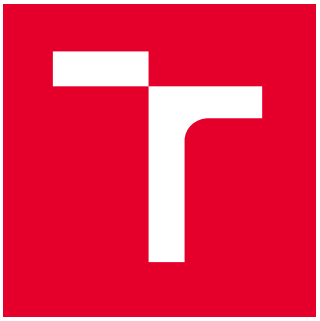

VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

BRNO UNIVERSITY OF TECHNOLOGY

FAKULTA CHEMICKÁ

FACULTY OF CHEMISTRY

ÚSTAV CHEMIE A TECHNOLOGIE OCHRANY ŽIVOTNÍHO PROSTŘEDÍ

INSTITUTE OF CHEMISTRY AND TECHNOLOGY OF ENVIRONMENTAL PROTECTION

STUDIUM DEGRADAČNÍCH PROCESŮ BIOPLASTŮ

STUDY ON DEGRADATION PROCESSES OF BIOPLASTICS

DIZERTAČNÍ PRÁCE
DOCTORAL THESIS

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VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ
FAKULTA CHEMICKÁ

Zadání dizertační práce

Ústav:	Ústav chemie a technologie ochrany životního prostředí	Akademický rok: 2022/23
Student:	**Ing. Jakub Fojt**	
Studijní program:	Chemie a technologie ochrany životního prostředí	
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Vedoucí práce:	**prof. Ing. Jiří Kučerík, Ph.D.**	

Název dizertační práce:

Studium degradačních procesů bioplastů

Zadání dizertační práce:

Zpracování rešeršní práce na téma analýza mikroplastů v půdách, bioplasty, biodegradace.
Vývoj metody pro analýzy mikrobioplastů v půdě.
Ověření vlivu mikrobioplastů na půdní orgacnikou hmotu.
Ověření vlivu biodegradace bioplastů na půdní kvalitu a růst rostlin.

Termín odevzdání dizertační práce: :

Ing. Jakub Fojt student	prof. Ing. Jiří Kučerík, Ph.D. vedoucí práce	prof. Ing. Jiří Kučerík, Ph.D. vedoucí ústavu
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Abstrakt

Biodegradabilní bioplasty jsou v současné době vyvíjeny jako ekologičtější náhrada za konvenční plasty, která má najít uplatnění v zemědělství nebo v obalových materiálech. Se vzrůstající produkcí těchto výrobků poroste i pravděpodobnost jejich průniku do životního prostředí. U biodegradabilních materiálů se předpokládá jejich rychlá biodegradace, čímž by měly snižovat množství mikroplastů v životním prostředí. Biodegradabilita je v současné době určována pomocí biodegradačních testů, jejichž parametry jsou definovány velmi obecně a často neodrážejí reálné podmínky, ve kterých se bude bioplast rozkládat v životním prostředí. O osudu a vlivu těchto částic v různých ekosystémech (především v půdě, kde předpokládáme nejvyšší kontaminaci) je toho v současné době známo velmi málo. Proto se tato disertační práci zabývá kritickým zhodnocením biodegradačního testu v půdě dle normy ISO 17556:2012, který doplňuje následným stanovením reziduí bioplastu. K tomuto účelu je zde prezentován vývoj analytické metody ke stanovení mikrobioplastů polyhydroxybutyrátu a polymléčné kyseliny založená na metodách používaných ke stanovení konvenčních mikroplastů. Dále je zde také diskutován vliv mikrobioplastů na půdu se zaměřením na efekty, které mohou představovat problémy v zemědělství. Je zde popsán vliv mikrobioplastů na abiotické (chování půdní vody, struktura půdy) a biotické (změna půdní diverzity, cyklus nutrientů) vlastnosti půdy ihned po jejich vstupu do půdy. Taktéž je zde studován vliv biodegradace a dlouhodobého působení mikrobioplastů na půdní organickou hmotu, obsah nutrientů a půdní mikrobiální diverzitu (popsanou pomocí aktivity 5 hlavních enzymů podílejících se na cyklech uhlíku, dusíku a fosforu). V poslední kapitole jsou pak demonstrované vybrané problémy, se kterými je možné se setkat při aplikaci bioplastových produktů v zemědělství, kdy může dojít k rychlejšímu růstů hub (plísní) přirozeně se vyskytujících v půdě. Výsledkem této disertační práce je širší pohled na problematiku bioplastů v životním prostředí, který může sloužit posouzení vhodnosti aplikace bioplastových produktů současné generace v zemědělství a možného nakládání s bioplastovým odpadem.

Klíčová slova

bioplasty, mikrobioplasty, mikroplasty, biodegradace, půda, půdní organická hmota, plastisféra, polyhydroxybutyrát

Abstract

Biodegradable bioplastics are currently developed as a more environmentally friendly replacement for conventional plastics to be used in agriculture or packaging materials. As the production of these products increases, so will the likelihood of introducing them in the environment. Biodegradable materials are expected to biodegrade rapidly, thereby they should reduce the number of microplastics in the environment. Biodegradability is currently determined by biodegradation tests, the parameters of which are defined in very general terms and often do not reflect the actual conditions under which the bioplastic will degrade in the environment. There is a lack of knowledge about the fate and impact of these particles in different ecosystems (especially in soil, where contamination is expected to be highest). Therefore, in this dissertation, we critically evaluate the ISO soil biodegradation test and extend it with a subsequent determination of bioplastic residues in soil. For this reason, an analytical method for the determination of polyhydroxybutyrate and polylactic acid microplastics based on methods used for the determination of conventional microplastics. The effect of microbioplastics on soil is also discussed, focusing on the effects that may pose problems in agriculture. The influence of microbioplastics on abiotic (soil water properties, soil structure) and biotic (soil diversity, nutrient cycling) soil properties immediately after their entry into the soil are described. The effect of biodegradation and long-term presence of microbioplastics in soil on soil organic matter, nutrient content, and soil microbial diversity (described by determining the activity of 5 key enzymes involved in carbon, nitrogen, and phosphorus cycling) are also studied. The last chapter demonstrates the potential problems that can be encountered when bioplastic products are applied in agriculture, where the growth of fungi and molds naturally occurring in the soil can be accelerated. As a result of this dissertation, a comprehensive view of bioplastics in the environment is presented, which can serve to assess the suitability of the application of current generation bioplastic products in agriculture and the possible management of bioplastic waste.

Key words

Bioplastics, micro-bioplastics, microplastics, biodegradation, soil, soil organic matter, plastisphere, polyhydroxybutyrate

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Prohlášení

Prohlašuji, že jsem disertační práci vypracoval samostatně a že všechny použité literární zdroje jsem správně a úplně citoval. Disertační práce je z hlediska obsahu majetkem Fakulty chemické VUT v Brně a může být využita ke komerčním účelům jen se souhlasem vedoucího práce a děkana FCH VUT.

……

Ing. Jakub Fojt

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OBSAH

Seznam použitých zkratek

Zkratka	Význam
ASAP	atmosférická sonda k analýze pevných částic
ASTM	Americká společnost pro testování a materiály – americká norma
ATR	zeslabená totální reflektance
CEN/TR	technická zpráva Evropského výboru pro normalizaci
DSC	diferenční kompenzační kalorimetrie
EN ISO	evropská norma Mezinárodní komise pro normalizaci
ESI/MS	hmotnostně-spektrometrická detekce s ionizací elektrosprejem
FID	plamenová ionizační detekce
FTIR	infračervená spektroskopie s Fourierovou transformací
HPLC	vysoceúčinná kapalinová chromatografie
IDMS	izotopické zřeďování s hmotnostní spektrometrií
IMS	spektrometrie iontové mobility
LC	kapalinová chromatografie
LOD	limit detekce
LOQ	limit kvantifikace
MIR	střední infračervená oblast
NIR	blízká infračervená oblast
P3HB	poly(3-hydroxymáselná kyselina)
PA	polyamid
PAN	polyakrylonitril
PBAT	poly(butylen adipát tereftalát)
PE	polyethylen
PES	polyester

PET	polyethylentereftalát
PHA	polyhydroxyalkanoáty
PLA	kyselina polymléčná
PMMA	polymethylmethakrylát
PP	polypropylen
PPC	polypropylen karbonát
PS	polystyren
PTT	polytrimethyléntereftalát
PVC	polyvinylchlorid
Py–GC/MS	pyrolýzní plynová chromatografie s hmotnostně-spektrometrickou detekcí
QTOF	analyzátor kvadrupól a doby letu
RDF	les náhodného výběru
RSD	relativní standardní odchylka
SDS	dodecylsulfát sodný
SOM	půdní organická hmota
TFA	trifluoroctová kyselina
TGA	termogravimetrie
UPLC	ultraúčinná kapalinová chromatografie
UV–VIS	spektrofotometrie v ultrafialové a viditelné oblasti

Seznam příloh

Příloha A

A critical review of the overlooked challenge of determining micro-bioplastics in soil

Autorův příspěvek: 70 %; literární rešerše, příprava manuskriptu, revize, definování pracovních hypotéz

Příloha B

A Simple Method for Quantification of Polyhydroxybutyrate and Polylactic Acid Micro-Bioplastics in Soils by Evolved Gas Analysis

Autorův příspěvek: 70 %; literární rešerše, vývoj analytické metody, provedení experimentů, vyhodnocení dat, grafické zpracování výsledků, příprava manuskriptu, revize

Příloha C

Influence of Poly-3-hydroxybutyrate Micro-Bioplastics and Polyethylene Terephthalate Microplastics on the Soil Organic Matter Structure and Soil Water Properties

Autorův příspěvek: 50 %; literární rešerše, vyhodnocení dat, grafické zpracování výsledků, příprava manuskriptu, revize

Příloha D

Effect of biodegradable poly-3-hydroxybutyrate amendment on the soil biochemical properties and fertility under varying sand loads

Autorův příspěvek: 20 %; příprava manuskriptu, revize, projektový management

Příloha E

Does the biodegradation of poly-3-hydroxybutyrate cause the degradation of soil organic matter?

Dosud nepublikovaný manuskript

Autorův příspěvek: 30 %; literární rešerše, vyhodnocení dat, grafické zpracování výsledků, příprava manuskriptu

Úvod

V roce 2016 bylo vyrobeno celosvětově 391 miliónů tun plastového materiálu [1]. Plasty jsou využívány především kvůli svým výborným užitným vlastnostem, mezi které patří například stabilita vůči chemikáliím, hydrolýze, mikroorganismům, světlu, teplu, nízká hustota a mechanické vlastnosti [2]. Některé tyto vlastnosti ale způsobují problémy při zpracování nebo likvidaci plastového odpadu. V důsledku toho pouze 9 % všech plastů, které byly za posledních 65 let vyrobeny, bylo zrecyklováno, 12 % bylo spáleno ve spalovnách a zbylých 79 % bylo uloženo ve skládkách. Zbytek se tedy nachází v přírodě [3]. Současné výzkumy odhadují, že v oceánech plave 5 biliónů plastových kousků vážících dohromady 250 miliónů tun a každý rok přibývá dalších 8 miliónů tun [4; 5].

V půdě jsou odhady koncentrace mikroplastů složitější, a to především kvůli komplikovanějšímu osudu mikroplastů v půdách, interakci s jednotlivými komponentami, silnému matričnímu efektu a problematické extrakci mikroplastů z půdy. Proto existuje v současnosti pouze limitované množství studií zaměřených na stanovení koncentrace mikroplastů v půdě [6]. Nicméně, některé odhady uvádějí, že koncentrace mikroplastů v terestrických ekosystémech může být 4–23krát vyšší než ve vodních [7].

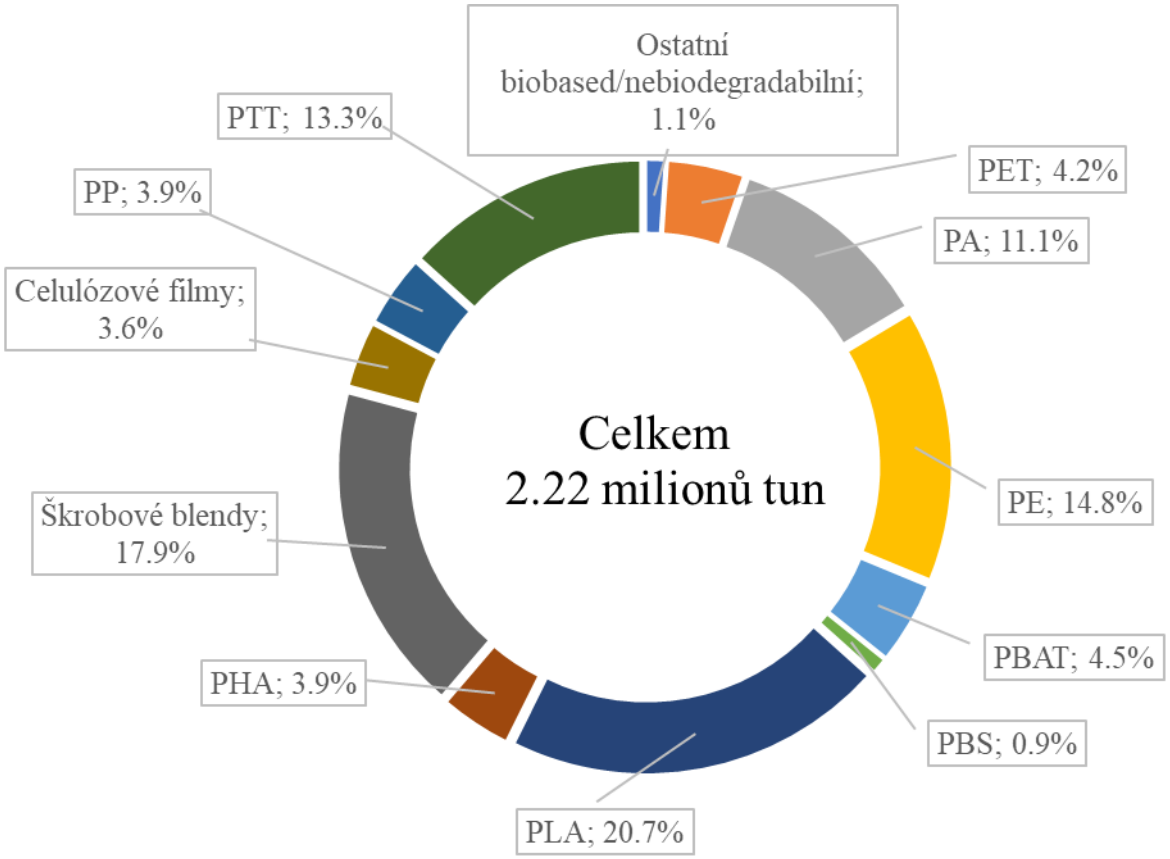
Předpokládá se, že konvenční (oil-based) plasty degradují velmi pomalu ve všech složkách životního prostředí [8], jejich postupný rozklad vede k tvorbě mesoplastů (velikost nejdelšího rozměru 1 až 10 mm), mikroplastů (1 až 1000 μm) a nanoplastů (1 až 1000 nm) [9]. Jakmile se mikroplast dostane do půdy, stává se součástí komplexní půdní organické hmoty a minerálních substituentů, díky čemuž se jeho perzistence ještě zvýší [10]. K transportu mikroplastů do půdy pak může dojít například atmosférickou depozicí [11], v půdě půdními živočichy, jakými jsou chvostoskoci [12] či žížaly [13], nebo aplikací čistírenských kalů a kompostů na zemědělské půdy apod. [14].

Obecně jsou mikroplasty rozdělovány na primární a sekundární. Primární mikroplasty jsou vyráběny záměrně a využívají se v kosmetice, čistících přípravcích a obráběcích směsích. Sekundární mikroplasty vznikají z plastů oděrem nebo rozpadem větších plastových předmětů nebo částic [15]. Kvůli tomu se v oceánech, ve sladkovodních ekosystémech a v půdách tyto částice akumulují, což vyvolává obavy z negativních vedlejších efektů [16; 17]. Již bylo prokázané, že nebezpečné jsou například svojí schopností adsorbovat a koncentrovat nebezpečné chemické látky z vody, jako jsou léčiva nebo pesticidy, a tím sloužit jako nosiče těchto sloučenin do těl organismů [18]. Při vysokých koncentracích mikroplastů ve vodních ekosystémech byla prokázána i chronická reprodukční toxicita u mikroorganismů, vyšší mortalita, potíže s příjmem potravy, endokrinní disrupce, oxidativní stres a poruchy imunity a nervového systému [19; 20; 21; 22]. Dále rostou obavy o možných efektech mikroplastů na lidi, protože tyto částice byly detekovány v lidském těle [23; 24]. Obecně platí, že s klesajícím poloměrem částic roste reaktivita a aktivní povrch, proto je možné předpokládat u nanoplastů vyšší biologickou aktivitu a schopnost adsorpce. Nanočástice plastů se také vyznačují

schopností pronikat buněčnou stěnou, čímž dochází k transportu nasorbovaných látek přímo do buněk [25].

