given contaminant. Right after, the results of these two simple calculations were combined to obtain theoretical amount (mass) of carbon in P3HB and cellulose based on the Equation 21 :

$$m_{\text{carbon}}\,(\text{mg}) = \frac{m_{\text{contaminant}}\,(\text{mg}) \times\ Carbon\,content\,(\%)}{100\,(\%)} \tag{21}$$

Eventually, the theoretical amount of CO_2 accumulated was calculated based on the Equation 22 using direct proportion once again.

$$\text{Theoretical}\,m_{CO_2}\,(\text{mg}) = \frac{M_{CO_2}\,(\text{kg} \times \text{mol}^{-1}) \times\ m_{\text{carbon}}\,(\text{mg})}{M_{\text{carbon}}\,(\text{kg} \times \text{mol}^{-1})} \tag{22}$$

The theoretical m_{CO_2} was subsequently compared with real results. To determine whether complete degradation had occurred, the results of respirometry were compared with the results of thermogravimetry. In samples with completed degradation the priming effect was possible to investigate.

Chapter 5

Analysis of residues and changes in soil properties

5.1 Thermal analysis of P3HB residues

5.1.1 Sample preparation

Thermal analysis was carried out for two reasons. First, to determine the level of degradation. Second, to outline possible changes in soil, that were caused by the presence of P3HB with focus on water retention and stable and labile carbon. To investigate these, the samples were subjected to thermogravimetry before and after degradation. This means that all, blank samples before and after degradation and samples that once included (or potentially still included) artificially added contaminants were prepared for analysis.

Prior to measurement, each sample was air-dried, homogenized and equilibrated in a desiccator to relative humidity of (43±2)% at 20 °C for two to four weeks. This was accomplished by adding potassium carbonate to the bottom of the desiccator and further adding its saturated solution between samples (placed in beakers). The specific relative humidity was chosen to unify the conditions and at the same time approach the ones close to the conditions in the environment. A hygrometer was used to indicate whether the samples were ready for analysis. In addition, the samples were stored in temperature-controlled room (20±2) °C.

5.1.2 Device construction, materials and methods

To analyze samples, a modified TGA 550 thermogravimeter from TA Instruments was used (see Figure 23). The main part of this device is an oven that is able to operate up to a temperature of 1000 °C. Samples were dosed into aluminum (Al_2O_3) pans, that were later placed on a twenty-five position automatic sampler. Once the samples were placed on the autosampler, they were passed by its movable arm to a platinum hook. This high temperature resistant hook is used to hang the samples in the oven. This process is not performed manually, but is programmed online using TRIOS software (from TA instruments), either directly on the device screen or on a computer. Based on the selected program (see Section 5.1.3), the samples were heated causing the change in their weight (in case of labile organic compounds). The weight was recorded by scales located inside at the top of the thermogravimeter. The scales consist of an internal balancing pan attached

to a platinum hook, that is balancing the weight of the sample. The movement of this double-sided hook is recorded. The platinum hooks thus serve at the same time as a holder for aluminum pans, but also as a detector of weight change during analysis. In addition, externally supplied nitrogen (gas bomb) was used to purify the scales.

Figure 23: Construction of modified TGA 550 (TA Instruments) with a lid [279].

To ensure the relative humidity of (43±2)%, while the samples are placed on the autosampler, specially modified lid (see Figure 23) was attached. In addition, this lid included a bypass, which was used as an aeration device. It was connected via tubes to two Drechsel's bottles. First was bubbling air through saturated potassium carbonate solution and the second one was filled with cellulose to prevent clogging of the tube leading directly to the lid and contamination of samples. Air with a relative humidity of (43±2)% was supplied to the lid in three directions.

Similarly, rather than connecting the air supply, necessary for the operation of the thermogravimeter (oxidation in an oxygen atmosphere), directly to the oven, the exact same bypass as in the previous case was connected. Here, the humidified air was blown into the oven instead of the lid to ensure the same conditions even during the analysis. Furthermore, the cellulose didn't prevent clogging of just a tube and contamination of samples but also prevented the oven from clogging.

The data was sent directly to the computer and hence it was possible to evaluate the results during the measurement.

5.1.3 Procedure

Firstly, the empty pans were tared. Subsequently, the samples were dosed into the aluminum pans and placed on an autosampler, in an amount of about 200 mg. Because the studied properties were not expected to change immediately right after the addition of contaminants, it was not necessary to analyze contaminated samples before degradation. Therefore, only blank samples (soil without artificially added contaminant) were prepared for the measurement. On the contrary, both blank and artificially contaminated samples after the respiromentry were prepared.

When samples were prepared on an autosampler covered with a modified lid, analysis was performed. The measurement was programmed so that the heating took place at a rate of 5 °C per minute from room temperature to 740 °C. Data were saved once every 0.1

minutes (6 seconds). The air flow rate of (43±2)% relative humidity was set at 90 mL per minute.

Before the start of measurement, the weight of the sample was stabilized (inside the oven), and only then did the analysis begin. The thermogravimeter recorded the change in weight loss in both μg and % as a function of temperature. Furthermore, the time in minutes was recorded. In that manner, about 1500 values of each variable were collected for each data set. To simplify the data manipulation, the derivative was calculated additionally in this program.

After analysis of the sample, the oven was cooling for about 55 minutes. One sample was thus measured for approximately 3 hours, including the cooling. Then the measurement of another sample was allowed. The samples were gradually transported to the oven. Each sample was measured at least 3 times. In case of suspected inaccurate measurement, the measurement was repeated once or twice more.

5.1.4 Data processing

As already mentioned, temperature, time and weight were monitored during the measurement. In this work, only the mass loss (in %) dependent on temperature was used for further analysis. These data indicated a decrease in the weight of the samples with increasing temperature during the measurement. Because the data were recorded in a specific time (each 0.1 min), the temperature was not identical (e.g. 30.84 °C, 30.52 °C, 30.07 °C etc). This was influenced by the ambient conditions, the type of soil and the proportion of the artificially added contaminant into the soil. Therefore, the data needed to be processed in a way, to make the results comparable. Given that this reaction was not linear, the '10 °C approach' had been used to 'filter' the data. The highest mass loss of each temperature interval (e.g. 30 °C – 40 °C) was 'filtrated' out of the data set. This one value was then assigned and plotted with the corresponding temperature decade (e.g. 30). Doing so, the accuracy of data remained high for each sample. This also made it possible to reduce the data from 1400 mass losses to 71 (decades from 30 °C to 730 °C). In case of several valid multiplications of measurements (of the same exact sample), the values were averaged to obtain as representative results as possible. This was possible due to the generally high complexity of the soil. To indicate in which decades the most dramatic declines occurred, each mass loss was subtracted (e.g. 40 °C) from the previous temperature decade (e.g. 30 °C). This was particularly useful because in the last step of data processing, the results of the contaminated samples were subtracted from the blank soils. The results were for clarity shown in graphs.

5.2 Enzymatic assays and elemental analysis

5.2.1 Sample preparation

To confirm once again, whether there are no residues of P3HB remaining and to possibly identify the impact of P3HB degradation on a microbial level, the exo-enzymes kinetics was studied. In addition, to identify possible impact of increased microbial activity on selected nutrients availability, elemental analysis was performed.

Samples taken out of the respirometer were placed into transporting zip bags and placed in a freezer to maintain current conditions until analysis. Both, enzymatic assays and elemental analysis, were performed at a laboratory of Mendel University in Brno.

5.2.2 Data processing

In the case of enzymatic test, each sample was determined 9 times based on the mentioned method. Out of it, an average value was calculated with respect to a standard deviation. The results were plotted and evaluated.

Chapter 6

Results and discussion

6.1 P3HB degradation in selected soils

As mentioned in the previous chapter, respirometry was used to examine P3HB's degradation. This experiment was based on my bachelor's thesis, where we noticed a possible priming effect [2]. However, due to the suspicion of inaccuracies caused by poor respirometer isolation and because confirmation of the priming effect (as mentioned in the theoretical part) cannot be determined by respirometry alone, this experiment had to be reproduced and further analyzed.

The first step was the degradation of P3HB in the respirometer. Beforehand based on our previous experiments, the equipment was optimized and the insulation was tested several times to ensure maximal elimination of external factors. In this way, the elimination of unwanted deflections, that we experienced before, was ensured.