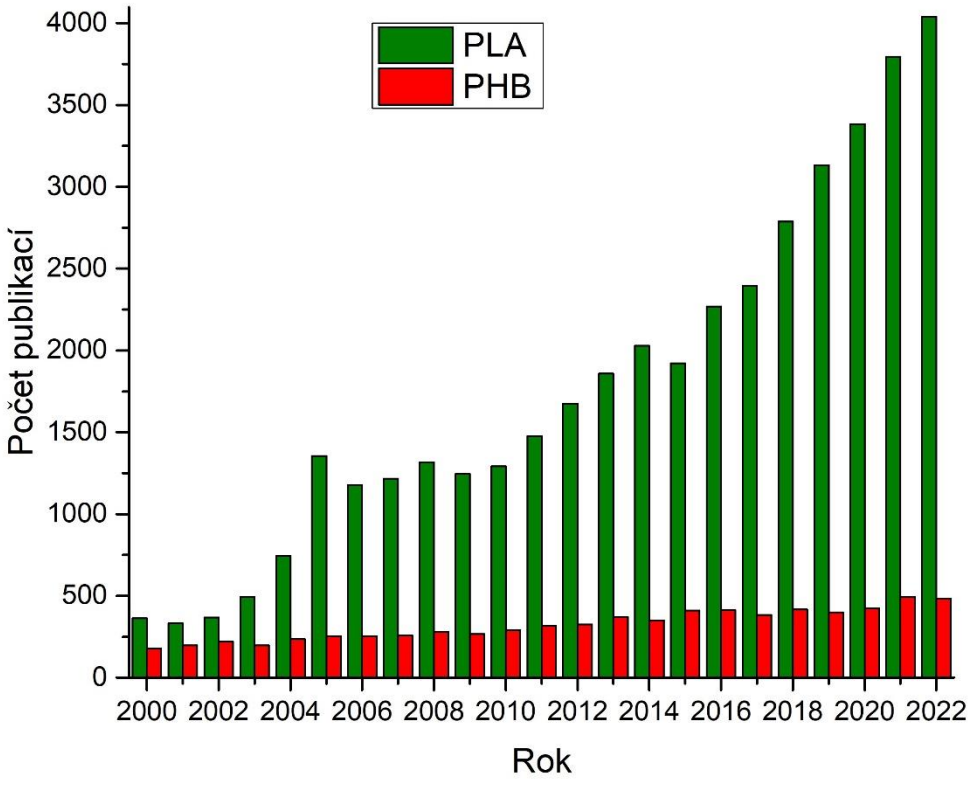
Na problémy s recyklací a likvidací plastového odpadu Evropská komise zareagovala v lednu roku 2018, kdy zveřejnila svoji vizi, kde mimo podpory recyklace plastů uvedla možné využití a rizika použití biodegradovatelných plastů [26; 27]. Evropská chemická agentura se také pokouší omezit množství mikroplastů v kosmetických přípravcích [28]. Mezi biodegradovatelné plasty patří obecně takové, u kterých dochází za stanovených podmínek (teplota, pH, vlhkost apod.) k rozpadu působením mikroorganismů. Rozpad takového plastu je doprovázen vznikem vody, methanu a/nebo CO_2 a biomasy. Mezi první obory využívající bioplasty se řadí medicína, která využívá biodegradabilní plasty již od konce 80. let a kde slouží jako specifické nosiče účinné látky, jako náhrada lidských tkání a chirurgické implantáty [29]. Velkou popularitu získaly bioplasty také v 3D tisku [30]. Jako materiály jsou biodegradabilní plasty slibná alternativa ke konvenčním, většinou nebiodegradabilním plastům [26]. Proto se biodegradabilní bioplasty začínají využívat pro výrobu obalů na jídlo, mulčovacích folií, geotextilií, tašek na odpad, kosmetiky a oblečení [29]. V roce 2021 bylo celosvětově vyprodukováno 2,22 miliónů MT biobased plastů, z čehož 51,5 % byly biodegradabilní bioplasty (Obrázek 1) [31]. Předpokládá se, že do roku 2027 se toto množství zvýší až na 6,30 milionů MT [32].

Nicméně, k úplné biodegradaci bioplastů dochází pouze za velmi specifických podmínek, mezi nejdůležitější pak patří vhodná teplota, pH a optimální vlhkost, bohaté prostředí na mikroorganismy a dostatek živin [33]. Jednotlivá prostředí, ve kterých dochází k biodegradaci, jsou od nejagresivnějšího kompostu (teplota, bakterie, houby) > půda (houby, bakterie) > sladká voda (bakterie) > až po mořskou vodu („zředěné bakterie“). Z pořadí vyplývá, že optimální a kontrolovatelné podmínky pro biodegradaci splňuje pouze průmyslový kompost. V reálných ekosystémech nejsou podmínky biodegradace často splnitelné, což vede ke značnému zpomalení biodegradace. Jedním z prvních nejdůležitějších kroků degradace je biofragmentace, která je závislá na množství enzymů. Enzymy jsou produkovány intracelulárně, a proto pro jejich exkreci jsou nutné poměrně specifické podmínky. V případě, že nejsou splněny, tak vznikají biodegradabilní mikroplasty (mikro-bioplasty) paradoxně mnohem rychleji u biodegradovatelných plastů než u běžných (oil-based) plastů [34]. Osud takto vzniklých mikroplastů [6] ale je, na rozdíl od mikroplastů z konvenčních plastů studován velmi málo [10]. První články týkající se této problematiky naznačují, že mikro-bioplasty mohou taktéž představovat nebezpečí pro životní prostředí [35; 36; 37].



Obrázek 1: Světová produkce biobased plastů v roce 2021 [32], polyhydroxyalkanoáty (PHA), poly(butylen adipát tereftalát) (PBAT), kyselina polymléčná (PLA), polybutylensukcinát (PBS), polyethylen (PE), polyamid (PA), polyethylentereftalát (PET), polytrimethylén tereftalát (PTT)

Proto, vzhledem k rostoucí produkci a využití bioplastů, je třeba toto téma otevřít a prostudovat. Prvním krokem pro rozhodnutí o přítomnosti mikroplastů jakéhokoliv typu v životním prostředí a následně o jejich možném vlivu na něj je jejich jednoznačná identifikace a kvantifikace. Nicméně, jak je poukazováno v dalších částech této práce, v současné době neexistují analytické metody pro stanovení biodegradabilních mikroplastů, ani ve vodách, ani v půdách případně v kompostu [38]. Jak je dále diskutováno, lze se inspirovat, alespoň metodicky, v pracích zabývajících se analýzou konvenčních mikroplastů. Jak už bylo řečeno dříve, největší výzvu představují analýzy mikroplastů v půdách, proto je tato práce zaměřena hlavně na tyto analýzy. Jako modelové představitele biodegradabilních plastů byly vybrány polyhydroxyalkanoáty a kyselina polymléčná, o které v posledních 20 letech vzrůstá zájem, což se projevuje i vzrůstajícím počtem publikací, které se jimi zabývají (Obrázek 2). Tato práce se nezabývá bioplasty ze škrobu a modifikovaných škrobů (byť představují cca 18 % všech bioplastů), protože zatím jsou poly(3-hydroxybutyrát) P3HB a PLA považovány za polymery s vyšším potenciálem ke tvorbě mikroplastů.



Obrázek 2: Množství publikací zabývající se PLA a PHB v databázi Scopus [39]

Současný stav poznání

1 Plastové zemědělství

Od té doby, kdy se začaly globálně používat v zemědělství mulčovací folie, vzrostlo riziko vzniku plastových reziduí v zemědělské půdě, které se s největší pravděpodobností rozpadají na mikroplasty [40]. Nejčastěji používanými plasty v zemědělství jsou plastové filmy, folie, netkané textilie pro mulčování, kolíky a trubky pro tunelové struktury, stínící sítě chránicí proti kroupám, klíčící nádoby, připínáčky a svorky pro uchycení rostlin anebo biodegradabilní kryty nebo disky pro semena. Přibližně 60 % plodin by v současné době nebylo možné efektivně pěstovat bez použití plastů v zemědělství. Na druhou stranu 1 ha zemědělské půdy může ročně přispět k tvorbě 80 až 200 kg mikroplastů za jeden rok [41]. Kvůli obavám z rostoucího množství plastového odpadu na polích roste popularita biodegradabilních plastů v zemědělství. Jejich biodegradabilita zřídka dosahuje 100 % [42] (anebo není možné zaručit jejich biodegradabilitu především z důvodu kolísající teploty a vlhkosti během roku) a tím pádem jsou podezřelé ze zhoršování kontaminace zemědělských půd mikroplasty s tím, že mikro-bioplasty vykazují podobné nebo stejné ekotoxikologické a sorpční vlastnosti a jsou považovány za perzistentní polutanty. Současným trendem v zemědělství je nahrazování konvenčních plastů

biodegradabilními [43], což by mělo ideálně vést kromě žádaného bezodpadového nebo téměř bezodpadového zemědělství také ke snížení půdní vlhkosti a zadržování vody pod mulčovací folií, čímž je docíleno snížení nebezpečí napadení houbovým onemocněním a také k omezení hypoxie kořenů [41]. Při tom se předpokládá možný vznik mikroplastů z těchto bioplastů [34].

2 Biodegradabilní polymery často používané v zemědělství či určené pro kompostování

V této práci byla provedena literární rešerše pomocí Clarivate Analytics Web of Science a Google Scholar se zaměřením na dva biopolymery s největším potenciálem uplatnění v zemědělství (P3HB a PLA), na jejich využití a jejich potenciální analytické metody vhodné k jejich stanovení v různých substrátech se zaměřením na komplikované a komplexní matrice jako je například půda. Pro porovnání byly vyhledány analytické metody vhodné ke stanovení konvenčních mikroplastů.

Mezinárodní unie pro čistou a užitnou chemii definuje biodegradabilní polymery jako „Polymery, které podléhají biodegradaci způsobené biologickou aktivitou, což se projevuje snižováním jejich hmotnosti“ [44]. Tato definice by mohla označit jako biodegradabilní polymery i polymery, které jsou pouze rozpustné ve vodě. Proto při definici biodegradabilních plastů vyžadují některé další organizace i úplnou mineralizaci plastu na oxid uhličitý, vodu a biomasu (CEN/TR 15351:2006) [26]. Tuto definici potom využívají pro normování testů biodegradability ve sladké a slané vodě, v půdě a kompostu různé národní a mezinárodní organizace jako je například Mezinárodní organizace pro standardizaci u norem EN ISO 14851:1999, EN ISO 17556 [45] nebo ASTM D 5338 a ASTM D 6400. Kombinací více relevantních norem vznikají certifikáty, které by měly zaručovat biodegradovatelnost certifikovaného materiálu jako je například belgický Vincotte OK Biodegradable nebo německý DIN CERTCO [46]. Cílem těchto certifikátů je eliminace slabin jednotlivých norem. Mimo informace o biodegradovatelnosti testovaného materiálu, která je získána kombinací biodegradačních testů v různých relevantních ekosystémech, je testována nebezpečnost produktů biodegradace pro životní prostředí pomocí ekotoxických testů. Také udávají formu a jednotlivé body, které má mít protokol výsledků testování. Tyto certifikáty naopak neberou v potaz různé podmínky, které jsou dány geografickou polohou místa, kde je produkt používán, a také změnou ročních období. Také mimo ekotoxikologické testy neřeší nijak charakteristiku produktů biodegradace.

Mezi vysoce biodegradabilní a biokompatibilní polymery patří polyhydroxyalkanoáty, které jsou syntetizovány bakteriemi jako zásobní energetické polymery [47; 48]. Nejrozšířenějším zástupcem PHA je poly(3-hydroxybutyrát) (P3HB). Tento polymer je produkován velkým množstvím gramnegativních i grampozitivních bakterií (*pseudomonas sp., bacillus sp., methylobacterium sp.*) ve stresových podmínkách vyvolaných nedostatkem živin [49]. P3HB je slabě hydrofobní materiál, který vykazuje velkou krystalinitu, vysokou teplotu tání a nízkou pružnost, což komplikuje jeho zpracování a omezuje možné využití v průmyslovém měřítku [47]. Mechanické vlastnosti P3HB mohou být vylepšeny

přídavkem dalších polymerů (kyselina polymléčná), vytvořením kopolymerů (s kyselinou 3-hydroxyvalerovou) nebo přídavkem plastifikátorů [49; 47]. Přídavkem aditiv se může výrazně zpomalit biodegradace výsledného produktu [2]. Proto se do těchto směsných plastů (blendů) přidávají látky usnadňující biodegradaci jako je například škrob. Dalším faktorem znesnadňujícím uplatnění PHB je jeho vysoká ekonomická náročnost při výrobě. Snížení ceny lze například dosáhnout výrobou z odpadních materiálů. Velmi dobrým zdrojem uhlíku pro bakterie syntetizující P3HB je například použitý řepkový fritovací olej, který má navíc vysokou výtěžnost, kdy lze teoreticky z jednoho gramu oleje získat více než jeden gram PHA [48]. Dále je možné využít pro výrobu P3HB biomasu, odpadní produkty cukrovarnického průmyslu nebo hydrolyzát kávových sedlin [47]. Problematická je extrakce P3HB z bakteriální biomasy, na které se v současné době používá chloroform a další neekologická rozpouštědla. Řešením by mohlo být část fritovacího oleje zmýdelnit, použít k extrakci PHB a poté opět rozložit na mastné kyseliny, které je možné využít jako substrát pro bakterie a výrobu dalšího PHB [50].

Druhým velmi rozšířeným biodegradabilním polymerem je kyselina polymléčná (PLA). Jedná se o biobased alifatický polyester. PLA je velmi dobře absorbovatelná těly lidí a zvířat, proto se výrazně rozvíjí její využití v medicínských implantátech. Na rozdíl od biodegradace v organismech dochází k neenzymatické degradaci tohoto biopolymeru [51]. Proto pro biodegradaci vyžaduje vyšší teplotu a vlhkost a je tedy možné ji biodegradovat pouze v průmyslovém kompostu [52]. Velkou popularitu si PLA drží také u 3D tisku [30; 53]. Vzhledem k tomu, že je snadné ho zpracovat a jeho vlastnosti jsou v porovnání s ostatními biodegradabilními polymery, a i některými polymery vyrobenými z ropy (vysoká pevnost a nízká propustnost pro plyny), lepší, je mu věnována posledních 20 let velká pozornost [54]. I přes tyto výhody je možné téměř vždy nalézt vhodnější, a především levnější konvenční plast. Cenu PLA (cena za pelety začíná v současné době na 500 $ za tunu) [55] lze snížit přídavkem levných aditiv na základě biomasy nebo jiných zemědělských produktů, například dřevní moučky nebo škroby. Přidávání některých z těchto materiálů do PLA není vždy v souladu s cíli udržitelného rozvoje, protože může docházet ke konkurenci o půdu jako systémový zdroj se zemědělskými produkty pro výrobu potravin [56]. Tímto dojde nejen ke zlevnění výroby, ale také se zrychlí biodegradace tohoto plastového materiálu, který jinak biodegraduje velmi pomalu [57].

3 Osud bioplastů v životním prostředí

3.1 Biodegradace bioplastů

K tomu, aby mohlo dojít k biodegradaci daného materiálu, je nutné, aby mohly mikroorganismy biodegradovaný materiál použít jako zdroj potravy nebo energie, tj. aby byla schopné ho metabolizovat svým enzymatickým aparátem. Tuto podmínku splňují jen velmi malé ve vodě rozpustné molekuly, mezi které polymery obecně nepatří. Proto biodegradace probíhá ve čtyřech krocích. Prvním krokem je biodeteriorace, při které je povrch materiálu pokrytý biofilmem, jehož působením dojde k fragmentaci na menší částice. V dalším kroku dojde k depolymerizaci působením extracelulárních enzymů

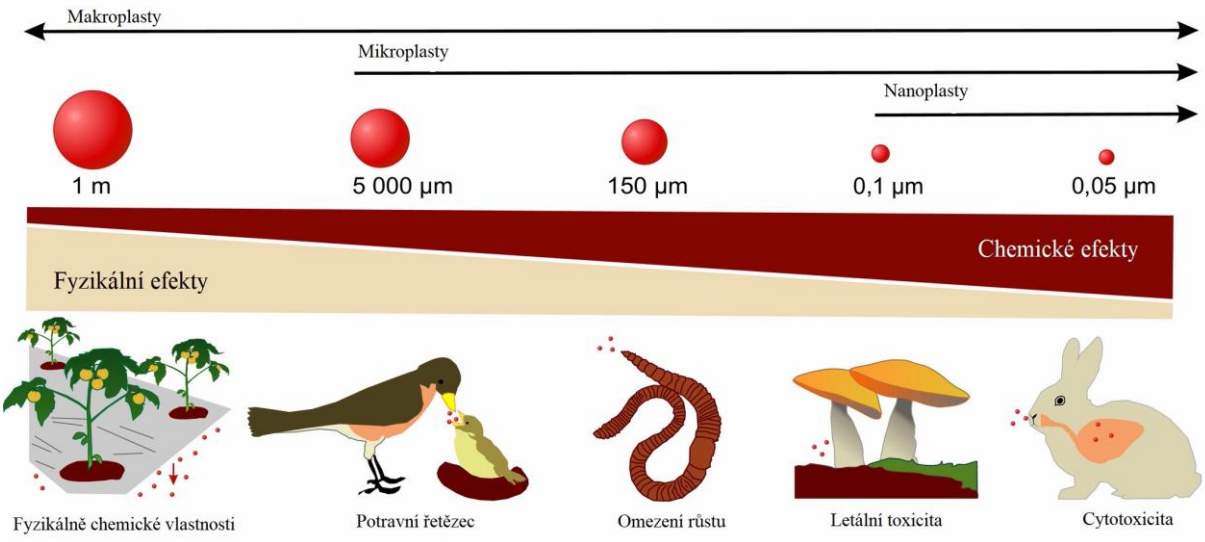
z mikroorganismů v biofilmu. Následně u vzniklých malých molekul může dojít k přestupu do buněk mikroorganismu, tomuto procesu se říká bioasimilace. V posledním kroku může dojít k mineralizaci, při které vznikají voda, dusík a v případě aerobní biodegradace vzniká oxid uhličitý a u anaerobní biodegradace vzniká hlavně methan [2; 26]. PLA může být biodegradován za aerobních i anaerobních podmínek bakteriemi i houbami (biodegradace uhlíku polymeru od 55 % do 95 %). Většinou není vhodné ke kompostování v domácích kompostech. P3HB může být biodegradováno aerobně, anaerobně a v kompostu, kdy v průmyslovém kompostu bylo dosaženo biodegradace uhlíku polymeru až 78 % [58].

Velmi častou metodou pro určení rychlosti aerobní biodegradace jsou respirometrické metody [59; 60; 61]. Tyto metody využívají skutečnosti, že při aerobní biodegradaci se spotřebovává kyslík a uvolňuje oxid uhličitý. Při anaerobní biodegradaci je měřen objem vzniklého methanu [62]. Dále je možné sledovat fyzikální změny testovaného materiálu, jako jsou například úbytek hmotnosti, vizuální změny vzorku, změny mechanických vlastností atd. [46].

Jednou ze slabin použitých norem je, že neberou v potaz žádné informace o prostředí, kde má probíhat biodegradace, proměnnost podmínek způsobenou geografickou polohou, kde bude produkt biodegradovat a změnou ročních období. Nejlépe je možné tento problém demonstrovat na teplotě, při které mají testy probíhat. Například ISO 14851 udává teplotu při testování 20 až 25 °C, což je teplota, která je snadno dosažitelná v laboratorních podmínkách, ale absolutně nekoresponduje s podmínkami, které se vyskytují v reálném životním prostředím České republiky. Dále se normy nedostatečně věnují rozsahu biodegradace (například ASTM D 6400 vyžaduje pouze biodegradaci z 60 %) a počítají biodegradaci z celkové hmotnosti vzorku a při tom neberou v potaz přítomnost nebiodegradabilních aditiv (například plnidlo uhličitan vápenatý). Velmi často je také prostředí pro testování biodegradability nadefinováno k určitému účelu nebo podmínkám zájmu, proto se například testují zvlášť aerobní a anaerobní biodegradace v odpadních vodách, sladkovodních ekosystémech a mořských ekosystémech. K dalšímu zkreslení výsledků může dojít díky různému přístupu k inokulu, nedostatečné reprodukovatelnosti výsledků a nedostatku způsobů, jak testovat v neřízeném prostředí. Dále se žádné normy nezabývají charakteristikou produktů biodegradace. Pro posouzení bezpečnosti biodegradace bioplastů je nutné provést testy ekotoxicity těchto produktů a také tyto produkty charakterizovat pomocí technik analytické chemie (velikost a tvar částic, jejich koncentrace a chemické složení, rezidua aditiv, vedlejší produkty biodegradace) [46]. Kvůli tomu je rychlost biodegradace silně závislá na mnoha parametrech a je nutná značná opatrnost při označení materiálu jako „biodegradabilní“ v otevřeném životním prostředí [46]. Při nepříznivých podmínkách může trvat biodegradace bioplastu i několik desetiletí, díky čemuž dochází k produkci mikroplastů podobně jako u konvenčních plastů [2; 46].

3.2 Vliv mikroplastů na fyzikální vlastnosti půdy

Vše, co vstoupí do půdy, narušuje určitým způsobem její rovnováhu. Proto je možné očekávat, že kontaminace půdního ekosystému vede k ovlivnění vlastností půdy. Některé z nich již byly pozorovány, patří mezi ně degradace půdní struktury, omezení vzniku půdních agregátů, zvýšení porozity a zakoncentrování polutantů v půdě [6; 63]. Vliv mikroplastů na půdu silně závisí na jejich velikosti (Obrázek 3). Například k degradaci půdní struktury dochází při kontaminaci makroplasty [64]. Při této kontaminaci se efekt postupně zesiluje, protože dochází ke zhoršování dalších parametrů jako je například snížení rychlosti infiltrace půdy následně vedoucí k negativnímu ovlivnění půdní vodní kapacity, a způsobující toxické anoxické podmínky [65; 66]. Mikroplasty interagují s různými komponenty půdy a tím se dostávají do půdních agregátů. Volné agregáty jsou tvořeny většími úlomky plastů zatímco kompaktní agregáty vznikají v přítomnosti mikrovláken [13; 67]. Toto se děje nejčastěji v zemědělských půdách, kde používání mulčovacích folií způsobuje úbytek půdních agregátů a omezuje půdní aeraci a průchodnost pro vodu, což vede k negativnímu ovlivnění růstu kořenů a tím snižování výnosu pěstovaných plodin [68; 69].



Obrázek 3: Vliv velikosti mikroplastu na změnu půdních vlastností [7].

Vliv mikroplastů na půdu taktéž silně závisí na chemickém složení a tvaru mikroplastu. De souza Machado a kol. publikovali ve své studii odlišný vliv čtyř různých mikroplastů (PP vlákna, PET vlákna, PE fragmenty a PA microbeads) na půdní vodní kapacitu, objemovou hmotnost a stabilitu půdních agregátů, kde například přítomnost PET vláken způsobila snížení objemové hmotnosti půdy a úbytek stabilních půdních agregátů, což se lišilo od efektů vyvolaných ostatními mikroplasty [7]. V další studii zjistil Boots a kol., že kontaminace půdy vysokohustotním PE změnila stabilitu půdních agregátů stejně jako PLA a textilních vláken [70].

Obecně lze tedy říct, že přítomnost mikroplastů v půdě ovlivňuje interakci půdy s vodou a atmosférou, což vede k omezení růstu plodin rostoucích v této kontaminované půdě [71]. Z toho vyplývá, že by

akumulace plastů v zemědělské půdě mohla narušit cyklování živin a vody v půdě a tím přímo ohrozit potravinovou produkci. V současné době jsou tyto efekty a vlastnosti spojené s přítomností mikroplastů v půdě málo prostudovány, a je tedy těžké odhadnout jaké vážné jsou rizika spojená s jejich přítomností v půdě.

3.3 Vliv mikroplastů na chemické vlastnosti půdy

Mikroplasty neovlivňují pouze fyzikální vlastnosti půdy, mohou mít také negativní efekt na chemické vlastnosti půdy. Nejčastěji mikroplasty ovlivňují pH půdy, obsah půdní organické hmoty (SOM, z anglického soil organic matter), transport půdních polutantů a živin. Lozano a kol. pozorovali zvýšení pH v půdě v přítomnosti mikroplastů, kdy tento efekt byl výraznější v půdě s nižším obsahem vody [72]. Vzrůstá tak obava, že residua plastových folii mohou v půdě zvyšovat pH. Také bylo pozorováno, že při degradaci mikroplastů v půdě dochází k uvolnění aditiv a oligomerů v nich obsažených, což také vede k ovlivnění pH [73]. Dalším možným mechanismem, kterým mohou mikroplasty mít vliv na půdní pH, je změna rychlosti a rozsahu kationtové výměny na velkém povrchu mikroplastů [70]. Bylo také prokázáno, že mikroplasty mají efekt na nitrifikační procesy v půdě, které vedou k uvolňování H^+ iontů a tím snížení půdního pH [74]. Různé výsledky z různých experimentů jsou nejspíše způsobeny tím, že mikroplasty můžou mít na každou půdu jiný vliv, který je ještě závislý na dalších parametrech jako je vlhkost půdy, velikost a typ mikroplastů a doba uplynulá od kontaminace.

Mikroplasty v půdě mohou také způsobit úbytek půdní organické hmoty SOM a depozici těžkých kovů [75]. Velké množství studií ukázalo, že v přítomnosti mikroplastů v půdě dochází ke snižování ale i zvyšování obsahu SOM způsobené ovlivněním mikrobiální aktivity a obsahem nutrientů [76; 77]. Většina studií í zabývajících se tímto tématem se zaměřuje nejčastěji na rozpuštěnou organickou hmotu (DOM z anglického dissolved organic matter), která je součástí SOM a má vliv na cyklus půdního uhlíku, transport polutantů a dynamiku N a P nutrientů [78]. Přestože přídavek mikroplastů do půdy vede ke snížení obsahu DOM, celkově závisí dynamika DOM na mineralizaci a produkci SOM [76; 79]. Liu a kol. popsal, že vysoké koncentrace PP mikroplastů (28 % w/w) vedou ke značnému zvýšení obsahu DOM a nutrientů N a P v porovnání s nižším koncentrací těchto mikroplastů (14 % w/w) [76]. To je možné vysvětlit zjištěním Fei a kol., že mikroplasty v půdě ovlivňují aktivity enzymů zapojených do cyklu C, N a P [80].

Mikroplasty v půdě mají také přímý a nepřímý vliv na obsah a transport půdních nutrientů, který závisí na jejich typu, tvaru a délce expozice půdy [74]. Pomineme-li přírodní zásoby nutrientů z půdních minerálu, pochází v zemědělství z externích zdrojů (např. z hnojiv). Stejně jako u SOM jsou efekty mikroplastů na půdní nutrienty pozitivní, negativní anebo neutrální [72; 74; 81]. Určení vlivu mikroplastů na obsah půdních nutrientů je nezbytné pro posouzení potencionálních výhod a nevýhod používání folií v zemědělství. Liu a kol. ve své studii ukázali, že vstup mikroplastů do půdy vede ke stimulaci enzymů, díky čemuž vzrostla dostupnost půdních nutrientů [76]. Na druhou stranu ve studii

Yan et al. bylo zjištěno, že 1% kontaminace půdy mikroplasty vedla ke snížení obsahu dostupného N a P o 13 respektive 30 % [82].

3.4 Vliv mikroplastů na biologické vlastnosti půdy

Používání plastových produktů v zemědělství může být přínosné pro rostlinnou produkci (například mulčování), na druhou stranu je ale nutné brát v potaz, že se nakonec mohou v půdě akumulovat fragmenty plastů. Z dlouhodobé perspektivy tedy mohou plastové částice v půdě zapříčinit ke snížení zemědělské produkce interakcí s rostlinami nebo semeny. To způsobuje sníženou klíčivost semen, omezený příjem vody rostlinou a menší růst kořenů [70; 83; 84; 85]. Byla také popsána akumulace mikroplastů v kořenech řeřichy seté, které poté vedlo k omezení jejich růstu, délky a biomase [20]. Podobně měly vliv primární mikroplasty (microbeads) na kořenový systém salátu setého nebo okurky seté [86; 87]. Akumulace mikroplastů a nanoplastů v kořenech má za důsledek distribuci těchto částic po celém těle rostlin a způsobuje nižší produkci listů a další biomasy [88; 89]. Na druhou stranu Sun a kol. popsal opačný jev, kdy byly mikroplasty zachycené z atmosféry na listech transportovány v rostlině ke kořenům [90]. Mikroplasty v rostlinách vedou také k oxidativnímu a genotoxickému stresu, který způsobuje snížení obsahu chlorofylu v rostlinách [91; 92].

Plasty, a především mikroplasty, mají povrch kolonizovatelný mikroorganismy, protože díky svým fyzikálně chemickým vlastnostem usnadňují mikrobiální adhezi [93]. Interakce mezi mikroplasty a mikroorganismy může způsobit případně zrychlit degradaci mikroplastů, anebo může ovlivnit organismy žijící v půdě [94; 95]. Mikroorganismy žijící přímo na povrchu mikroplastu benefitují ze snadného přístupu nutrientů, který stimuluje jejich růst [96]. Mikroorganismy pak vytváří na povrchu biofilm, do kterého jsou uvolňovány extracelulární enzymy, které urychlují degradaci mikroplastu a uvolňují rychleji nutrienty z mikroplastu [97; 98; 99]. Takto vzniklý biofilm pak vede k ovlivnění mikrobiální komunity a její funkce [100; 101]. Správný cyklus nutrientů v půdě vyžaduje vyváženou druhovou skladbu mikroorganismů, která je kvůli mikroplastu narušena [102]. Mikroplasty v půdě také mohou mít efekt na vlastnosti bakterií, kdy jejich kontaminace může způsobit rozšíření genů antibiotické rezistence a změny v jejich metabolismu [103; 104]. Mikroplasty můžou také v půdě způsobit rozšíření invazních druhů mikroorganismů [105].

Při studiu ekotoxikologického vlivu mikroplastů na půdní organismy jsou popsány různorodé výsledky, které neumožňují jednoznačnou odpověď na to, jaký vliv mají tyto částice na živočichy žijící v půdě [13]. Z dostupných studií ale plyne, že pozření menších mikroplastů a nanoplastů může negativně působit na růst živočichů, a způsobit histopatologické změny v jejich tělech, oxidativní stres, silnou imunitní odpověď nebo dokonce úmrtí [22; 63; 106].

4 Stanovení mikroplastů na bázi konvenčních plastů ve složkách životního prostředí

Působením lidské aktivity byly mikroplasty kontaminovány všechny složky životního prostředí včetně těl živočichů [107; 108; 109; 110; 23]. Vzhledem ke zvyšujícímu se množství mikroplastů v životním prostředí a prokázané škodlivosti účinků pro živé organismy [19] jsou vyvíjeny kvantitativní a kvalitativní metody pro jejich stanovení [18; 20; 111; 112]. Díky tomu je možné posoudit míru kontaminace různých lokalit primárními sekundárními mikroplasty a schopnost čistíren odpadních vod mikroplasty zadržet [109].