The new experiment lasted longer (approximately 9 months) compared to the previous one (4 months). The reason for extending the duration of the experiment was the longer degradation of P3HB, which was probably caused by the utilization of different soil types and thus it was caused by different soil properties. However, due to the longevity of the new experiment, several unwanted deflections had occurred. As a result, mathematical adjustments (that are not expected to affect the accuracy of the results) were made. Nevertheless, due to yet not explained voltage change on electrodes, some inaccuracies still had occurred. The hypothesis is that it was caused by sudden degradation of the electrodes, since it had happen only on several of them. This change however is suspected to happened in the second half of the measurement, which is not essential for priming effect investigation, since the selected samples were already degraded. Therefore, the priming effect will be discussed in data, that were already degraded. The data of non-degraded samples will only be used to monitor the trend of the degradation, since their accuracy cannot be ensured.

Based on our previous experiments, it was expected that addition of P3HB substrate will accelerate decomposition of soil organic matter (positive priming effect). This paragraph accordingly explains the processes that occurred during the P3HB degradation. The substrate behaviour was discussed closer in detail in Section 3.4. The Table 4 shows theoretical and real amount of CO_2 evolved. If the added substrate did not disturb the soil balance, the theoretical amount of CO_2 produced would be approximately equal to the real values. From our data, it can be seen that both 0.5 % and 1 % of P3HB were fully degraded in all soil types. However, the amount of CO_2 evolved from all samples (taken at the point of termination of degradation) was significantly higher than expected

Table 4: Comparison of CO_2 evolved from P3HB spiked samples with theoretical values.

Concentration of P3HB added (%)	**Theoretical amount of CO_2 evolved (mg)**	**Real amount of CO_2 evolved (mg)**		
		Phaozem (černice)	**Chernozem (černozem)**	**Cambisol (kambizem)**
0.5	50.9	66.5	60.8	62.1
1	102.3	110.0	107.7	112.2
3	313.1	215.3	305.8	179.1

(compared to the theoretical values). This is believed to be caused by the fast-growing community of microorganisms, that was capable to obtain less available carbon compounds (complex molecules) from soil organic matter compared to the normal conditions (positive priming effect). In other words, with enough nutrients (especially nitrogen and phosphorus) and substrate addition (P3HB in this case) the community of microorganisms grows and produces extracellular enzymes, capable of decomposition of complex molecules. These exo-enzymes decompose substrate along with soil organic matter (provides them with additional nutrients). Soil organic matter is hence decomposed together with P3HB and therefore is the CO_2 evolved much higher than expected. What is more, even after depletion of a P3HB, the dead microbial biomass can serve as an easily accessible substrate for other organisms. Dying microbial biomass can then support production of extracellular enzymes and continue to influence the decomposition of soil organic matter. This is not an unusual phenomenon. The soil already includes microorganisms that decompose simple molecules. The addition of a high amount of carbon-rich substances (capable of biodegradation) causes an imbalance in the soil to which microorganisms try to adapt (taking advantage of it). Speaking of the third concentration, as it will be graphically represented and described in following paragraphs, the highest concentration (3 %) had not been degraded in nine months of this experiment (see Figure 26 on page 58).

The Figure 24 represents a respiration of chernozem soil with addition of 0.5 % P3HB compared to a blank sample. The red line then indicates the end of P3HB degradation calculated by blank subtraction from the contaminated sample as shown in Figure 25. The subtraction indicates the highest variations in respiration compared to the blank and hence the end of P3HB degradation. In other words, the end of degradation is indicated by a plateau in Figure 25, which indicates the period when only soil organic matter degraded (same respiration as blank) and hence the exact point of the termination of P3HB degradation (see the trend similarity at the end of degradation in Figure 24). As can be seen on both figures, the degradation lasted less than 2 months. Based on our calculations, the amount of CO_2 exceeded the theoretical value by 19.6 %. The theory was that the P3HB supported acceleration of soil organic matter decomposition (positive priming effect). To confirm this, elemental analysis was performed (for more details see Table 6 on page 96). The result showed that the total carbon in soil decreased by 11.6 % compared to the original values. In addition, compared to our control treatment the carbon content in soil samples with P3HB addition was higher after degradation. This means that bacteria preferred carbon acquisition from P3HB over soil organic matter, but the soil organic matter was still decomposing along with P3HB degradation (to obtain

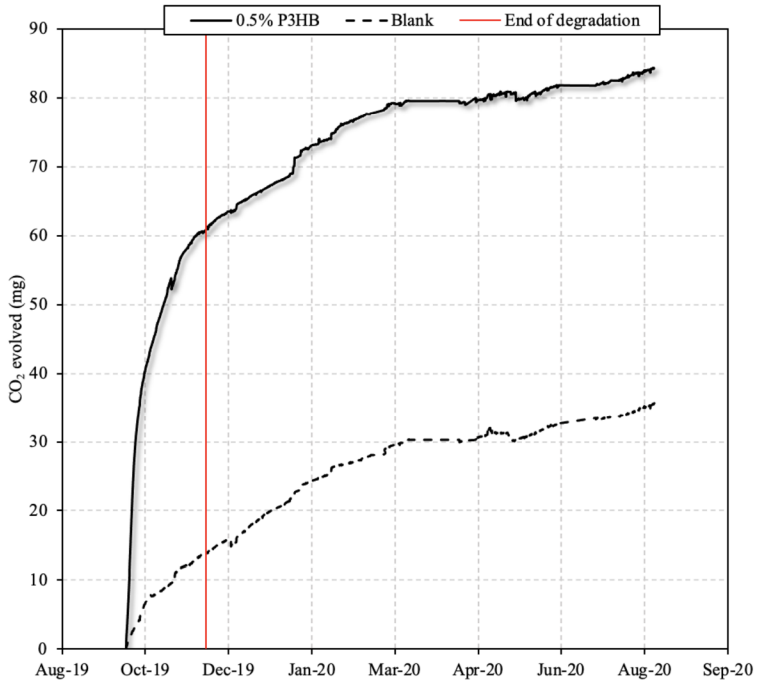

Figure 24: Respiration of blank chernozem soil and soil with addition of 0.5 % P3HB.

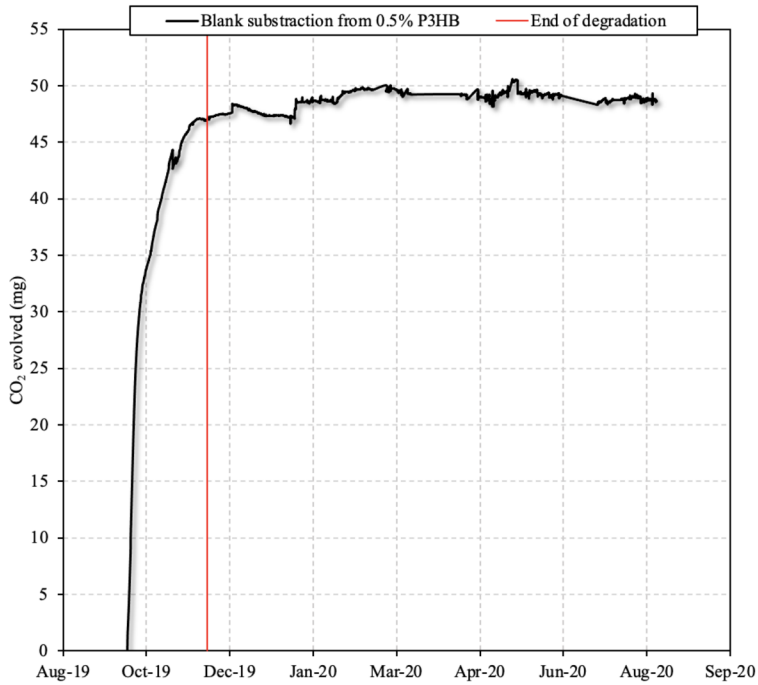

Figure 25: Subtraction of blank values from chernozem soil spiked with 0.5 % P3HB.

other nutrients as well). Therefore, to confirm the positive priming effect theory, the nitrogen concentration in soils before and after degradation had to be observed. The results showed that the concentration decreased. This means that microorganisms were trying to acquire more nitrogen, than in case of non-contaminated soil (to cope with

P3HB addition). The nitrogen concentration was decreasing gradually with increasing concentration of P3HB in a soil (non-degraded samples were not counted). In this case, it decreased by 1 % (more significant results yielded for higher concentrations; will be discussed later in the text). This indicated acceleration of decomposition of soil organic matter (positive priming effect) insomuch as microorganisms preferred decomposition of P3HB substrate over soil organic matter. However, the substrate couldn't provide them with all essential nutrients. In other words, as it was displayed in the theoretical part in Figure 19 (page 42), in case of positive priming effect the soil organic matter is degraded along with a substrate and thus the amount of CO_2 evolved is higher (as well as the amount of mineral nitrogen) than non-contaminated sample. The information of sulphur was unfortunately missing in case of a blank soil before degradation due to a lack of soil samples. However, compared to a control treatment (blank sample after degradation), we observed its decrease in the soil. The decrease in sulphur is caused by microorganisms drew (of sulphur) as an essential element for their survival (chemotrophic sulphur oxidation - reduces sulphur compounds as electron donors for respiration) [280].