4.1 Extrakce a purifikace

Matrice obsahující mikroplasty je velmi často tvořena organickými a anorganickými příměsemi [107]. Nejjednodušší a nejlevnější způsob, jak odstranit ze vzorku větší částice, je sítování [107; 109; 10]. Obsahuje-li vzorek nadbytečné množství vody je možné ji odstranit filtrací nebo jejím odpařením. Pro separaci mikroplastů ze vzorku je také možné využít jejich rozdílné hustoty od zbytku matrice a použít hustotní frakcionace. K tomuto účelu se používají roztoky solí (NaCl, $CaCl_2$, $ZnCl_2$, NaI) [107; 109; 113]. Při extrakci nízkohustotních plastů, jako jsou například PE a PP, je možné použít pouze destilovanou vodu v kombinaci se sonikací ultrazvukem [69]. Pro extrakci mikroplastů s vyšší hustotou jako je PES použil Liu a kol. nasycený roztok NaCl s hustotou 1,19 $g \cdot cm^{-3}$ [114]. Pro extrakci PVC a PET použili Scheuer a kol. nasycený roztok $CaCl_2$, který má díky vyšší hustotě i vyšší efektivitu. Nevýhodou je ale interakce vápenatých iontů s organickou složkou půdy, kvůli čemuž vznikají agregáty komplikující další analýzu [115]. Imhof a kol. a Liebezeit a kol. vyvinuli separační metodu pro extrakci pomocí roztoku chloridu zinečnatého s hustotou 1,6 až 1,7 $g \cdot cm^{-3}$ [116; 117]. Dekiff a kol. a Van Cauwenberghe a kol. použili pro extrakci roztok NaI s hustotou až 1,8 $g \cdot cm^{-3}$ [118; 119]. Použití roztoků NaI a $ZnCl_2$ je velmi efektivní díky vysoké hustotě roztoku, ale je dražší a vznikají odpady, které jsou nebezpečnější než při použití NaCl nebo $CaCl_2$.

Organické sloučeniny přítomné ve vzorku, které by mohly negativně ovlivnit stanovení, je možné odstranit jejich rozkladem [107]. Vzhledem k tomu, že jsou konvenční plasty až na výjimky odolné vůči agresivnímu chemickému prostředí, je možné použít pro rozklad organické složky vzorku silná oxidační činidla, báze a kyseliny. Velmi častá je metoda rozkladu pomocí katalytické mokré oxidace [107; 109; 113]. Pro tuto metodu jsou nejčastěji použity peroxid vodíku, chlornan sodný a Fentonovo činidlo nebo roztoky alkalických hydroxidů a silných kyselin [109; 120]. Tento typ rozkladu není vhodný pro velké objemy vzorku, protože by celý proces trval příliš dlouho. Použití koncentrované kyseliny dusičné není vhodné pro plasty citlivé na velmi nízké pH, jako jsou polystyren (PS) nebo nylon [121]. Při rozkladu peroxidem vodíku se používá 30% roztok, tato metoda je ale velmi pomalá, rozklad trvá 7 dnů. Použitím Fentonova činidla místo peroxidu vodíku je možné dobu rozkladu snížit až na 10 minut bez zničení mikroplastů ve vzorku [122]. K rozkladu je možné také použít směs technických enzymů jako jsou lipázy, amylázy, proteinázy, chitinázy a celulázy [109; 113]. Cole a kol. použil pro rozklad Proteinasu-

K, se kterou dosáhl rozkladu vyššího než 97 % (pro porovnání 2 M HCl 72,1±9,2 %; 10M NaOH 60 °C 91,3±0,4 %) [123]. Löder a kol. vyvinul vícekrokovou metodu pro rozklad kombinující více enzymů (proteáza, chitináza, celuláza), povrchově aktivní látky (dodecylsulfát sodný SDS) a peroxid vodíku s účinností 98,3±0,1 % [124].

4.2 Semikvantitativní stanovení konvenčních mikroplastů

Za kvalitativní metody stanovení mikroplastů považujeme takové, které jsou schopny rozlišit mikroplasty od matrice a jiných částic (celulózy, písku, skla...) a identifikovat typ polymeru u nalezených mikroplastů. Semikvalitativní metody pak dokážou určit např. počet částic mikroplastů vztažených na objem či hmotnost matrice, případně podíl plochy zaujímané mikroplasty na mikroskopickém substrátu. Velmi často využívanou metodou pro stanovení mikroplastů je vizuální identifikace pomocí mikroskopu. Jedná se o rychlou a jednoduchou metodu. Touto metodou je možné zjistit tvar, texturu a typ povrchu částice. Na druhou stranu tímto způsobem není možné identifikovat částice menší než ~100 μm [125; 126]. Dále pak při této metodě může dojít k falešně pozitivní identifikaci mikroplastu nebo naopak mikroplast nemusí být rozpoznatelný od jiných složek matrice [107; 113]. Také je možné udělat chybu při počítání částic, kterou omezil Carr a kol. použitím Petriho misky rozdělené na sektory [127]. Pro potvrzení složení mikroplastu a vůbec jeho odlišení od jiných částic je nutno tuto metodu zkombinovat s infračervenou spektroskopií s Fourierovou transformací (FTIR) nebo s Ramanovou spektroskopií. Browne a kol. identifikoval pomocí FTIR v mořských sedimentech, odtocích z čistíren odpadních vod a z použité vody z praček PA, PES, PE, PP a polyvinylchlorid (PVC) [128]. Lares a kol. použila pro konfirmaci mikroplastových částic a vláken v čistírenském kalu a vodě z jezera FTIR a Ramanovu mikroskopii, kterou identifikovala polyester, PE, polyamid, PP [129]. Další zpřesnění analýzy matrice je možné dosáhnout použitím elektronového mikroskopu.

Pro kvalitativní stanovení mikroplastů o rozměrech větších než 10 μm je vhodné použít FTIR. Pro charakterizaci částic větších než 1 μm lze použít Ramanovou spektroskopii. Touto metodou ale nelze stanovit polymery citlivé na fotodegradaci, jako je například PVC. Obě metody jsou komplementární a je potřeba je volit i s ohledem na strukturu mikroplastu očekávaného ve vzorku. Metodou FTIR je možné stanovit mikroplasty se strukturou, kde se vibrací atomů v molekule mění její dipólový moment, zatímco Ramanovou spektroskopií je možné stanovit mikroplasty se strukturou molekuly, kde se vibrací atomů mění polarizovatelnost molekul [107]. Pro zrychlení analýzy Primpke a kol. použil při stanovení mikroplastů v sedimentech z Německa automatizovanou FPA (focal plane array) FTIR mikroskopii, kde každé pole slouží jako infračervený senzor umožňující měření velkého množství polí jednoduše dohromady s vyvinutým automatickým zpracováním dat [130] a Renner a kol. využil pro analýzu standardů mikroplastů v referenčních vzorcích software pro automatizované detekovaní píků při použití ATR/FTIR [131]. Serranti a kol. vyvinula pro charakterizaci mořských mikroplastů hyperspektrální zobrazení sestávajícího se ze spektrografu pracujícího s krátkými infračervenými vlnami a z čoček

a softwarem k automatizovanému vyhodnocení dat a porovnávání s databázemi pomocí analýzy hlavních komponent [132].

Stále častěji se pro kvalitativní analýzu mikroplastů v různých složkách životního prostředí využívají destruktivní metody, mezi které patří Py–GC/MS (pyrolýzní plynová chromatografie s hmotnostně-spektrometrickou detekcí), desorpční-GC/MS a LC (kapalinová chromatografie). Dümichen a kol. stanovil pomocí TED–GC/MS PP, PE a PS ve fermentačních reziduích odebraných z bioplynové stanice a PE, PS a PET ve vodě z řek. Tato metoda je výhodná svojí rychlostí, malou spotřebou vzorku (20 mg) a možností vynechat časově náročnou úpravu vzorku, mezi nevýhody patří vysoké nároky na homogenizaci vzorku [133]. Fries a kol. identifikoval v sedimentu z ostrova Norderney, na kterém díky silným slapovým jevům dochází k vysoké akumulaci sedimentů PE, PS a PVC a určil i aditiva v nich obsažená [134], touto metodou také stanovil Nuelle a kol. PP, PET a PVC v mořských sedimentech [135]. Piehl a kol. testoval zemědělské půdy na přítomnost mikroplastů (za použití vzorkování, sítování, filtrace a ATR–FTIR) a našel 0,3 ± 0,4 částic·ha^{-1}, ale musel vyloučit částice menší než 1 mm kvůli problematické předúpravě vzorku [136]. Pro zvýšení robustnosti vibračně spektrometrických mikroskopických metod a jejich zrychlení použil Hufnagl a kol. chemometrickou metodu využívající kombinaci spektrálních deskriptorů s algoritmem Náhodného lesa (random decision forest, RDF) dohromady s FPA FTIR mikroskopií jako detektorem. V této předběžné studii byli autoři schopni identifikovat PE, PP, PMMA (polymethylmethakrylát), PS a PAN (polyakrylonitril) částice v uměle mikroplasty fortifikovaném vzorku sladkovodního planktonu [137].

4.3 Kvantitativní stanovení konvenčních mikroplastů

Kvantitativní stanovení mikroplastů (zejména v půdě a jiných komplexních pevných či koloidních matricích) je v současné době velká analytická výzva. Právě komplexita odebraného vzorku znesnadňuje analýzu díky možným interferencím s analytem, vytvářením falešného pozitivního nebo negativního signálu [138]. Za kvantitativní stanovení považujeme takové, které je schopno odlišit plast a matrici či jiné materiály, dává informaci o druhu plastu (alespoň přibližně) a zároveň uvádí koncentraci mikroplastů v chemickém smyslu (tedy zejména hmotnostní či objemovou).

Jednou ze slibných kvantitativních metod je stále Py–GC/MS. Při této metodě je vzorek pyrolyzován v inertní atmosféře a vzniklé plynné produkty jsou zachyceny v rozpouštědle nebo vedeny (nejčastěji kapilárou) přímo do GC-MS. Výhodou je možné stanovení směsí více plastů a je možné analyzovat vzorek bez jakékoliv úpravy. Nevýhodou je, že neposkytuje informace o počtu, velikosti a tvaru mikroplastů [107].

Scheurer a Bigalke vytvořili metodu pro kvantifikaci mikroplastových částic v půdě pomocí hustotní separace a oxidativního rozkladu matrice následované FTIR analýzou. Autoři uvádějí výtěžky separace 93–98 % a průměrnou koncentrací nalezených mikroplastů ve švédské záplavové oblasti 5 mg·kg^{-1}. Limity detekce a kvantifikace nebyly uvedeny [115].

Fischer a Scholz-Böttcher využili Py–GC/MS techniky (Curie-point pyrolýza a microfurnace pyrolýza) pro stanovení mikroplastů v mořské vodě, soli a sedimentech (v posledních dvou matricích za použití hustotní separace a rozkladem pomocí H_2O_2) s přístrojovými limity od 3 do 200 ng [139]. Dříve stejní autoři použili Curie-point Py–GC/MS pro stanovení mikroplastů (nejníže 0,4 µg pro PMMA nebo 0,6 µg pro PP (a nejvýše >290 µg pro PVC)) v rybích tkáních, ale po významné úpravě vzorku jako je třeba několik různých rozkladů [140]. Dümichen a kol. použil TED–GC/MS ke stanovení PE spikovanou blíže nespecifikovanou městskou vrchní vrstvu půdy z Berlína s přístrojovým limitem 0,2 % (<0,04 mg) [141] a později stejnou techniku předělal Eisentraut a kol. k analýze PE (s limitem 0,07 %), PP (0,15 $mg \cdot g^{-1}$) a PS (0,25 $mg \cdot g^{-1}$) ve splachu z Berlínských ulic dohromady s mikrogumovými částicemi z pneumatik (limit detekce 3,9 $mg \cdot g^{-1}$) stanoven jako styren-butadienová guma [142].

Jako další termoanalytická technika vhodná ke kvantifikaci mikroplastů v půdě je termogravimetrie (TGA), kterou použil David a kol. ke stanovení PE, PS, PVC a PET pomocí metody Univerzálního modelu půdy, nicméně využití této metody pro reálné vzorky se směsmi mikroplastů je, jak sami autoři uvádějí, zatím ve vývoji. LOD se pohyboval u různých mikroplastů od 0,33 % u PET do 2,22 % u PVC, LOQ byly stanoveny na 1,15 % pro PET a 3,08 % pro PS. Pro PVC bylo naměřené LOQ příliš vysoké a bylo zjištěno, že tato metoda nebyla prozatím vhodná ke stanovení PE [138]. Yu a kol. vyvinul TGA–FTIR metodu ke stanovení PS a PVC v mořských sedimentech a vodě, ovšem pouze pro vzorky, které obsahuji více než 0,49 mg plastu s výtěžkem od 98 do 108 % [143]. David a kol. také vyvinul metodu pro stanovení PET v půdě pomocí TGA–MS založenou na analýze pyrolýzních produktů a jejich přiřazení k teplotě rozkladu polymeru odlišné od teploty rozkladu půdní matrice. LOD byly stanoveny od 0,06 do 0,58 %, LOQ od 1,52 do 51,00 % u PET v půdě, R^2 od 0,870 do 0,987 a relativní standardní chybou od 3,21 do 12,76 % [144]. Steinmetz a kol. vyvinul metodu Py-GC/MS ke stanovení PE, PP a PS extrahovaných 1,2,4-trichlorbenzenem z jílovitých půd s výtěžností 70 až 128 % a s limity detekce 1 až 86 $\mu g \cdot g^{-1}$ [145].

Hintersteiner a kol. vytvořil metodu pro stanovení PE a PP mikročástic (v koncentracích od 0,07 do 3,07 %; relativní standardní odchylka (RSD) od 2,3 do 13,4 % a výtěžnost 92–96 %) v kosmetických produktech pomocí extrakce, rozpuštěním a vysokoteplotní permeační chromatografie [146].

5 Stanovení biodegradabilních mikroplastů P3HB a PLA

S příchodem produktů z biodegradovatelných plastů na trh je možné předpokládat, že bude nutné řešit problematiku mikroplastů stejně jako u konvenčních plastů, které mohou být kvůli nedokonalé nebo příliš pomalé biodegradaci zdrojem velkého množství mikroplastů v krátkém časovém období [34]. Přestože by mělo být setrvání mikroplastů z biodegradabilních materiálů v životním prostředí kratší než u konvenčních plastů, mikroplasty z biodegradabilních plastů mohou mít podobné negativní vlastnosti jako u konvenčních mikroplastů [26]. Metody pro kvalitativní a kvantitativní stanovení těchto částic v současné době až na výjimky neexistují, přestože by mohly být užitečné pro zhodnocení kontaminace

životního prostředí, ale i pro verifikaci testů biodegradability, kdy v současnosti není možné uspokojivě analyzovat testovací medium na rezidua těchto materiálů.

Pro extrakci zmíněných bioplastů z půdy nebo dalších tuhých matric je možné použít jen některé metody používané u konvenčních mikroplastů. Jde především o fyzikální procesy jako filtrace, sítování nebo sedimentační frakcionace [109]. Kvůli špatné odolnosti vůči agresivním chemickým podmínkám není možné použít pro rozklad organických složek matrice žádné agresivní chemikálie. Jako jedna z alternativ se jeví rozpuštění bioplastů v chloroformu [147]. Při tomto kroku se však vyextrahují i další organické látky, především frakce huminových látek, proto by musel být extrakt náročně přečištěn. Slibnou alternativou by mohla být extrakce pomocí iontových kapalin, které jsou pro rozpouštění více specifické. V tomto případě je možné použít dvě různé strategie. První z nich je rozpuštění biomasy a uvolnění mikroplastů do roztoku, odkud mohou být snadno filtrovatelné [148; 149; 150], kde Kobyashi a kol. udává výtěžnost až 98 % [148]. Takto mohou být analyzovány pouze vzorky se specifickou matricí (například matrice tvořené těly mikroorganismů, jako je například aktivovaný kal – není jasné, zdali tyto metody budou fungovat i pro matrice typu půda atd.), další nevýhodou je velká spotřeba iontové kapaliny s poměrně malými výtěžky a malou možností recyklace rozpouštědla. Další možností je rozpustit vhodnou iontovou kapalinou PHA bioplast a po odfiltrování zbylé biomasy ho opět vysrážet, což je popsáno v patentu USOO7763715B2 [151].