The middle P3HB concentration (1 %) is considered degraded as well (see Figure 26). As it can be seen, the degradation time increased gradually with increasing concentration of P3HB added. The addition of P3HB lead again to the acceleration of native soil organic matter mineralization (and as expected in the case of 3 % P3HB addition). Furthermore, the induced microbial immobilization of essential nutrients was more pronounced at this concentration compared to the 0.5 % (for more details see Table 6 on page 96). Particularly, the nitrogen decreased by 3.2 % compared to our control treatment, suggesting that the presence of P3HB affected nitrogen retention. This is believed to be caused by alteration of microbial community composition and functioning during degradation [272]. To illustrate, a presence of P3HB as a compound rich in carbon but poor on nutrients, can cause an overtake by specific bacteria that dominate oligotrophic environments where nitrogen availability is

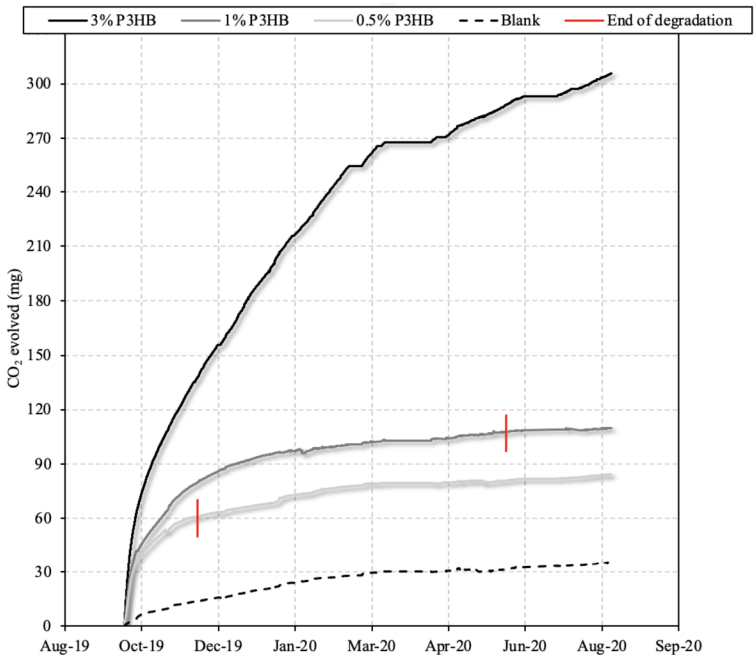

Figure 26: Respiration of blank chernozem soil and soils with 0.5 %, 1 % and 3 % of P3HB added.

low [272, 281]. Higher concentrations of P3HB can therefore cause excess nitrogen removal and hence have a negative impact on plants.

Overall, the rate of decomposition is dependent on the amount of substrate (see Figure 26). In this sense, all soil types with addition of 3 % P3HB were not fully degraded. In the Figure it is visible, that several problems affecting the continuity of the curve have occurred. They were either caused by electrode problems or by system blackouts. Therefore, the respiratory record of 3 % P3HB concentration is only used to observe the degradation trend. The degradation however hasn't been finished. Microorganism either required more time to degrade the remaining P3HB or in the latter case they didn't finish their job due to a nutrient depletion. Based on our results, we assume that only more time was required to finish the degradation, since the degradation could be considered almost complete (probably less than 2.3 % remains to degrade) as the degradation trend suggests. Furthermore, as in the previous cases, the possibility of priming effect cannot be neglected. The proof of non-termination of degradation was proven by elemental analysis (for more details see Table 6 on page 96). From the data it is visible, that the carbon content was about 9.4 % higher compared to the blank before degradation (original conditions). These 9.4 % suggest, that there is still some P3HB remaining to degrade. Besides as it is presented in Figure 27, the remaining P3HB can be observed visually (focus on white particles). These bigger particles were difficult to degrade, since microorganisms on the surface of microplastics need to acquire nitrogen from the surrounding soil to support growth [272, 282].

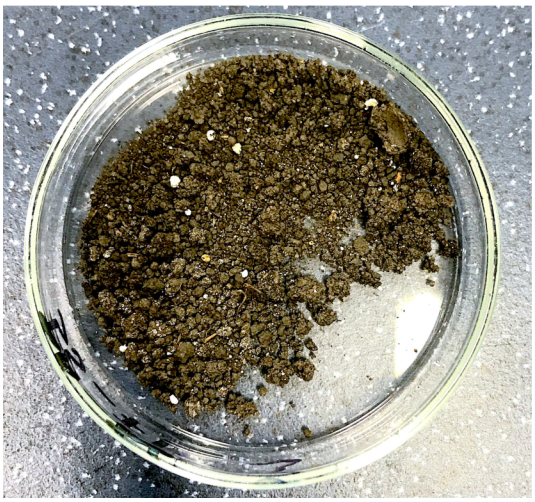

Figure 27: The chernozem soil originally containing 3 % of P3HB after 9 months of degradation.

Each concentration could accelerate the growth of other microbial communities, which consequently can have a different impact on the biodegradation process. For example, for higher concentrations, the P3HB can significantly reduce the pH (P3HB is broken down to 3-hydroxybutyric acid), thus favour the growth of acidobacteria [218]. What is also important, that each soil type has a different soil properties and hence contains different communities of microorganisms. In this sense, we can observe that phaeozem (see Figure 28) and cambisol soils' (see Figure 29) respiration was visibly different. Compared to the chernozem soil, both phaeozem and cambisol soils degraded slower. This might be caused by relatively low humus quality in both cases compared to the chernozem soil. Based on elemental analysis, the nitrogen concentration was decreasing with increasing concentration

of P3HB in case of phaeozem (same as in the previous case). The nitrogen level decrease by 1.5 % in sample with 0.5 % of P3HB added. This concentration was similar to the concentration of a control sample. The 1 % concentration was significantly more affected by P3HB addition. The decrease was about 3.7 % compared to the original condition and was about 3.1 % lower than our treated control sample. As in case of chernozem soil, it was observed, that the carbon content was higher in samples with addition of P3HB compared to our control treatment (however still much lower than original conditions) and the nitrogen level significantly decreased (positive priming effect).

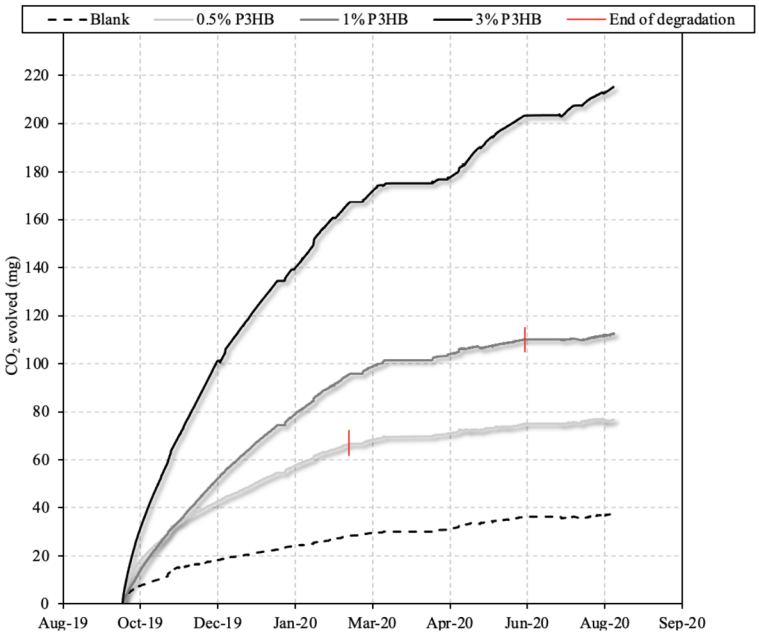

Figure 28: Respiration of blank phaeozem soil and soils with 0.5 %, 1 % and 3 % of P3HB added.

The contrast cambisol soil as a soil with different physicochemical properties compared to previous types significantly different results. It was registered that the P3HB didn't have a negative effect on nitrogen consumption. On the contrary it is visible, that with increasing concentration, the soil maintained its original nitrogen level. For the lowest concentration (0.5 %) the nitrogen level was the same as in case of control treatment. The carbon content decreased in case of 0.5 %, however this decrease was not significant. Therefore, we conclude that a retardation of soil organic matter decomposition occurred (negative priming effect). Such different results are possible due to a different composition and properties of cambisol soil compared to the previous cases. As it was displayed in the theoretical part in Figure 19 (page 42), in case of negative priming effect the soil organic matter is still degraded along with a substrate, however in a lower amount than in case of a positive priming effect and soil without substrate. This occurs in soils, where carbon substrate utilization is more easily accessible by the microorganisms. The microorganisms preferably utilized a substrate and thus a decomposition of less utilizable soil organic matter was retarded compared to the initial state [283]. In case of 1 % P3HB addition the nitrogen content was even similar as for initial sample (before degradation). Such finding suggests, that it is likely that the bacterial community associated with nitrogen processing was not visibly affected by P3HB addition. As the elemental analysis revealed, the carbon content for a concentration of

1 % P3HB was higher compared to non-treated sample. This could possibly mean that the degradation was not complete, however both the respiration trend and thermal analysis (will be discussed later) did not refute this theory. Our hypothesis therefore is that particles of P3HB can be incorporated in aggregates and thus protected against microbial attack. The data also suggested a positive priming effect. As in the previous cases, 3 % concentration of P3HB was excluded from the evaluation due to incomplete respiration and inaccuracies (as described earlier).

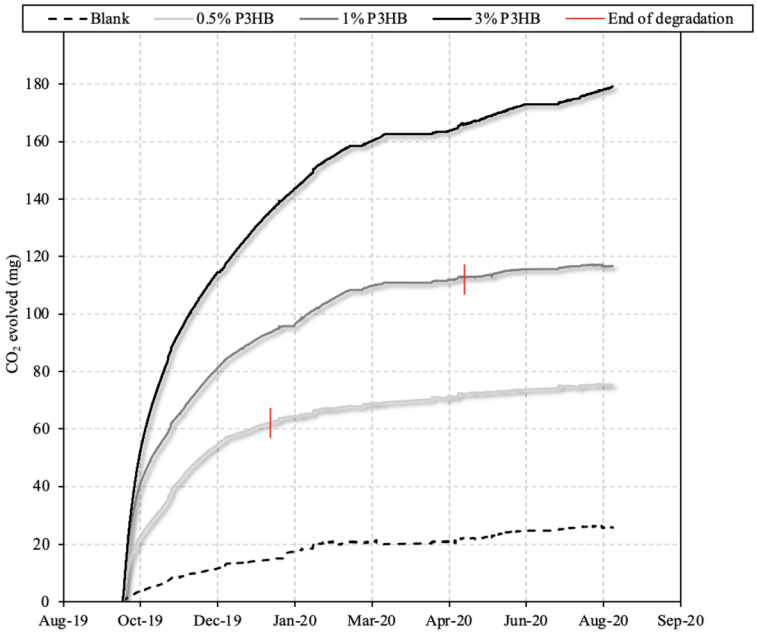

Figure 29: Respiration of blank cambisol soil and soils with 0.5 %, 1 % and 3 % of P3HB added.

6.2 Analysis of residues

6.2.1 Thermal analysis

The thermal analysis was performed to analyze the residues and to investigate the impact of P3HB addition on selected soil properties. Mass loss as a function of temperature was observed. Any deviation from blank sample indicated changes in soil properties, that will be discussed in this chapter.

The properties discussed above, are typical for specific temperature zones (intervals) highlighted in Figure 30. While the temperature in a thermogravimeter increases, some compounds remain intact and some undergo changes. As it is visible in the Figure, during this oxidative process, the mass decreases nonlinearly. Therefore, each zone is characterized by a visible change in the course of the curve (considered for normal atmospheric pressure). These changes are associated with specific compounds/elements. To demonstrate these changes in soil properties based on the approximate zones, the Figure was separated colourfully. The first blue zone, is typical for water loss. It contains two visible changes in the curve course, first characteristic for weakly bound water in the soil (up to 100 °C) and second is characteristic for water strongly bound in the soil and

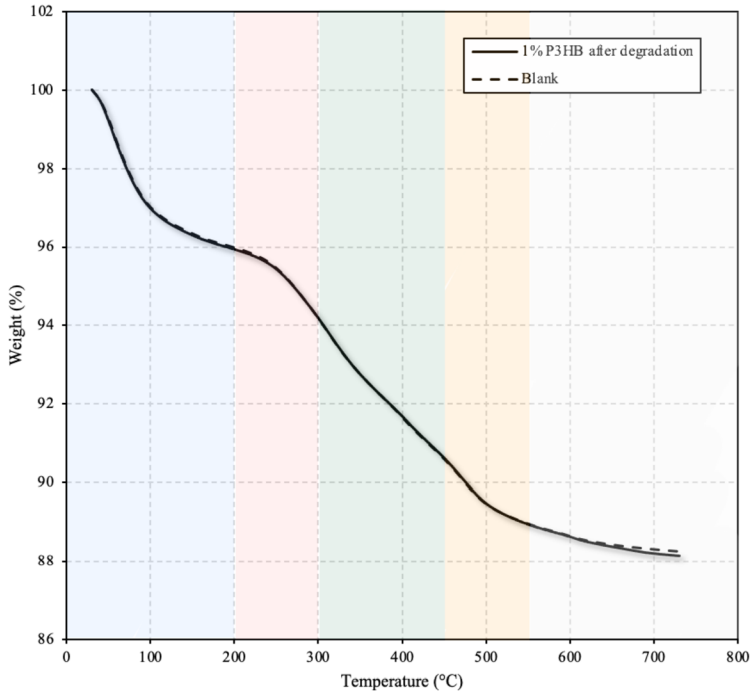

Figure 30: The typical observed temperature zones (blue zone is characteristic for water loss, red zone for labile fractions or carbon, nitrogen and phosphorus, green zone for labile and yellow zone for stable organic matter and gray zone is characteristic for carbonates).

organomineral complexes (100 °C – 200 °C) [284]. This interval is indicative for clay content and at the same time with microbial activity. To continue, the mass loss in the red 200 °C – 300 °C interval/zone is associated with thermally labile fractions correlating with organic carbon and partially with organic nitrogen (interacts from 200 °C) [284]. The green 300 °C – 450 °C interval is associated with transformation of labile organic matter and the yellow 450 °C – 550 °C interval with stable organic matter [284]. In addition, the weight loss in the zone of 450 °C – 550 °C correlates with the clay content indicating the association of organoclay complexes [284]. Above 550 °C the decomposition of carbonates takes place (indicated with light gray colour in the Figure) [284]. The delimitation of these zones depends on bound compounds and/or chemical properties and therefore the borderlines of these intervals are just approximate.

Overall, the effect of P3HB addition was seen on all samples. It differs in the concentration of P3HB added and matrix at which degradation was observed. The degradation/oxidation of P3HB can be observed in Figure 31. It is notable by a significant deviation from the blank trend between 230 °C and 300 °C. In this area, about 2 % of soil organic matter were oxidised. It means, that P3HB in this sample was not fully degraded. The oxidation interval of polymers (but not only) is dependent on their properties and varies significantly within each compound. Accordingly, the non-degraded samples were registered (see Table 5). Any other deviation from blank sample signified changes in soil properties mentioned in the previous paragraph.

Table 5: An overview of samples that were evaluated as (non)degraded using thermogravimetry.

Concentration of P3HB added (%)	Degradation finished?		
	Phaeozem	**Cambisol**	**Chernozem**
0.5	Yes	Yes	Yes
1	Yes	Yes	Yes
3	No	No	No

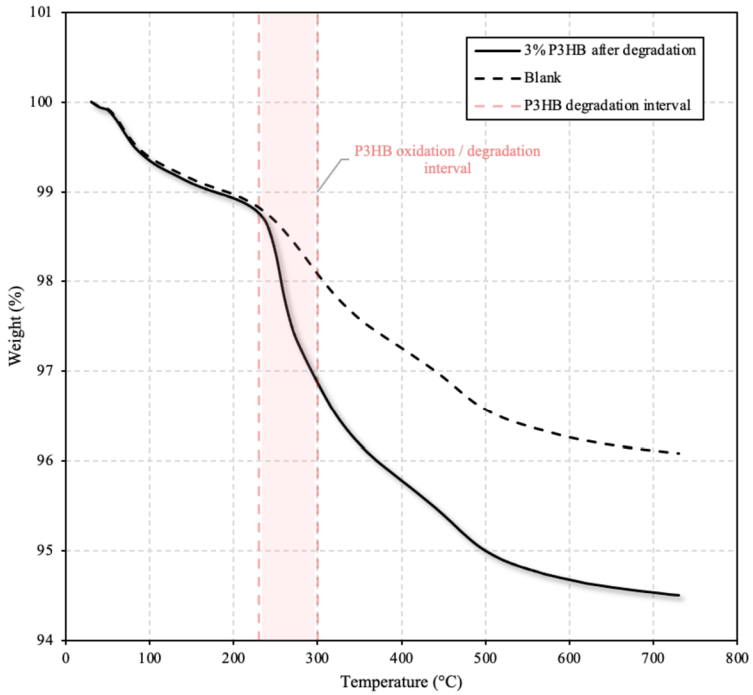

Figure 31: Weight loss of contaminated cambisol soil with addition of 3 % P3HB as a function of temperature.