5.1 Poly(3-hydroxybutyrát)

Aby mohl být P3HB stanoven pomocí separačních metod, je nutné ho depolymerizovat a stanovit jako monomer, tj. hydroxymáselnou kyselinu. Jedna z možností je použít methanolýzu za přítomnosti zředěné kyseliny sírové. Tato metoda ale není kvantitativní, může docházet ke vzniku vedlejších produktů jako je například kyselina krotonová nebo derivátů propenu. Výtěžky této metody se pohybují mezi 30 až 40 % [152; 153]. Další možností je štěpení pomocí tetraethoxytitanátu a ethylesteru kyseliny trifluoroctové (TFA). Tato metoda je vhodná při další analýze pomocí GC, přičemž může dojít ke ztrátě TFA řetězce a je nutná derivatizace [152]. Nejvhodnějším postupem pro depolymeraci P3HB je použití tetrahydridohlinitanu lithného. Výsledný monomer je pak možné stanovit pomocí vysoceúčinné kapalinové chromatografie s hmotnostně-spektrometrickou detekcí s ionizací elektrosprejem (HPLC–ESI/MS; limity detekce se pohybují od 4,2 % z 10 µL alikvotu z 2 mg polymeru) [152].

V případě P3HB existují metody pro stanovení intercelulárního P3HB v buňkách aktivovaného kalu, které by mohly být taktéž použity ke stanovení mikroplastů obsahujících tento polymer. Tato metoda je založena na propanolytické nebo hydrolytické depolymerizaci plastu a stanovení vzniklých produktů iontovou chromatografií (semikvantitavní, limity detekce neuvedeny) [154]. Stanovení mikroplastů v aktivovaném kalu při těchto metodách komplikuje právě přirozený výskyt P3HB v buňkách mikroorganismů v kalu, který může tvořit až desítky procent hmotnosti buněk mikroorganismů [155].

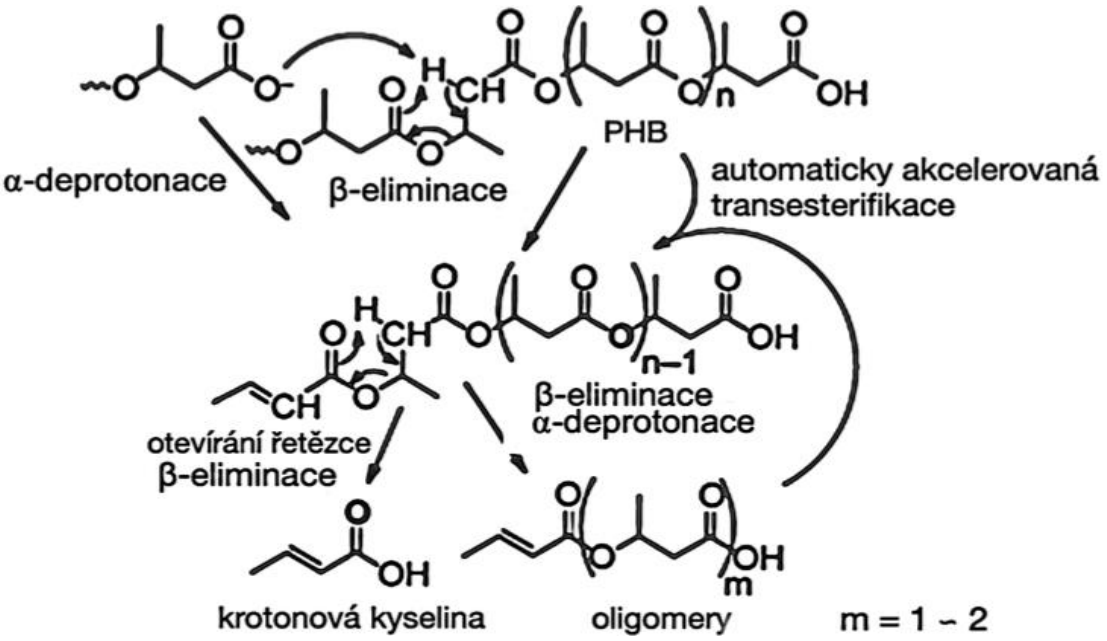
P3HB v bakteriální biomase lze kvantifikovat pomocí FTIR spektrometrie a následného statistického zpracovaní dat [156], například regresní analýzou metodou nejmenších čtverců a dalšího matematického modelování. Arcos-Hernandez a kol. vyvinuli FTIR metodu pro stanovení PHB v různých buňkách v koncentracích od hmotnostního zlomku 0,011, přičemž R^2 se pohybovalo od 0,911 do 0,985 [157], a Samek a kol. využili Ramanovu spektrometrii ke kvantifikaci obsahu P3HB v *Cupriavidus necator* H16 mediu, nejnižší kalibrační bod byl 1,395 μmol·l^{-1}. Tuto metodu lze použít pouze pro amorfní P3HB [158]. Autoři posledních dvou článků neudávají limity detekce. Tyto metody jsou však optimalizovány pro stanovení P3HB v buňkách a není jasné, zdali by fungovaly i pro stanovení P3HB v půdě.

Vyextrahovaný P3HB je také možné rozpustit v chloroformu, po přídavku kyseliny sírové dojde ke vzniku kyseliny krotonové a zbarvení roztoku dohněda, obsah P3HB je pak možné stanovit spektrofotometricky [147]. Limity detekce nebyly uvedeny, množství P3HB by mělo být od 5 do 50 μg [159]. Pro stanovení P3HB v bakterii *Synechocystis sp.* je možné využít komerční set, který využívá enzymatickou depolymeraci a vzniklý roztok se analyzuje spektrofotometricky [160]. Tato metoda vyžaduje pro použití k analýze mikrobioplastů v půdě velmi účinnou extrakci, protože půdní organická hmota může vytvořit v roztoku zákal nebo látky s podobným absorpčním spektrem, které mohou interferovat se signálem analytu.

Karr a kol. použil HPLC/UV–VIS pro stanovení P3HB *Rhizobium japonicum* po komplikovaném rozkladu pomocí chlornanu sodného a následné extrakci, rozkladu a depolymeraci na krotonovou kyselinu. Nejnižší změřená koncentrace byla 140 nmol·l^{-1}. Další limity detekce nebyly stanoveny [161]. Elhottová a kol. využili GC/MS–MS ke stanovení derivatizovaných produktů methanolýzy glykolipidového extraktu u stanovení obsahu P3HB v půdě, čistírenských kalech a bakterii *Bacillus megaterium* CC M 1464. Nejnižší naměřená koncentrace byla 1,16 nmol·l^{-1}, další detekční limity nebyly publikovány [162]. Monteil-Rivera a kol. využili methanolýzy i kyselé hydrolýzy P3HB k dosažení produktů depolymerizace – hydroxyalkenových kyselin – a jejich následné analýze pomocí SPME–GC/FID, dosažené LOD se pohybovaly od 1,6 do 53 mg·l^{-1}, LOQ se pohybovaly od 5,5 do 175 mg·l^{-1}, výtěžnost 98–100 % a RSD 2–8 [163], podobně Werker a kol. použili GC/FID po kyselé alkoholýze a hexanové extrakci ke stanovení obsahu PHB v čistírenských kalech pocházejících z laboratorních bioreaktorů k čištění odpadní vody s nejnižším detekovatelným množstvím 14 μmol 3-butylesteru hydroxymáselné kyseliny odpovídající 1,2 mg P3HB [164]. Torri a kol. vyvinul offline Py–GC/FID ke stanovení 2–alkenových kyselin v bakteriální biomase s LOD od 100 μg (1 % P3HB v biomase) a RSD ≤ 15 %, čímž je tato metoda porovnatelná s metodami založenými na methanolýze [165]. Abbondanzi a kol. dále předělal a vylepšil tuto metodu na termolýzu uvnitř vialky vhodnou pro stanovení v rozsahu 0,5–2,5 mg P3HB (10–50 % P3HB v mikrobiálním vzorku) detekovanou pomocí GC/MS [166]. Velasco Alvarez a kol. vyvinul metodu využívající plynovou chromatografii–izotopické zřeďování s hmotnostní spektrometrií (GC/IDMS) ke stanovení P3HB v bakteriální biomase

Escherichia coli (po propanolýze a derivatizaci) s ^{13}C–P3HB jako vnitřním standardem a LOD od 0,01 $\mu g \cdot g^{-1}$ (nebo 0,11 $nmol \cdot l^{-1}$) [167].

Další možností jsou metody založené na rozkladu vzorku s následnou analýzou plynných produktů, umožňující přímé stanovení P3HB v matrici, podobně jako u konvenčních mikroplastů [135] [143] [144]. V případě P3HB, které má nízkou odolnost vůči tepelné degradaci, dochází k řetězovému štěpení, což vede k poklesu molekulární hmotnosti (*Obrázek 4*). P3HB se rozkládá při teplotách těsně nad teplotou tání okolo 180 °C, v případě přídavku stabilizátoru pak při teplotách vyšších. Produktem termické degradace je krotonová kyselina s molekulovou hmotnostní 86,09 $g \cdot mol^{-1}$ a její oligomery. Okoffo a kol. využil k analýze vzorků s bioplasty PLA, PHA, PBAT, polybutylensukcinát a polykaprolakton, které nejdříve extrahoval pomocí extrakce podporované tlakem s methanolem, a následně je analyzoval pomocí Pyr-GC/MS. Touto metodou dosáhl LOQ pro PLA 0,05 $mg \cdot g^{-1}$ (*m/z* 118); pro polykaprolakton 0,03 $mg \cdot g^{-1}$ (*m/z* 160); pro PBAT 0,03 $mg \cdot g^{-1}$ (*m/z* 194); pro polybutylensukcinát 0,04 $mg \cdot g^{-1}$ (*m/z* 146); pro PHA 0,02 $mg \cdot g^{-1}$ (*m/z* 100) [168].



Obrázek 4: Mechanismus tepelné degradace PHB [169]

5.2 Polymléčná kyselina

U PLA je mechanismus termické degradace komplikovanější a je připisován náhodnému štěpení hlavního řetězce [170], depolymerizaci [171] a transesterifikačním reakcím [172]. Jako primární pyrolýzní produkty jsou CO, CO_2, methylketen, acetaldehyd, H_2O a cyklické oligomery. Sekundární produkty jsou methan a butadion, které jsou pravděpodobně produkty degradace acetaldehydu. Navíc, degradace je také ovlivněna reaktivními koncovými skupinami, zbytky katalyzátorů, nezreagovanými monomery a dalšími nečistotami. V tomto případě je využití pyrolýzy problematičtější. Nicméně, v obou případech je použití podmíněno tím, že degradační produkty bioplastu nejsou překryty

degradačními produkty matrice. Pak by musel být vzorek předupraven, což by mohlo vést k dalším problémům.

Pro stanovení PLA ve formě folie, bez matrice, vyvinula Arrieta a kol. metodu sekvenční pyrolýzy (pomocí Py–GC/MS) s limity detekce od 0,1 μg dle analytu, R^2 od 0,841 do 0,985, RSD od 4,68 do 2,65 [173]. Spíše než kvantitativně-analyticky zde bylo postupováno s cílem popsat pyrolýzní produkty PLA pro možnou pyrolýzu a možné energetické využití PLA odpadu. Spojením více sofistikovaných metod používaných při analýzách potravin, jako je ultraúčinná kapalinová chromatografie – hmotnostní spektrometrie s analyzátorem doby letu a kvadrupólem (UPLC–QTOF–MS) nebo ultraúčinná kapalinová chromatografie – hmotnostní spektrometrie iontové mobility s analyzátorem kvadrupólem a doby letu (UPLC–IMS–QTOF–MS) je možné analyzovat cyklické a lineární oligomery PLA (molekulové hmotnosti od 257 do 1175) v potravinářských náhražkách v koncentracích v tisících $ng \cdot g^{-1}$ [174]. Atmosférická sonda k analýze pevných částic využívající hmotnostní spektrometrii iontové mobility (ASAP–IM–MS) umožňuje kvalitativně odlišit PLA, PBS a PE v PLA–PBS a PLA–PE směsných plastech po termickém rozpuštění vzorku a desorpci analytů [175].

Cíl disertační práce

Jak vyplývá z předcházejících částí, v současnosti je největší zájem o bioplasty typu PLA a PHA. Při jejich aplikaci je velmi pravděpodobný vznik sekundárních mikrobioplastů, ačkoliv životní prostředí může být samozřejmě kontaminováno i jejich primárními mikrobioplasty. Vzhledem k tomu, že problematika kontaminace životního prostředí mikrobioplasty je nová (o čemž svědčí minimální množství publikovaných prací), je třeba se zamyslet nad jednotlivými fázemi výzkumu v této oblasti. Proto bylo právě PHB zvoleno jako modelový bioplast pro všechny experimenty uvedené v této disertační práci a pro porovnání výsledků byl použit v některých experimentech další bioplast PLA a konvenční plast PET. Zkušenosti s mikroplasty z konvenčních plastů v půdě napovídají, že výzkum bude směřován několika provázanými směry: 1) nalezení metod pro analýzu mikroplastů v půdě případně v jejích částech (nebo analogických substrátech), 2) studiem osudu mikrobioplastů v půdě (tzn. transport, biodegradace, biofragmentace…), 3) objasněním, jak mikrobioplasty ovlivňují ekosystémové funkce půdy (například zádrž vody, sekvestraci uhlíku, primární produkci atd.). Je zřejmé, že směr „1“ determinuje úspěch směru „2“, směr „3“ lze pak úspěšně řešit jen pokud jsme vyřešili směr „2“.

V této disertační práci se těchto témat dotýkáme přímo i nepřímo, pro pochopení osudu biodegradabilních plastů jsou často využívány normy pro biodegradaci v různých prostředích (nejčastěji OECD, ISO, ASTM). Proto jsou tyto normy v této práci brány jako výchozí bod, jsou diskutovány podmínky a výsledky a je poukázáno na jejich nedostatky, protože zdaleka neodrážejí reálné problémy biodegradabilních plastů (které například mohou vést k nevhodnému nakládání s bioplastovým odpadem nebo nesprávnému využití bioplastů v zemědělství a následné tvorbě mikrobioplastů). Další kapitolou jsou analytické metody využívané pro extrakci, purifikaci a kvalitativní a kvantitativní stanovení konvenčních mikroplastů v komplexních pevných matricích, jako je půda nebo aktivovaný kal. Nicméně, jak vyplývá z literární rešerše, v současné době téměř neexistují analogické robustní, rychlé a citlivé analytické metody ke stanovení mikrobioplastů v půdě, které by pomohly verifikovat testy biodegradability analýzou produktů biodegradace, objasnit osud potencionálně vzniklých mikrobioplastů a jejich vliv na životní prostředí. Jestliže by použití bioplastů nevedlo k redukci vzniku mikroplastů jak se všeobecně předpokládá, ale naopak při jejich biodegradaci by mikroplasty vznikaly rychleji, je důležité zjistit jaký vliv mají na životní prostředí (tj. zda se liší nebo je stejný ve srovnání s konvenčními plasty). Tyto efekty jsou v této práci sledovány se zaměřením na vlastnosti důležité v zemědělství, protože zemědělská půda je jedním z hlavních receptorů mikrobioplastů, kde poškození jejich vlastností může vést ke snížení efektivity zemědělské produkce. Na konci této práce jsou proto také prezentovány výsledky z testování prototypu „inteligentního“ potahovaného hnojiva, na kterém jsou demonstrovány možné vlivy takovéhoto výrobku na půdu. Tím se tedy všechny zmíněné směry prolínají a z nich pak vyplývají některé dílčí výzkumné otázky:

- **O1:** Jak relevantní je využití norem pro biodegradaci pro pochopení osudu biodegradabilních plastů v půdě?
- **O2:** Je možné stanovit mikrobioplasty v půdě podobnými metodami jako konvenční mikroplasty?
- **O3:** Je vliv mikrobioplastů na životní prostředí srovnatelný s doposud známým vlivem konvenčních mikroplastů?
- **O4:** Existují nějaká rizika využití biodegradabilních plastů v zemědělství?

V následujících kapitolách jsou diskutovány výsledky dosažené během doktorského studia. Část z výsledků již byla publikována (viz přílohy na konci této práce), část se „nevešla“ do publikací a část tvořila výsledky projektů, na nichž jsem posledních několik let pracoval. Cílem následujícího textu je tedy seznámit čtenáře s podstatnými výsledky disertační práce, ale také se zkušenostmi, které byly získány a cestami, které byly v průběhu práce testovány.