Figure 32 on the contrary highlights in blue changes in other properties. The first interval, as it has been shown in Figure 30, refers to weakly bound water. It could possibly mean, that the sample was not air dried properly and/or incubated under proper conditions before measurement, however this behaviour was also observed for other samples of the same concentration. What is more, the elemental analysis (see Table 6 on page 96) also didn't suggest changes in hydrogen composition and hence more water was not present in the soil (it was equal to the blank sample). The hypothesis therefore is that the addition of smaller concentrations (0.5 % P3HB) affect water retention. It is possible, that presence of P3HB allowed faster water evaporation as it was more accessible (water in interval to 100 °C is weakly bound). This is probable, because soil is naturally rich on pores and interactive compounds. Addition of P3HB could prevent these interactions and hence the soil binds water less. In total the amount of water does not change, just its form changes from strongly to weakly bound. In the following paragraphs, the changes in properties will be discussed altogether. The graphs separately are given in the appendices (see page 99 and above).

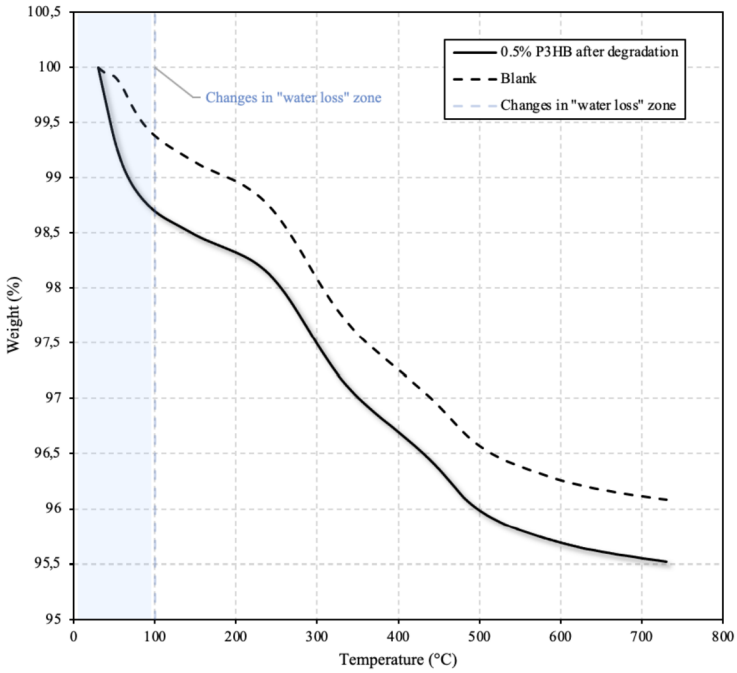

Figure 32: Weight loss of contaminated cambisol soil with addition of 0.5 % P3HB as a function of temperature.

As it was registered in the previous chapter, the impact of degradation is influenced by the soil type and P3HB concentration added. To observe the exact changes, thermal weight (mass) losses were calculated and all concentrations of one soil type were simultaneously plotted in a graph. The chart axis were set the same, to see more clearly the difference in behavior of P3HB in different soils. Overall, the curves were similar for both chernosols (for chernozem see Figure 33 and for phaeozem see Figure 34). On the contrary, the results of the samples containing cambisol soil were significantly different to these (see Figure 35). All samples were significantly deviating from blank in lower concentrations (0.5 %). This trend may be best observed in first Figure 33. It can be seen (as discussed in the previous paragraph), the addition of lower concentration of P3HB has a notable impact on soil water retention (interval up to 100 °). With increasing concentration, the effect of P3HB addition on water retention decreases in case of chernozem soil. Other intervals were not affected by biopolymer addition. The only deviation was observed in case of soil with 3 % P3HB added, where P3HB was not fully degraded (see blue curve in interval of P3HB oxidation).

In phaeozem soil (see Figure 34), all concentrations of P3HB added were nearly degraded, with exception of 3 % (slightly deviating from blank in a P3HB oxidation interval) suggesting small amount of P3HB was not degraded. However, the effects of P3HB addition on soil can already be observed. The water retention in phaeozem soil was affected by higher concentrations (all three) of P3HB added. Water was likely to evaporate in lower temperatures, suggesting that P3HB degradation caused water to less bound to soil and to less infiltrate into the pores of the soil. What is more, for 0.5 % P3HB added, slight change in the interval 300 °C to 500 °C occurred (typical for degradation of labile fractions of carbon, nitrogen and labile and stable organic matter) [284]. The weight loss was significantly lower, than in other cases (especially compared to our blank sample), this means, that nutrients were drawn from the soil and hence can lead

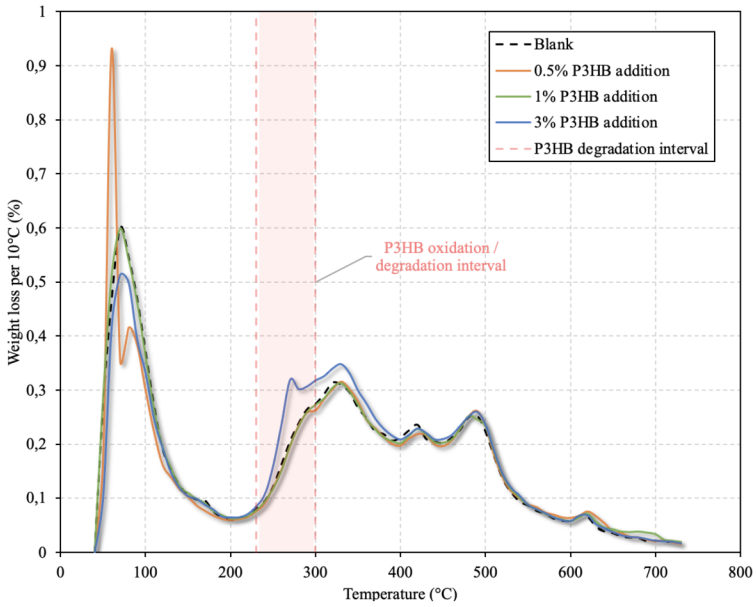

Figure 33: Weight loss of contaminated chernozem soil samples as a function of temperature.

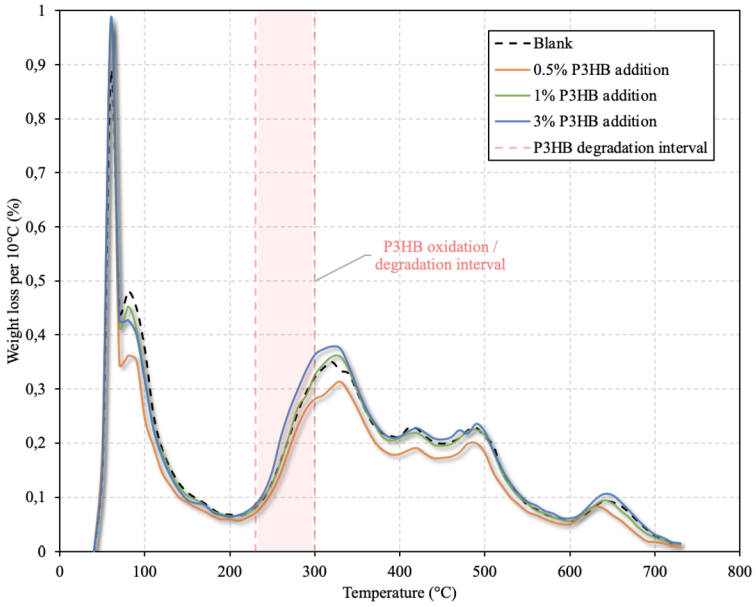

Figure 34: Weight loss of contaminated phaeozem soil samples as a function of temperature.

to a confirmation of a priming effect (however, this method is not usually applied). This trend can also be seen in other soils, however it is not so pronounced. Overall, the soil organisms degraded this soil the easiest (even higher concentrations were nearly degraded, see interval of P3HB oxidation).

And again, the cambisol soil (see Figure 35) yield strikingly different results. The weight loss was in general much lower than for chernosols (the total carbon content was much lower than in previous cases). It can be seen that water retention in case of cambisol was much lower. Further, as in case of chernozem, the P3HB affected soil's water retention only in case of 0.5 % P3HB addition. Other concentrations of P3HB added were nearly not affecting

this soil. It is also visible that in the case of cambisol soil, higher concentration of P3HB did not degrade well because a large amount remained to be degraded.

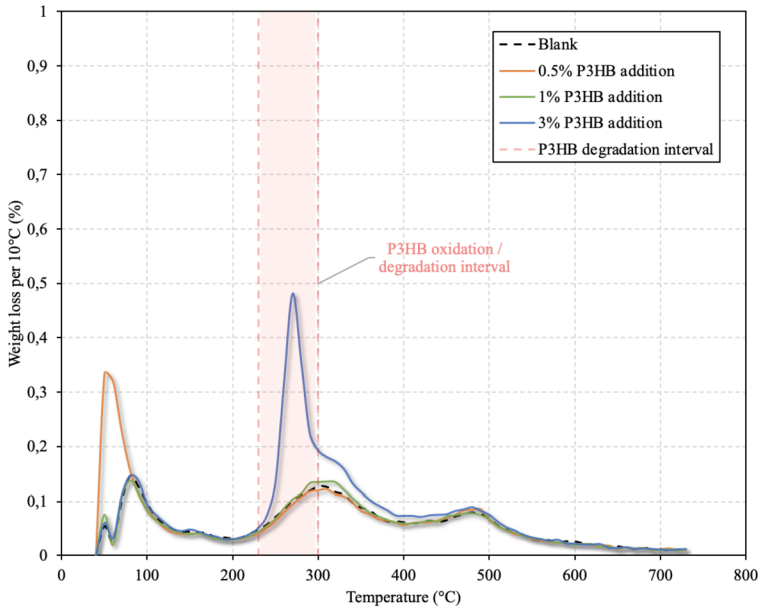

Figure 35: Weight loss of contaminated cambisol soil samples as a function of temperature.

Altogether, lower concentration of P3HB (0.5 %) had negative effect on water retention in the soil. It was more difficult to retain water in the soil and hence it might cause quicker evaporation. The impact was decreasing with increasing concentration. The cambisol soil was an exception. Here, the water retention was only affected by 0.5 % P3HB added. There was also a slight deviation in interval of 300 °C to 500 °C occurred. It was linked to degradation of labile fractions of carbon, nitrogen and phosphorus and further for labile and stable organic matter. The theory is, that this may indicate a priming effect.

6.2.2 Enzymatic assays

Soil enzyme production is dependent on soil properties, energy and nutrient availability [272, 285]. This was observed in our measurements of five key enzymes: arylsulfatase, urease, phosphatase, NAG and glucosidase. Their main functions were discussed before. In all cases, there was a significant response to bioavailable carbon in form of P3HB, compared to our control treatment (blank sample). This increase in microbial activity is expected given that P3HB is a common storage compound produced by a wide range of microorganisms, particularly in response to nitrogen deficiency and cold stress [272, 286]. In addition, the enzyme production after addition of P3HB to soil, was compared with cellulose samples (1 % of cellulose addition). The comparison of P3HB with cellulose was crucial because cellulose is naturally present in the cell walls of plants (therefore it is gradually added to the soil) and hence simulates the way in which microorganisms cope with substrate addition. Furthermore, the combination of both P3HB and cellulose addition were studied. However, the results for cellulose mixed with P3HB were not considered in most cases, because the degradation was not complete. Overall, the results of each enzyme activity were plotted separately (see Figure 36) and for better understanding of interrelations, each enzyme was plotted in 3D graph altogether with all soil types (see Figure 37). For more of

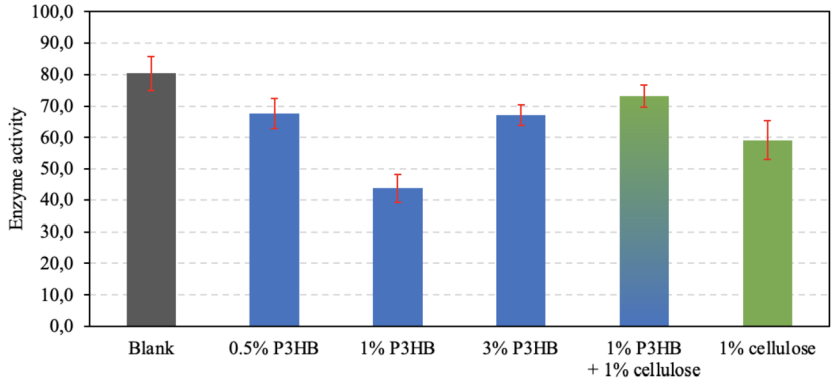

Figure 36: Glucosidase activity (in $\mu g \cdot g^{-1} \cdot h^{-1}$) for phaeozem soil samples with addition of P3HB and cellulose (blank sample represents treated control sample).

the detailed graphs see appendices (page 57 et seq). In this chapter, enzymatic activities are observed to understand their changes after P3HB addition. Due to lack of chernozem soil, the enzymatic analysis was not preformed in one case (see missing data for chernozem soil before degradation in 3D figures). However, based on the common trend with phaeozem soil, the results are expected to be similar. The results for samples with 3 % P3HB were expected to be affected by non-termination of degradation.

Glucosidase activity (see Figure 37) serves as a mean of carbon acquisition investment and decomposition [287]. Based on our results, the glucosidase activity increased in both chernosols after P3HB addition. This suggests that the addition stimulated degradation of other common polymers, such as cellulose and hence caused a positive priming effect. In other words, more enzymes were produced to cope with the substrate. In contrast, the addition of P3HB to cambisol soil caused increase in enzymatic activity, however this increase was not higher that in case of treated sample and hence retardation of decomposition of soil organic matter occurred (negative priming effect). This might be due to a production of a different microorganism, that took over the degradation of soil organic matter. Different soil properties might be also more hostile to some organisms

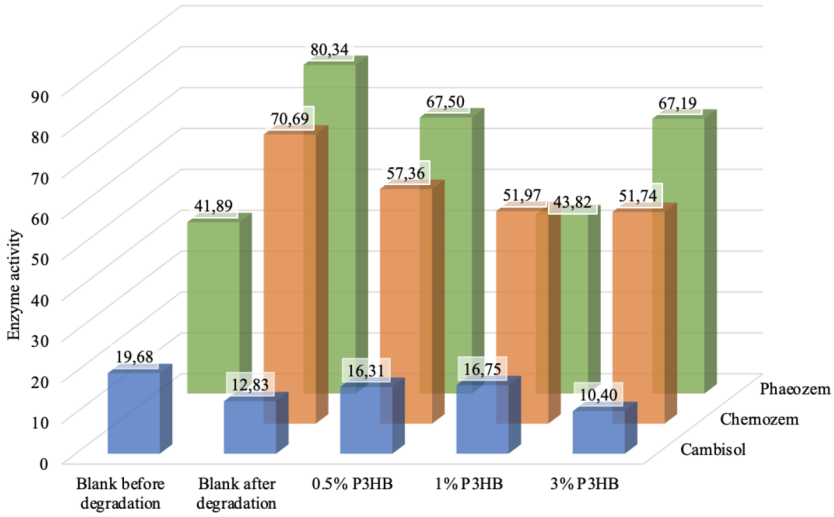

Figure 37: Glucosidase activity (in $\mu g \cdot g^{-1} \cdot h^{-1}$) for all soil samples with addition of P3HB.

and thus the addition of P3HB might have caused shift in a community (glucosidase is produced mostly by fungi). The addition of cellulose did not have a large effect on glucosidase activity (see appendices). The results differed slightly from the treated blank sample based on the soil type, however the deviation was not significant. Addition of P3HB and cellulose simultaneously, didn't have much of an impact on soils, except for cambisol soil, where the level of enzyme activity remained the same as in case of samples before degradation.