Diskuze výzkumných otázek

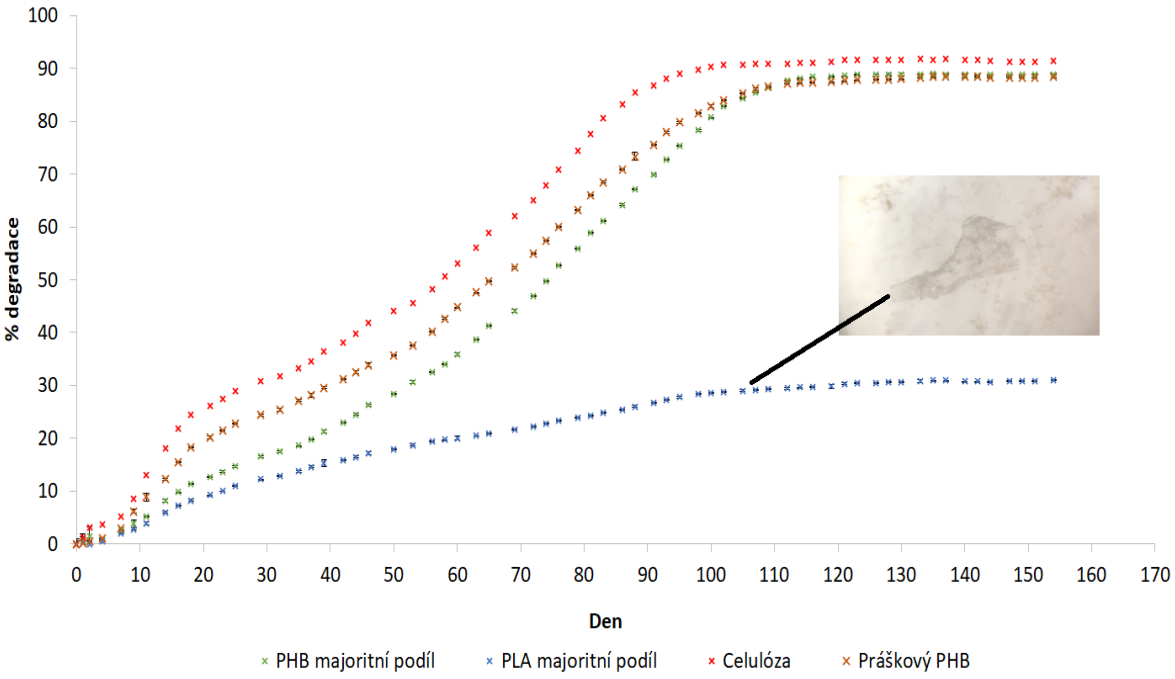
1 Výzkumná otázka 1: Jak relevantní je využití norem pro biodegradaci pro pochopení osudu biodegradabilních plastů v půdě?

První téma, se kterým jsme se začali zabývat při práci s biodegradabilními bioplasty bylo testování jejich biodegradability. Ve spolupráci s Firmou NAFIGATE a kolegy z Ústavu chemie materiálů jsme začali vyvíjet nové materiály a výrobky na bázi PHB v rámci projektu MPO TRIO „Smart fertilizers“. Při těchto experimentech jsme prostudovali většinu dostupných norem a provedli první biodegradační experimenty. Jejich výsledkem bylo překvapující zjištění, že biodegradabilní polymer kompletně zdegradoval jen zřídka během definované doby, a proto jsme se důkladně zaměřili na různé typy biodegradačních experimentů, a jak jsou experimentální podmínky relevantní pro životní prostředí (například koncentrace mikroorganismů ve vodě, která v ISO normě může být mezi 30 až 1000 $mg \cdot l^{-1}$, nebo teplota experimentu s půdou mezi 20 až 28 °C).

Rychlost biodegradace a biodegradabilita bioplastových materiálů je obvykle měřena pomocí biodegradačních testů, které byly diskutovány výše a v **Příloze A**. V těchto testech je testovaný materiál vystaven vlivu mikroorganismů za kontrolovaných podmínek (většinou nastavených tak, aby byly ideální pro biodegradaci). Rychlost biodegradace pak silně závisí právě na množství mikroorganismů, na teplotě, vlhkosti, pH, velikosti částic testovaného materiálu a dalších parametrech [26]. Biodegradaci taktéž ovlivňuje typ bioplastu a aditiva v něm obsaženého. Například ve směsi dvou různě biodegradovatelných bioplastů dojde přednostně k biodegradaci lépe biodegradovatelného bioplastu, čímž se výrazně zhorší mechanické vlastnosti materiálu, který se poté rychleji rozpadne na mikrobioplasty [37]. Ty jsou ale tvořeny hůře biodegradovatelným bioplastem, a proto se biodegradace výrazně zpomalí, anebo i zastaví.

Toto chování bioplastového „blendu“ jsme pozorovali právě v prvním kompletním biodegradačním experimentu, který byl proveden s folií obsahující majoritní podíl PHB (snadno degradovatelného bioplastu) a folií obsahující majoritní podíl PLA (hůře biodegradovatelného bioplastu). Obě folie byly nastříhány na čtverečky s velikostí strany 0,5 cm a pro porovnání byl experiment proveden i s práškovým PHB, kde velikost částic byla mezi 64 a 125 μm, a s práškovou celulózou. Experiment proběhl v artificiální vodě inokulované čistírenským aktivovaným kalem dle normy ISO 14851:1999 a biodegradabilita byla měřena metodou stanovení spotřeby kyslíku. Z výsledku na Obrázku 4 vyplývá, že nejrychleji zdegradovala celulóza; práškové PHB a folie s majoritním podílem PHB zdegradovaly téměř ze 100 % přibližně za 115 dnů. Folie s majoritním podílem PLA zdegradovala přibližně ze 30 % za stejnou dobu, kde většinu zdegradované části nejspíše tvoří PHB a další plniva. Zpomalení biodegradace je možné pozorovat zhruba po 100 dnech experimentu, kdy do konce experimentu (54 dnů) zdegradovalo pouze 2,7 % fólie. Z tohoto důvodu je možné předpokládat (nebo i extrapolovat), že kompletní biodegradace, tj. mineralizace této fólie by trvala i za těchto ideálních podmínek několik let

a v reálném prostředí životního prostředí pak tedy ještě déle. Na dně reakční nádoby byl po ukončení experimentu odebrán sediment, na kterém byly pod mikroskopem detekovány mikrobioplasty vzniklé z folie (Obrázek 5). Jejich povrch byl zvrásněný důlky vzniklými biodegradací snadno biodegradabilního materiálu, kvůli kterým došlo během experimentu k rozpadu folie (k rozpadu také přispělo neustálé míchání na třepačce, které bránilo sedimentaci aktivovaného kalu) na mikrobioplasty s ostrými hranami.



Obrázek 5: Výsledky biodegradačního experimentu bioplastových folií ve vodě

Nejčastějším způsobem měření biodegradace jsou biodegradační testy založené na respirometrii (měření spotřeby kyslíku nebo uvolněného oxidu uhličitého), které jsou zatíženy velkou experimentální chybou způsobenou manipulací s respirometrem (doplňování kyslíku, výměnou KOH nasyceného oxidem uhličitým), nevydýcháním veškerého CO_2 (mikroorganismy část uhlíku používají pro stavbu svých těl) a i změnou chování mikroorganismů v přítomnosti mikrobioplastu způsobujícího stres, který vede k odlišné biodegradaci uhlíku v matrici vzorku a blanku.

V této práci proto navrhuji neurčovat biodegradovatelnost pouze z biodegradačních experimentů, ale také analýzou biodegradačního media po ukončení experimentu (bude diskutováno v další části). Tuto problematiku demonstrují výsledky experimentů z článku v **Příloze E**, kde je vidět, že například u půd s nejnižší počáteční koncentrací PHB není ani v jednom případě dosaženo 100 % biodegradace měřené respirometricky, i když z biodegradačních křivek vyplývá, že u všech půd s počáteční koncentrací 0,5 došlo k ukončení biodegradace téměř v polovině experimentu a u půd s počáteční koncentrací PHB 1 %

byla ukončena chvíli před ukončením experimentu. V půdách obsahující 3 % PHB stále probíhala biodegradace a mikrobioplasty PHB zde byly viditelné i bez použití mikroskopu (Obrázek 6). Při analýze PHB v půdách pomocí termogravimetrie byla zjištěna ve všech půdách biodegradace vyšší než při respirometrickém stanovení, což naznačuje, že část uhlíku z bioplastu nebylo mineralizováno, ale že bylo imobilizováno v SOM (patrně ve formě mikrobiální biomasy).

Obrázek 6: Půda s původní koncentrací PHB 3 % po 300 dnech biodegradačního experimentu. Bílé částice jsou mikrobioplasty PHB

Odpovědí na výzkumnou otázku 1 tedy je, že normy využívané pro testování biodegradability bioplastů nereflektují dostatečně environmentálně relevantní podmínky vyskytující se v životním prostředí. Navíc i za těchto ideálních podmínek nebylo vždy dosaženo 100 % biodegradace a nebylo jasné, zdali z bioplastu vznikly stabilnější mikrobioplasty, anebo byla část uhlíku imobilizována v SOM (a tím došlo k ovlivnění půdy). Proto se ukázalo, jako nezbytné analyzovat rezidua bioplastů. Nicméně, jak ukázala literární rešerše, v současné době téměř neexistují vhodné metody pro tyto analýzy.

2 Výzkumná otázka 2: Je možné stanovit mikrobioplasty podobnými metodami jako konvenční mikroplasty?

Při vývoji vhodné metody pro stanovení mikrobioplastů v půdě jsme provedli literární rešerši zaměřenou na stanovení konvenčních mikroplastů a pokoušeli se zjistit, zda by některé z nich šly využít ke stanovení mikrobioplastů. Výsledkem této práce bylo critical review (**Příloha A**).

Publikované review ukázalo, že při vzorkování je možné použít stejné postupy jako u konvenčních plastů, protože zde bude docházet ke stejným problémům. Analýzy může nejvíce ovlivnit homogenita vzorku, které je komplikovanější dosáhnout v půdách s většími fragmenty mikrobioplastů. Tyto velké částice dále mohou způsobovat problémy při redukci množství vzorku kvartací, anebo při dávkování vzorku při analýze (například do termogravimetrických pánviček). Proto je výhodné půdu před analýzou přesítovat (alespoň na úroveň tzv. jemnozemě, tj, podsítný podíl při sítování se síty s oky 2 mm) a větší mikroplasty analyzovat separátně. Tímto krokem se ze vzorku odstraní i větší části organických reziduí rostlin, které by mohly interferovat se signálem mikroplastů při analýzách. Vzhledem k tomu, že se mohou mikrobioplasty rozložit v přítomnosti silných kyselin, hydroxidu a dalších agresivních chemikáliích, není vhodné odstraňovat SOM, která může být zdrojem interferencí se signálem analytů. Pro úpravu vzorků půd při analýze mikrobioplastů je tedy možné použít pouze sítování, hustotní frakcionaci a extrakci do organických rozpouštědel (zde je ovšem problém s přečištěním extraktu). Metoda FTIR hojně využívaná při analýzách konvenčních mikroplastů je proto značně limitovaná v případě stanovení mikrobioplastů a je použitelná pouze v případě, že organická složka vzorku neinterferuje se signálem analytu nebo nelze signál interferencí odečíst od signálu vzorku (nejčastěji odečtem signálu blanku) [112]. Při analýzách je výhodné měřit co největší množství vzorků najednou, čímž se sníží příspěvek nehomogenity vzorku k experimentální chybě. Proto lze dosáhnout lepších výsledků při měření v módu FTIR DRIFT (Diffuse reflectance infrared Fourier transform).

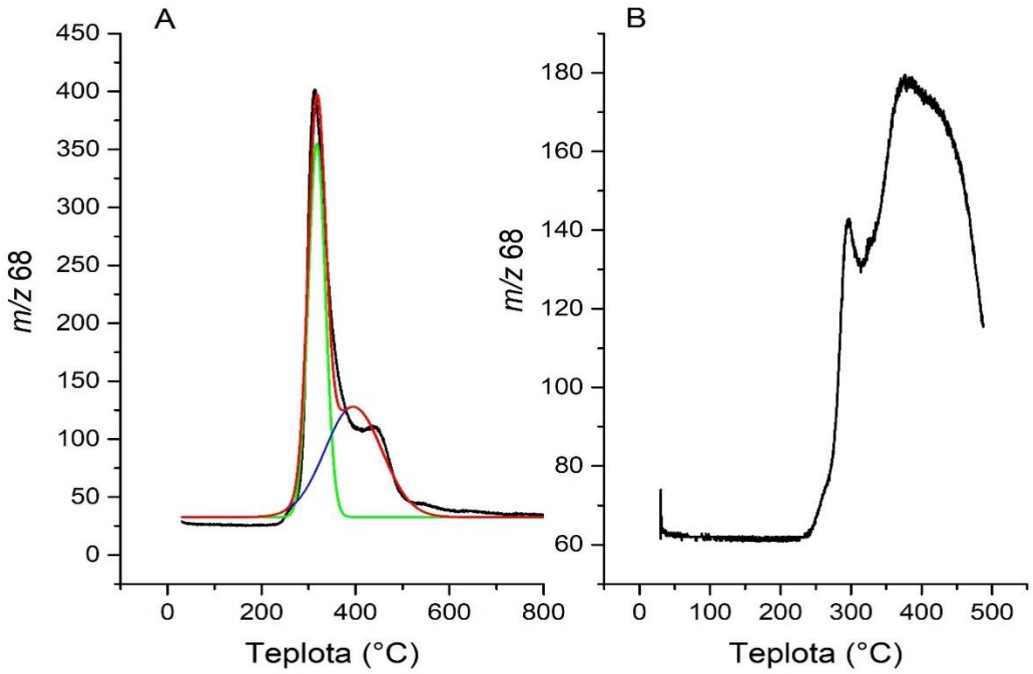
Předúprava vzorků je velmi často časově náročná, a proto je výhodnější zvolit metody stanovení mikrobioplastů, kde je možné analyzovat vzorek přímo bez nějaké další manipulace. Mezi takovéto metody patří metody založené na termickém rozkladu vzorku a následné analýze uvolněných plynů. U těchto metod je možné stanovit pouze množství mikrobioplastu ve vzorku. Tímto postupem tedy není možné zjistit velikost částic, jejich tvar a počet (jako například u FTIR mikroskopie) [112]. Nejjednodušší je analyzovat vzorky, když je k dispozici nekontaminovaná matrice (blank). Toto je právě případ vzorků z biodegradačních experimentů. Pro detekci i stanovení mikrobioplastů v těchto půdách je pak možné využít pouze samotné termogravimetrie, kdy je množství mikrobioplastu určeno přímo z úbytku hmotnosti v teplotním intervalu typickém pro daný mikrobioplast odečteného od úbytku hmotnosti v blanku ve stejném teplotním intervalu (Obrázek 2 v **Příloze E**). V **Příloze E** jsou takto analyzovány vzorky půd po biodegradačním experimentu PHB v různých typech půdy. Při této metodě je limitujícím faktorem malý objem měřících pánviček (200 µl), a také odlišné složení SOM v půdách

se vzorky a v půdách se slepým pokusem z důvodu odlišného chování mikroorganismu v přítomnosti cizích částic.

U reálných environmentálních vzorků není často nekontaminovaná matrice k dispozici, proto je potřeba využít sofistikovanějších metod. V **Příloze B** je využita metoda TG-MS pro stanovení mikrobioplastů PLA a PHB v půdě, při které byly nejdříve určeny charakteristické *m/z* změřeným čistých bioplastů (PHB = 68 a 86; PLA = 29 a 43). Poté byly vytvořeny kalibrační směsi. Pro odstranění experimentální chyby z důvodu nehomogenity vzorků byly půdy spikovány přímo v pánvičkách. Při experimentech nebyly pozorovány plynné produkty termického rozkladu s vyššími *m/z*, což bylo vysvětleno nízkou teplotou transfer kapiláry (maximální teplota vyhřívání u použitého přístroje byla 300 °C), kvůli které došlo ke kondenzaci těchto plynných produktů před tím, než se dostaly do hmotnostního spektrometru. V této práci byl ověřený minimální vliv signálu matrice stanovením závislosti poměru obou charakteristických *m/z* na koncentraci analytu (Obrázek 6 a 7 v **Příloze B**). V případě vyššího obsahu interferující SOM by nebylo možné tuto metodu použít. Kritické to je v případě PLA, u které jsou zvolené m/z tak nízké, že by mohla být matrice snadno jejich zdrojem. V tomto případě by pak bylo nutné použít přístroj s kapilárou vyhřívanou na vyšší teplotu, anebo přístroj s přímým vstupem z termogravimetru do hmotnostního spektrometru. LOQ u tohoto experimentu se pohybovalo v rozmezí 1,82 až 4,72 %, takže je tato metoda vhodná pouze pro silně kontaminované půdy. Tento přístup může být, ale použit pro vývoj metody, kde je místo termogravimetrie použita pyrolýza, při které se analyzují výrazně vyšší množství vzorku (TG-MS využívá typicky desítky mg vzorku u analytické pyrolýzy to můžou být až gramy vzorku).