Soil arylsulfatase (see Figure 38) affects the acquisition of organic sulphur and thus the soil sulphur cycling. In the Figure it was observed, that the activity of all soils increased compared to treated blank sample. In case of chernosols, the activity even increased compared to non-treated blank sample (see blank before degradation; the chernozem soil was expected to follow the same trend as the phaeozem). On the contrary in case of cambisol soil, the activity decreased. However, the enzymatic activity after P3HB addition was still significantly higher than in case of treated sample and hence still indicates an increase in enzymatic activity. In addition, non-degraded samples had visibly lower arylsulfatase activities than degraded ones (see 3 % compared to others). This was probably due to various rates of reaction. While degrading P3HB, several microorganisms are involved. They decompose organic matter through a process called 'substrate succession'. During this process, the composition of the decomposing community gradually changes, as does the quality of the decomposed substrate. Hence, if the substrate isn't degraded, some enzymes might not have been produced yet (or not in such an amount). Overall, the results prove increased acquisition of soil organic sulphur and hence negative effect on soil organic matter (higher sulphur acquisition). In addition, arylsulfatase was not significantly affected by cellulose addition (see appendices).

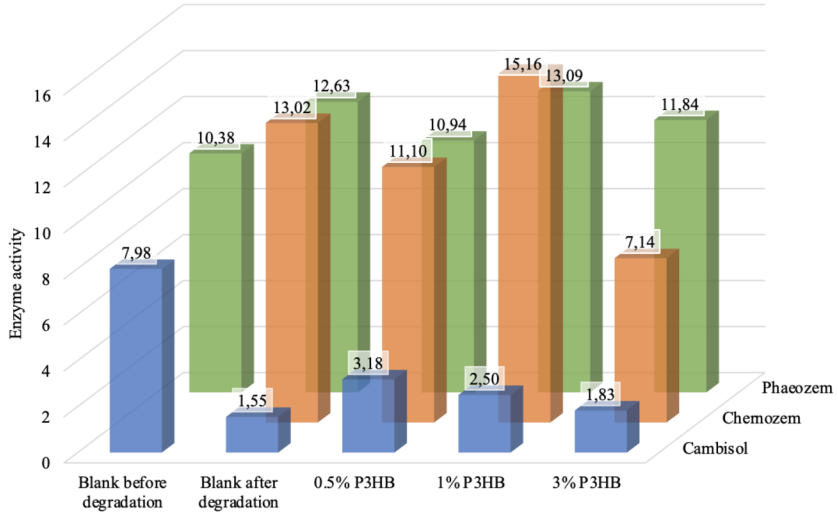

Figure 38: Arylsulfatase activity (in $\mu g \cdot g^{-1} \cdot h^{-1}$) for all soil samples with addition of P3HB.

NAG (see Figure 39) catalyzes chitin hydrolysis. It was studied, because of its importance in carbon and nitrogen cycling (it participates in chitin conversion to amino sugars, which are major sources of mineralizable N in soils) [88]. In other words, it was studied to understand nitrogen mineralization in soils after P3HB addition. Our results had reversed overall trend to the previous enzymes. The chernosols have much lower NAG

activities compared to both blanks (before and after degradation; the chernozem's trend was expected to follow the same trend as phaeozem's). This is likely for fertilized soils. On the contrary, the enzymatic activity significantly increased for cambisol. This was due to its nature. The microorganisms needed to obtain more nitrogen to balance the carbon income and therefore produced more NAG. This need of nitrogen was increasing with concentration. The only exception was 3 % concentration of P3HB in cambisol soil, where the reason of sudden decrease might have the same cause as mentioned in the previous paragraph (substrate succession). Overall, it can be seen that microorganisms required higher amount of nitrogen to degrade higher concentrations of P3HB.

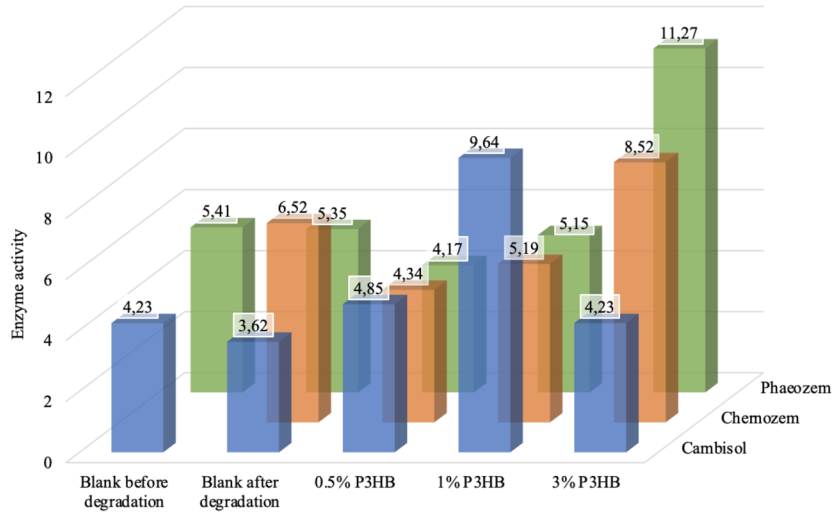

Figure 39: NAG activity (in $\mu g \cdot g^{-1} \cdot h^{-1}$) for all soil samples with addition of P3HB.

Another enzyme that serves as a source of nitrogen is urease (see Figure 40). It was studied due to its involvement in nitrogen mineralization to ammonium nitrogen (which is easily accessible to plants). As in the previous case, the urease activity was gradually increasing with concentration. In both chernosols, the treated blank sample had the same

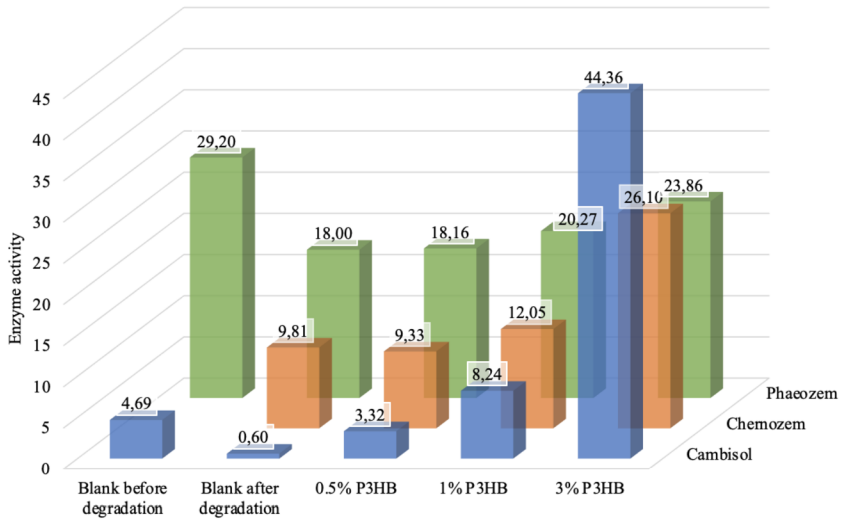

Figure 40: Urease activity (in $\mu g \cdot g^{-1} \cdot h^{-1}$) for all soil samples with addition of P3HB.

urease activity as sample with 0.5 % P3HB. On the other samples the lack of nitrogen shown and hence the activity was higher. In case of cambisol soil, the activity increased gradually as well. The urease activity was even nearly 10 times higher than control sample in case of 3 % P3HB, suggesting increased microbial activity due to a lack of nitrogen (microorganisms tried to obtain it by urease production). Nitrogen limitation in both cases (NAG and urease) was connected to microbial nitrogen immobilization due to stimulated microbial growth after carbon supply from P3HB [272]. The enzyme involved in nitrogen mining is dependent on soil properties, microbial community and P3HB concentration. In both cases (NAG and urease), the enzyme activities were affected by addition of 1 % cellulose (see appendices) about the same as in case of 1 % P3HB.

Phosphatase (see Figure 41) is essential for phosphorus cycling in phosphorus-deficient soils. It is produced to provide the microorganisms with nutrients for growth and enzyme synthesis [287]. From our results, it is visible that all soils had similar behaviour; with increasing concentration the production of phosphatase increased. Compared to non-treated samples, the activity of the treated ones was either decreasing (cambisol) or stagnating (chernosols; the chernozem's results were expected to be similar to phaeozem). From the results it is visible, that enzymes in chernosols lacked phosphorus for P3HB processing with increasing concentration. This trend was again disturbed by the highest concentration (3 % P3HB), which was explained in previous paragraphs. The results for cambisol soil suggested, that microorganisms had relatively enough phosphorus while processing 0.5 % P3HB and hence needed to produce less phosphatase. The concentration of 1 % P3HB was higher than treated blank and thus according to this results the need of microorganisms to obtain phosphorus was higher. In addition, phosphatase was affected by addition of 1 % cellulose (see appendices) about the same as in case of 1 % P3HB.