Nejkomplexnější matricí, se kterou jsme se setkali, byl aktivovaný kal, na kterém jsme také testovali možnosti metody vyvinuté pro půdy. Výsledky této práce již nebyly publikovány v **Příloze B**, budou publikovány separátně později. Tyto vzorky mohou pocházet přímo z čistíren odpadních vod, anebo z vysušených vzorků biodegradačních experimentů ve vodě, kde se aktivovaný kal používá jako zdroj mikroorganismů. Problematické je, že se v buňkách aktivovaného kalu PHB vyskytuje jako zásobní zdroj energie mikroorganismů a je proto nutné při analýze odlišit přírodní PHB od PHB antropogenního původu. V tomto případě je možné využít toho, že přírodní PHB je vázané na proteiny přímo v buňkách a termálně degraduje při vyšších teplotách než PHB antropogenního původu, které se nachází volně ve vzorku. Při těchto experimentech je nezbytné postupovat při vysušování vzorku a homogenizaci velmi opatrně aby došlo k co nejmenšímu porušení buněk bakterií aktivovaného kalu, a tím pádem uvolnění přírodního PHB. Experiment se spikovaným čistírenským kalem byl proveden za stejných podmínek jako u experimentu v **Příloze B**. Na Obrázku 7 jsou výsledky tohoto experimentu, kde v části A je výsledek analýzy aktivovaného kalu spikovaného 0,7 % PHB. Černou čarou je zde označen signál získaný z přístroje pro *m/z* 68. Vzhledem k tomu, že se píky přírodního a antropogenního PHB překrývají, je nutné je oddělit matematickou dekonvolucí. Lze tím získat ostrý pík antropogenního PHB (zelená čára) a přírodního PHB (modrá čára). Červená čára poté znázorňuje sloučení obou píků, u

kterého je vidět nepřesnost při fitování píku přírodního PHB, a proto je zde potřeba zvolit pokročilejší způsob oddělení těchto píků. Na Obrázku 7B je záznam z analýzy blanku, kde je možné také vidět pík antropogenního PHB. PHB se uvolnilo z buněk kvůli nešetrné manipulaci s aktivovaným kalem, která vedla k narušení některých buněk bakterií aktivovaného kalu. Bude proto nutné vyvinout lepší způsob sušení a homogenizace těchto vzorků, které povede k menšímu narušování buněk mikroorganismů.



Obrázek 7: A) záznam z experimentu s vysušeným aktivovaným kalem spikovaným 0,7 % PHB (černá čára), zelená čára je fitovaný pík antropogenního PHB, modrá čára je fitovaný pík přírodního PHB a červenou čárou je označený součet obou píků. B) záznam nespikovaného aktivovaného kalu.

Nakonec jsme tedy vyvinuli metodu založenou na TG případně TG-MS, která může otevřít cestu pro relativně snadné a rutinní analýzy mikrobioplastů v půdě. Její největší slabinou jsou vysoké limity detekce a kvantifikace, které je ale možné snížit analyzováním většího množství vzorku najednou a použití přístroje s kapilárou vyhřívanou na vyšší teplotu nebo, jak předpokládáme, přístroje s přímým vstupem z TG do MS. Pro analýzu mikrobioplastů PHB v aktivovaném kalu je potřeba vyřešit problém s uvolněním přírodního PHB mimo buňky aktivovaného kalu použitím méně agresivní sušící metody a problém se sloučenými píky. Je nutné tedy najít buď vhodné podmínky analýzy (například optimalizovat rychlost ohřevu), při kterých budou odděleny, anebo využit lepší matematický model pro jejich dekonvoluci.

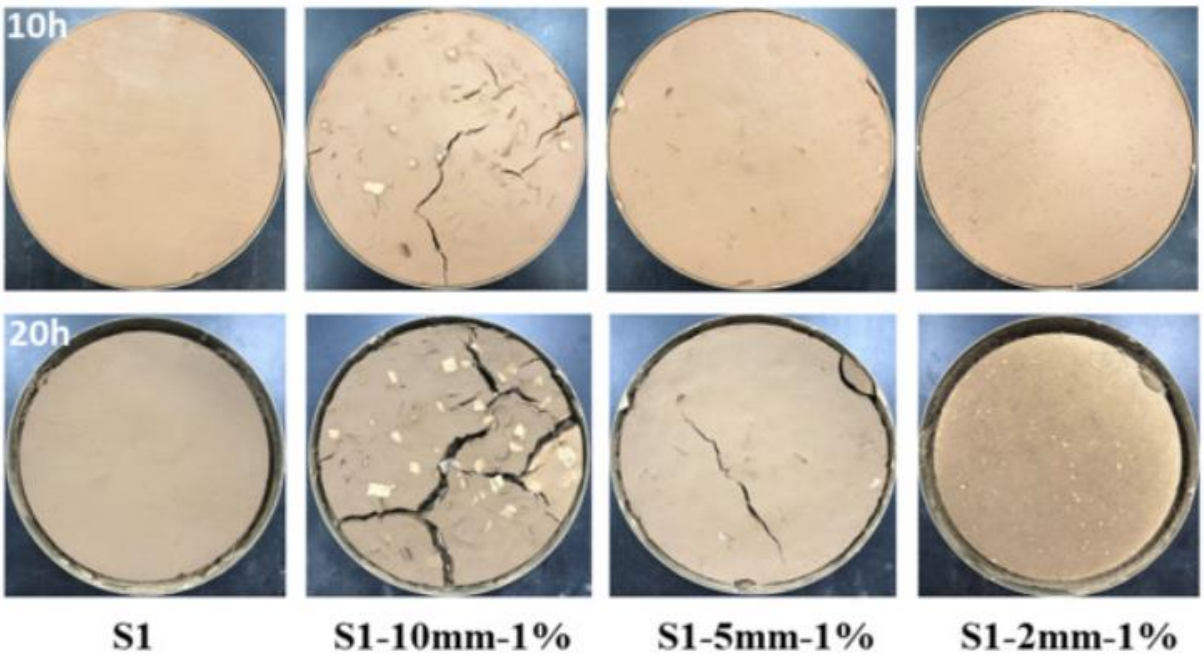
Odpověď na výzkumnou otázku 2 tedy je, že některé metody používané pro stanovení konvenčních mikroplastů je možné použít pro stanovení mikrobioplastů. Je nutné se vyhnout předúpravě vzorků agresivními chemikáliemi, a proto jsou vhodné metody pro tyto analýzy ty, které umožňují analýzu vzorků bez potřeby čištění, extrakce a zakoncentrování analytů. Taktéž nesmí analytické signály matrice

vzorků interferovat se signálem analytů. Obě tyto podmínky splňuje vyvinutá TG, respektive TG-MS metoda, u které je potřeba snížit limity detekce.

3 Výzkumná otázka 3: Je vliv mikrobioplastů na životní prostředí srovnatelný s doposud známým vlivem konvenčních mikroplastů?

Vzhledem k tomu, že se produkce bioplastů neustále zvyšuje a předpokládá se jejich využití v zemědělství, je pravděpodobné, že se tento typ materiálu brzy stane jedním z půdních kontaminantů. Bioplasty jsou také často prezentovány jako ekologičtější náhrada konvenčních plastů pro omezení vzniku mikroplastů. V předchozích kapitolách bylo ale diskutováno, že naopak mohou v některých případech vést k rychlejšímu vzniku mikrobioplastů a proto je potřeba zjistit jaký vliv budou mít mikrobioplasty na půdu v porovnání s mikroplasty. Díky tomu bude možné posoudit, které aplikace bioplastů dávají smysl, a které by mohly být problematické. Vliv mikrobioplastů na půdu může být sledován z různých pohledů, například by tyto částice mohly ovlivnit fyzikálně chemické vlastnosti půdy, půdní živočichy, rostliny a mikroorganismy. Chování mikrobioplastů v půdě se kvůli jejich biodegradabilitě bude měnit s časem, proto je potřeba popsat jejich efekty v době, kdy vstoupí do půdy, v době, kdy bude probíhat jejich biodegradace, a také jak ovlivní půdu po tom, co kompletně zdegradují.

V experimentech v **Příloze C** jsme zjistili, že ihned po vstupu do půdy bude vliv mikrobioplastů a mikroplastů na SOM podobný, protože se při jejich interakcí s půdou uplatňuje pouze jejich velikost a hydrofobicita (která je u zkoumaného PET a PHB podobná) a nikoliv jejich biodegradabilita. Tyto efekty jsou popsány v **Příloze C**, kde jsou experimenty s PET mikroplasty a PHB mikrobioplasty v rašelině vystavené různým relativním vlhkostem. Rašelina byla zvolena z důvodu vysokého podílu SOM, díky kterému je možné lépe pozorovat efekty mikročástic na půdu a eliminovat vliv interakcí mezi mikrobioplasty a minerální částí (minerální) půdy. Jejímu vystavení různým vlhkostem bylo dosaženo různé rigidity struktury SOM a tím pádem i různých forem půdní vody, na kterých byly následně pozorovány efekty mikročástic. Vliv na obsah půdní vody nebyl u obou mikročástic v rámci experimentální chyby pozorován, takže není možné říct, zdali mikroplasty, anebo mikrobioplasty mají na tento parametr nějaký efekt. Na druhou stranu, bylo zjištěno, že přítomnost mikro(bio)plastů v půdě snižuje desorpční entalpii vody a ovlivňuje vodní molekulové můstky. Z tohoto důvodu může dojít k narušení struktury půdy a vysychání půdy, které se v makroskopickém měřítku projevuje vznikem prasklin v půdě. Podobné efekty v experimentech s PE folií v půdě pozoroval i Wan a kol., který následně publikoval Obrázek 8 zobrazující tyto půdní praskliny [176]. Vliv PHB na živé organismy byl studován v rámci interního projektu KInG na žížalách *Eisenia fetida*, u kterých bylo pozorováno nepatrné snížení hmotnosti v akutním testu a snížení porodnosti nových jedinců v reprodukčním testu v testech dle metodiky OECD.



Obrázek 8:Půda vystavena vlivu 1% kontaminaci PE folie o různé velikosti po dobu 10 a 20 hodin [176]

Bioplasty jsou většinou tvořeny pouze atomy C, H a O, vazby mezi nimi jsou snadno metabolizovatelné, a proto bioplasty představují snadno dostupný zdroj uhlíku pro půdní organismy. Výsledkem pak je, že jejich vstup do půdy a následná biodegradace vede k porušení rovnováhy živin z důvodu prudkého nárůstu obsahu půdního uhlíku. V **Příloze D** jsou publikovány výsledky květináčových experimentů se salátem, který rostl v půdě kontaminované různými koncentracemi PHB a s různým obsahem písku po dobu 8 týdnů. Následně bylo analyzováno množství sklizené biomasy, mikrobiální aktivita a druhové složení mikrobiálního biomu. Bylo zjištěno, že v půdě s nízkým obsahem písku (a tedy i vyšším obsahem živin) vedlo přidání PHB do půdy k přednostní spotřebě PHB uhlíku a tím pádem k ochuzení půdy o dusík, což se negativně projevilo na množství vyprodukované biomasy. Přídavek PHB také způsobil nárůst množství mikroorganismů schopných degradovat PHB na úkor ostatních mikroorganismů. Na druhou stranu u půd s větším množstvím písku (60 až 80 %) byl pozorovaný nárůst enzymatické aktivity, který mohl být způsobený tím, že PHB uhlík částečně nahradil funkci SOM, které bylo v této půdě nedostatek. Tudíž nelze říct, že by měla přítomnost PHB v půdě vždy pouze negativní efekty a vždy bude záležet na typu půdy a na množství živin v ní obsažených a na časovém měřítku.

V **Příloze E** jsou uvedeny výsledky experimentu s třemi různými půdami spikovanými třemi koncentracemi PHB (0,5; 1 a 3 % PHB) inkubovanými po dobu 300 dnů v respirometru. Z termogravimetrického stanovení zbytkového obsahu PHB vyplynulo, že v půdě s nejnižším obsahem PHB veškeré PHB zdegradovalo po méně než 200 dnech a je tedy možné pozorovat ovlivnění SOM biodegradací z dlouhodobého hlediska. V půdách s obsahem 1 % PHB dle termogravimetrie právě v době ukončení experimentu došlo ke kompletní biodegradaci a je tedy možné posoudit přímý vliv biodegradace na půdy a v půdách s nejvyšší koncentrací není PHB zcela zdegradováno a je tedy možné

pozorovat vliv dlouhodobé biodegradace na půdu. Analýza půdní vlhkosti v testovaných půdách potvrdila vysychání půd s vyšším obsahem SOM zjištěné v **Příloze C**. U půdy s nižším obsahem SOM došlo při experimentu k mírnému zvýšení půdní vodní kapacity. Termogravimetrické stanovení celkové SOM a jejich frakcí zase ukazuje jaký má vliv na půdu dlouhodobá zvýšená mikrobiální aktivita způsobená mikrobioplasty, došlo zde totiž ke snížení celkové a stabilizované SOM oproti blanku, což naznačuje, že by mikrobioplasty mohly v půdě způsobovat priming effect, kvůli kterému přichází půda o část půdního uhlíku, který je do atmosféry vydýchán mikroorganismy ve formě CO_2.

Podobné jevy byly také popsány v některých studiích s experimenty s konvenčními mikroplasty [96; 102]. To znamená, že příčinou těchto jevů nemusí souviset pouze s biodegradací bioplastů. Při vstupu těchto částic do půdy může dojít k oxidativnímu stresu mikroorganismů, který následně vede ke zvýšení jejich aktivity a tím pádem k degradaci SOM a narušení cyklů nutrientů [102]. Taktéž na povrchu konvenčních mikroplastů dochází k selekci mikroorganismů, čímž vzniká na mikroplastech biofilm (nika) označovaný jako plastisféra [17; 101]. Tyto organismy pak intenzivně produkují extracelulární enzymy, které taktéž ovlivňují vlastnosti půdy [102]. Tyto negativní efekty jsou v těchto článcích popsané často v případech vysokých koncentracích mikroplastů (na rozdíl od našich experimentů, kde je možné pozorovat vliv mikrobioplastů už při koncentraci 0,1 %) a jsou pozorovány jen v některých půdách [76]. Pro určení toho, které způsobují pouze biodegradabilní mikroplasty, a které jsou naopak společné pro mikroplasty a mikrobioplasty je potřeba experimenty zopakovat se zástupci konvenčních mikroplastů.