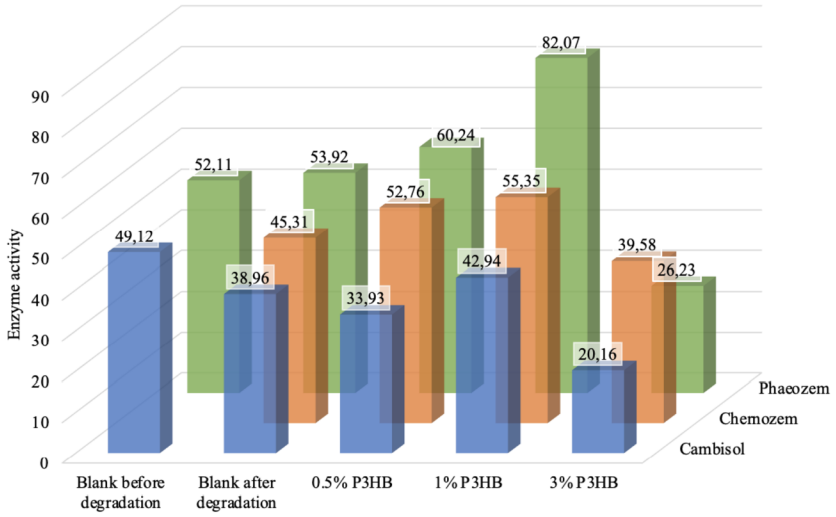

Figure 41: Phosphatase activity (in $\mu g \cdot g^{-1} \cdot h^{-1}$) for all soil samples with addition of P3HB.

Chapter 7

Conclusion

The aim of this work was to investigate the influence of bioplastics' biodegradation on soil quality. To do so respirometry, elemental analysis, thermogravimtery and enzymatic assays were applied. The study was held in three model soils; two chernosols (chernozem and phaeozem) and one cambisol. As a representative of biodegradable plastics P3HB was selected.

Respirometry and elemental analysis were combined to understand the effect of P3HB on carbon and partially even nitrogen cycling. Respirometry discovered, that higher amount of carbon dioxide is evolved during P3HB degradation than the calculated theoretical value. This amount was significant enough to state that during P3HB degradation even soil organic matter decomposed. This is a principle of the priming effect (however, this is not enough information to state it for sure and hence other experiment took place). The duration of respiration differed based on soil type and P3HB concentration added. Lower concentrations were degraded in much shorter period. On the contrary, the highest concentration (3 %) was not degraded during 9 months of this experiment. The experiments with higher concentrations are therefore recommended for further investigation to find out, whether they cause the degradation process to stop completely. Compared to elemental analysis, we indicated the mutual relations in carbon and nitrogen. As expected, the nitrogen and carbon concentrations in soil were decreasing after P3HB addition. This was another evidence of the priming effect as the microorganisms were forced to obtain nitrogen from soil to continue processing P3HB. Based on the evidence, it was additionally possible to indicate a specific type of the priming effect. When comparing contaminated samples with blank samples before and after treatment, it was found that both chernosols were affected by a positive priming effect and, conversely, cambisol soil was affected by a negative priming effect. In principle, both positive and negative priming effects yield higher volumes of CO_2 evolved compared to non-contaminated soil, however, positive priming effect was more demanding on availability of other nutrients. Such a limitation of nitrogen might cause either change in intrinsic properties of microorganisms hydrolytic enzymes or shift in the soil microbial community (along with types of enzymes produced) [272, 288]. Therefore, it is recommended to include experiments with the structure of soil bacterial communities (composition and diversity) to further investigate the priming effect. Furthermore, as priming effect caused by the decomposition of carbon-rich compounds affected nitrogen (and probably even phosphorus) immobilization, the plant growth might have been negatively affected (increased competition between plants and soil microorganisms for nutrients) [272, 289, 290, 291].

The third method utilized was thermogravimetry. It helped to reveal/confirm which samples were degraded and the effect of P3HB on water retention. As already suggested by respirometry, the degradation was not complete in any of our soil samples with addition of 3 % P3HB. On the contrary, samples with addition of 0.5 % and 1 % P3HB were fully degraded. Furthermore, it was observed that P3HB addition negatively affected water retention. It can be seen, that degradation of all samples with 0.5 % P3HB had significant impact in thermal interval of 0°C – 100°C. In this interval a weakly bound water is evaporated and hence it shows that P3HB addition causes fewer interactions of soil with water. This reaction was highly affected by soil type and P3HB concentration. Degradation of all concentrations of P3HB in chernosol soils highly affected this 'water loss' zone and on the contrary cambisol soil was affected only in samples with 0.5 % P3HB. The probable cause of this phenomenon was the retardation of soil organic matter degradation. It was linked to degradation of labile fractions of carbon and nitrogen and further for labile and stable organic matter. The theory is, that this may indicate a priming effect. Any other change in physico-chemical properties is likely to happen close to the microplastic particles and therefore, further investigation around these 'hot spots' is advised [272].

Enzymatic activities were studied in the last method. In all cases, there was a significant response to bioavailable carbon in form of P3HB, compared to our control treatment (blank sample). An increase in enzymatic activity was due to nutrient deficiency and hence, based on this (and previously mentioned methods) we conclude that increased microbial activity caused priming effect. The difference between negative and positive priming effect was observed even when measuring enzymatic activities, especially in case of glucosidase enzyme. Here, the enzymatic activities were significantly higher compared to treated blank sample. In case of both chernosol soils, the activities were even higher than original values, suggesting rapid decomposition of soil organic matter. The cambisol soil had slightly higher activities compared to treated blank sample but still lower values than blank before degradation. This suggests that partial retardation of soil organic matter decomposition occurred (negative priming effect). Enzymatic activities were decreasing with increasing concentration, which also had an effect on other physio-chemical properties (mentioned in previous paragraph). From the results, the tendency to obtain other essential elements was also visible. The acquisition of organic sulphur (using arylsulfatase) also increased. The trend was similar to glucosidase and hence confirmed our priming effect theory as well. Urease and NAG, as sources of mineralizable nitrogen in soils, also showed changes in enzymatic activities. The activity of urease increased compared to treated blank samples (especially in higher concentrations). NAG activity was especially high for cambisol samples. The nitrogen was either available freely in soil (cambisol soils) or was acquired via mentioned enzymes (cambisol soil). The last enzyme, phosphatase, again showed increased phosphorus acquisition in case of chernozem soils and a retardation of soil organic matter decomposition in case of cambisol soil.

Last but not least, we conclude that P3HB degradation in soils might have serious impact on water retention and carbon and nutrient cycling. These effects may hence lead to long-term impact on a range of soil ecosystem services [272, 292]. Consequently, P3HB microplastics negatively affect plant growth due to lack of nutrients. And with this in mind any application should be taken into consideration.

List of Abbreviations

AAS atomic absorption spectroscopy. 14

AEPAT AgroEcosystem Performance Assessment Tool. 20

Al aluminium. 12

AMF Arbuscular mycorrhizal fungi. 18

ASTM American Society for Testing and Material. 33

ATP adenosine triphosphate. 15

B boron. 13

BD Bulk Density. 12

C carbon. 11

Ca calcium. 12

CEC Cation-Exchange Capacity. 12

CEN European Committee for Standardisation. 33

CFE chloroform fumigation extraction. 15

CFI chloroform fumigation incubation. 15

CIC DIN-Gerprüft Industrial Compostable and Consorzio Italiano Compostatori. 32

Cu copper. 13

DOM dissolved organic matter. 41

DTPA Diethylenetriaminepentaacetic acid. 15

EC Electrical Conductivity. 13

EDTA Ethylenediaminetetraacetic acid. 14

FAO Food and Agriculture Organization of the United Nations. 6

Fe iron. 13

ISO International Standardisation Organisation. 20

IUSS International Union of Soil Sciences. 6

K potassium. 12, 13

KOH potassium hydroxide. 48

MDS Minimum Data Set. 21

Mg magnesium. 12

Mn manganese. 12

Mo molybdenum. 13

N nitrogen. 13

NAG beta-1,4-N-Acetyl-glucosaminidase. 17

P phosphorus. 13

P3HB poly-(R)-3-hydroxybutyrate. 30

PC-CRFs polymer-coated controlled-release fertilizers. 35

PCA Principal Component Analysis. 21

PHA polyhydroxyalkanoates. 30

POPs persistent organic pollutants. 39

PVC polyvinyl chloride. 40

S sulphur. 13

SIR substrate-induced respiration. 15

SMAF The Soil Management Assessment Framework. 19

SOC Soil Organic Carbon. 13

SOM Soil Organic Matter. 13

SQI Soil Quality Index. 20, 21

TCA tricarboxylic acid cycle. 30

TPS thermoplastic starch. 42

WHC Water Holding Capacity. 12

WRB World Reference Base for Soil Resources. 6

Zn zinc. 13

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