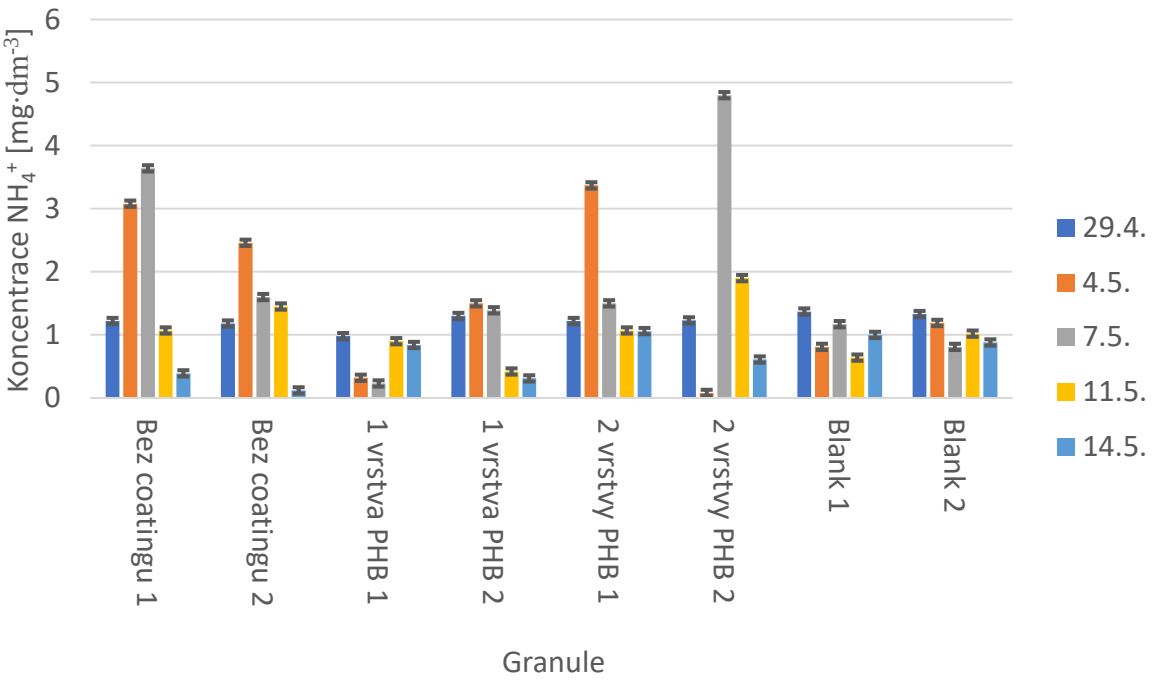
Odpovědí na výzkumnou otázku 3 je, že mikrobioplasty mohou mít podobné efekty na půdní abiotické vlastnosti, především na půdní vodu a strukturu. Stejně tak na obou typech těchto částic se daří jen některým druhům mikroorganismů, a tím ovlivňují mikrobiální diverzitu v půdě v okolí částice. Na rozdíl od konvenčních mikroplastů se u mikrobioplastů prosazují taktéž efekty způsobené jejich biodegradací, tzn. narušení rovnováhy nutrientů v půdě a rychlejší degradace SOM (priming effect).

4 Výzkumná otázka 4: Existují nějaká rizika využití biodegradabilních plastů v zemědělství?

Jak je patrné z odpovědi na výzkumnou otázku 3, otázka 4 již byla částečně zodpovězena. Je zřejmé, že používání bioplastových výrobků může mít za určitých podmínek negativní vliv na zemědělskou půdu.

Dalšími aspekty, které je nutné brát v úvahu je fakt, že již samotné biodegradabilní produkty mohou obsahovat zárodky cizorodých mikroorganismů přímo z výroby. Lze také uvažovat, zda biodegradabilní výrobky nemohou způsobit narušení diverzity půdních mikroorganismů, tím že se na jejich povrchu bude dařit jen některým z nich, anebo může dojít biodegradací ke vzniku mikrobioplastu, který ovlivní fyzikálně chemické vlastnosti půdy a tím i půdní organismy. Tím pádem může jejich aplikace do zemědělské půdy teoreticky vést k nižším výnosům zemědělských plodin, a případně také ke zvýšení objemů použitých pesticidů, což je proti logice ekologického zemědělství.

Jako příklad testování zemědělských výrobků uvádíme v této práci vývoji inteligentního hnojiva, které bylo vyvinuto ve formě granulí obsahujících přibližně 50 % močoviny a 50 % PHB pokrytých různým počtem vrstev PHB (vyvinuto v rámci projektu MPO TRIO Smart Fertilizers) Tyto granule se vyznačují postupným uvolňováním močoviny a mají omezit odplavování části hnojiva dešťovou vodou a tím snížit potřebné množství hnojiva použité na polích a průnik nutrientů do povrchových vod, kde způsobují eutrofizaci. Nejdříve bylo sledováno uvolňování močoviny z granulí v půdním výluhu pomocí stanovení amoniových iontů vzniklých hydrolýzou močoviny. Byly použity granule bez coatingu, s jednou vrstvou PHB a dvěma vrstvami PHB. Experiment trval po dobu 16 dnů. V pravidelných intervalech byl odebírán 1 ml suspenze, který byl přefiltrován a analyzován pomocí analytické sady Merck 114752 Ammonium test (LOQ uvedené výrobcem 0,05 $mg \cdot dm^{-3}$). Na Obrázku 9 jsou výsledky tohoto experimentu. Jsou zde uvedeny všechny granule bez průměrování výsledků, protože u každé granule došlo k uvolnění a spotřebě močoviny v jinou dobu. U granulí bez coatingu je vidět okamžitý nárůst koncentrace amoniových iontů, protože zde měla voda přímý přístup k močovině. U ostatních granulí je možné vidět nejdříve úbytek amoniových iontů, které byly spotřebovány při biodegradaci coatingu a následný nárůst koncentrace těchto iontů uvolněných z granule. U většiny granulí pak byl pozorován dramatický úbytek amoniových iontů pod úroveň blanku, což naznačuje, že veškeré hnojivo bylo spotřebováno na biodegradaci granule.

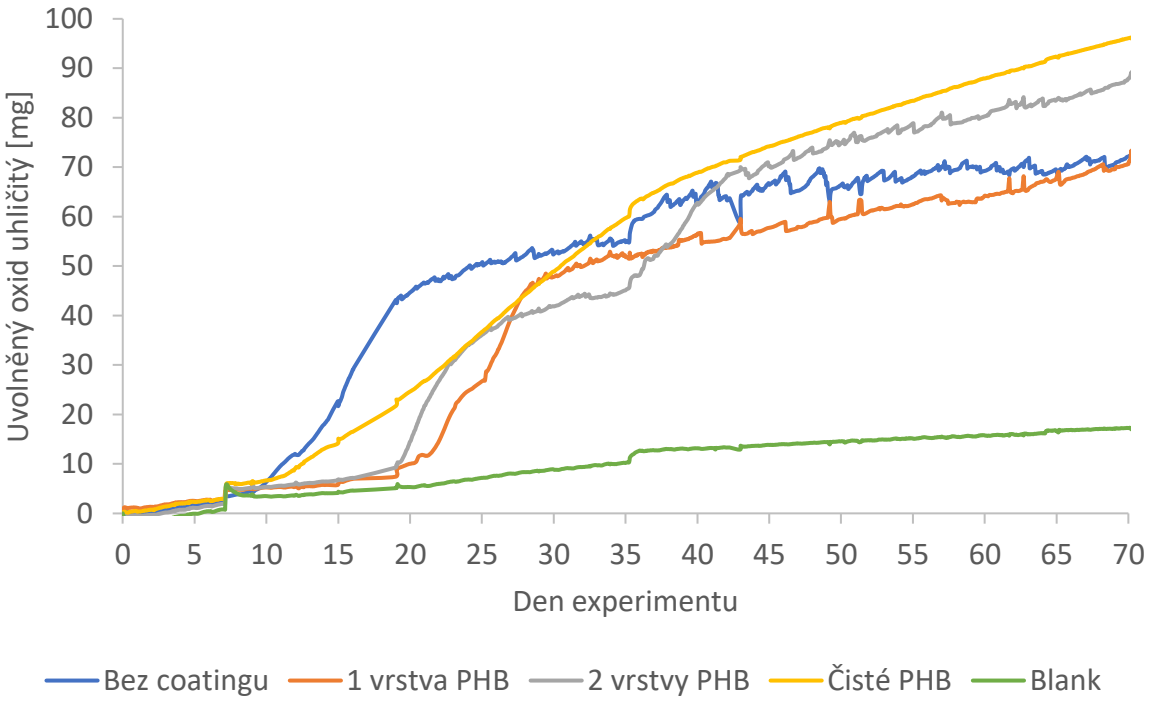


Obrázek 9: Výsledky experimentu s granulemi inteligentního hnojiva. Legenda tohoto grafu obsahuje datum, kdy bylo provedeno stanovení koncentrace amoniových iontů. Experiment začal dne 29.4.

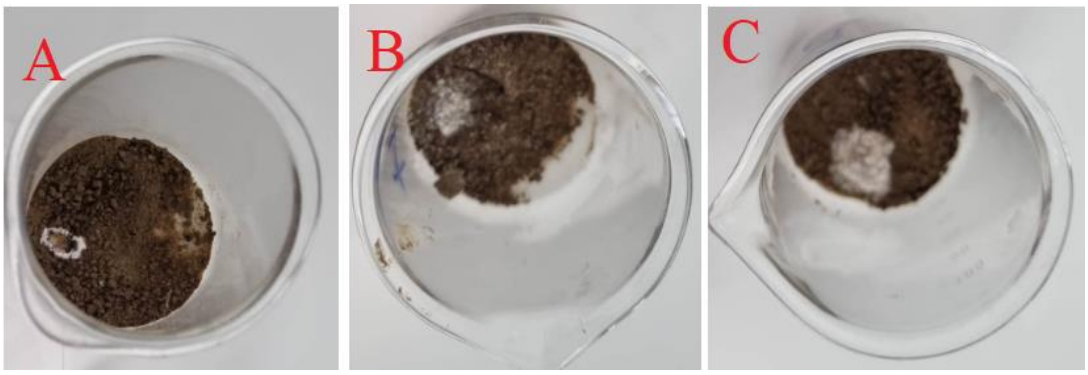
Pro posouzení reálného chování granulí v půdě byly granule inkubovány v půdě v respirometru za stejných podmínek jako při biodegradačních experimentech. Půda byla nejdříve inkubována po dobu 7 dnů, a potom byla přidána granule do každé nádobky s výjimkou nádobek s čistým PHB a blankem. Uvolnění močoviny indikoval prudký nárůst uvolněného CO_2 z důvodu vyšší aktivity mikroorganismů v nadbytku půdního dusíku. Na Obrázku 10Obrázek 10 je zobrazeno uvolňování CO_2 v čase, každá granule, blank a čisté PHB bylo inkubováno ve dvou různých nádobkách a výsledky byly pro přehlednost zprůměrovány. U granulí bez coatingu je pozorován okamžitý nárůst uvolněného CO_2 ihned po vložení granule do půdy. U granulí s jednou vrstvou coatingu je pozorován podobný nárůst se 14denním zpožděním způsobeným degradací coatingu. U granulí se dvěma vrstvami coatingu je jasně vidět, že jedna z granulí měla defektní vrstvu a uvolnila močovinu již po 12 dnech (první prudký nárůst v produkci CO_2), což je srovnatelné s granulemi s jednou vrstvou coatingu a u druhé granule došlo k uvolnění močoviny až po 28 dnech od vložení granule. Je tedy možné říct že jedna vrstva coatingu zpomalila uvolnění močoviny o 14 dnů. Tento experiment probíhal dále po dobu 468 dnů, během něhož nedošlo k úplnému rozložení granulí.

Považujeme za velice důležité, že při vyjmutí půd z přístroje byla ve většině nádobek pozorována plíseň v okolí zbytků granulí (Obrázek 11). Z toho je možné usuzovat, že PHB v půdě upřednostňuje růst hub (plísní), které se v půdě běžně vyskytují a vede k jejich přemnožení. To je v souladu s výsledky z experimentů v **Příloze D**, kde byl zjištěn nárůst plísní oproti ostatním mikroorganismům ve většině vzorků.

První náznaky možnosti snadné kontaminace PHB výrobků jsme také pozorovali v laboratoři při sušení PHB po mokrém sítování, kdy byly některé vzorky napadeny plísní. Vzhledem k tomu, že výskyt této plísně v laboratoři způsobil masivní úhyn v chovu okřehku menšího, bylo nutné sušit PHB v jiné laboratoři. Vizuální analýza naznačila, že se mohlo jednat o *Fusarium oxysporum*, která je přirozenou součástí půdního edafonu a má na některé rostliny silně fytotoxické účinky [177]. V kombinaci s efekty bioplastů na fyzikální a chemické vlastnosti půdy, které byly popsány v předchozí kapitole se ukazuje jako důležité, veškeré bioplastové produkty určené pro zemědělství před použitím důkladně otestovat a aktivně bránit napadení patogenními mikroorganismy. Podobně je tomu samozřejmě i s obalovými a dalšími materiály. Nicméně, toto jsou témata, která přesahují rámec této disertační práce.



Obrázek 10: Uvolňovaný CO_2 při inkubaci granulí chytrého hnojiva v respirometru



Obrázek 11: Granule chytrého hnojiva po ukončení experimentu. A) granule bez coatingu, B) granule s jednou vrstvou coatingu, C) granule s dvěma vrstvami coatingu

Odpověď na výzkumnou otázku 4 tedy je, že využíváním bioplastových produktů by mohlo docházet ke vstupu invazních mikroorganismů žijících na těchto výrobcích, a také ovlivnění mikrobiální diverzity a vznik mikrobioplastů, jejíž vliv na půdu je popsán v Odpovědi na výzkumnou otázku 3. Nejzásadnějším problémem se zdá případ, kdy bioplasty v půdě mohou podporovat růst plísní, které následně povede k větší aplikaci fungicidů na zemědělské půdy.

Závěr

Produkty vyrobené z biodegradabilních bioplastů začínají nahrazovat produkty vyrobené z konvenčních plastů kvůli tlaku ze strany organizací a zákazníků. Spotřebitelé se potom mohou mylně domnívat, že tyto materiály nepředstavují pro životní prostředí žádnou hrozbu, čímž se může zvýšit odhazování odpadků v životním prostředí (littering), bioplastové produkty můžou používat špatným způsobem, a tím mohou neúmyslně zatížit životní prostředí [178]. Kvůli Jevonsovu paradoxu (jev, při kterém úsporné opatření vyvolá větší spotřebu) může také dojít k vyšší spotřebě biobased-plastu a tím i vyšší produkci (bio)plastového odpadu [179], pro jehož recyklaci v současné chvíli neexistuje infrastruktura. Velmi častým způsobem jeho likvidace je kompostování. Takto vzniklý kompost může obsahovat velké množství mikrobioplastů vzniklých nekompletní biodegradací, kterými se může kontaminovat půda. Proto některé kompostárny separují bioplasty z bioodpadu, které poté končí ve spalovnách nebo na skládkách [180]. Taktéž nesprávné využívání bioplastů v zemědělství může být významným (byť relativně krátkodobým) zdrojem mikrobioplastů v půdě [37].

Není zcela objasněno, zdali bude tento odpad stát za masivní tvorbou mikročástic s velkou sorpční schopností, chronickou toxicitou pro živé organismy nebo negativním vlivem na půdu. Současné normy, na základě, kterých jsou nové materiály testovány a certifikovány, neodráží reálné podmínky, kterým budou bioplasty vystaveny v životním prostředí a není v nich kladen důraz na detekci reziduí vzniklých při biodegradaci. Proto je potřeba vyvinout spolehlivou a rychlou metodu pro stanovení těchto částic ve všech složkách životního prostředí, a především v půdě. Jak vyplývá z této práce, metody pro stanovení mikrobioplastů mohou být inspirovány již vyvinutými přístupy, nicméně bioplasty mají svá specifika, která je nutné při volbě metody brát na zřetel. Jde především o jejich chemickou nestabilitu, díky které není možné použít při rozkladu matrice agresivní chemikálie, čímž se významně komplikuje celá analýza a je nutné použít metody, které nevyžadují žádnou předúpravu vzorku. A také přirozený výskyt analyzovaného polymeru nebo jeho monomerů může interferovat s analytickým signálem, který dává mikrobioplastová kontaminace. Zde představené metody (**Příloha B a E**) mají potenciál být základem pro takovouto metodu a je zde představena cesta, kterou by mohly být vylepšeny, aby byly dostatečně citlivé k analýzám reálných vzorků.

Při vývoji nových bioplastových materiálů, anebo výrobků je potřeba důkladně ověřit jejich biodegradabilitu v podmínkách, kde se předpokládá jejich end-of-life a ideálně po biodegradačním experimentu výsledky verifikovat analýzou reziduí bioplastů. Nedílnou součástí certifikace nových produktů by měla být analýza jejich vlivu na životní prostředí, a to při jejich vstupu do testovaného ekosystému, během jejich biodegradace a po jejich biodegradaci. Bez těchto experimentů není možné zaručit bezpečnost jejich používání.

Přestože jsou v této práci prezentovány možné problémy plynoucí z používání bioplastů, musíme zmínit, že jsou právě bioplasty možnou nadějnou cestou k omezení vzniku mikroplastů, které sami o

sobě představují riziko pro životní prostředí. Tato práce má poukázat na slabiny při testování biodegradability a možný vliv bioplastů na životní prostředí, které je nutné brát v úvahu při vývoji nových materiálů. Při tom je tedy nutné nejen zabezpečit jejich konec života (biodegradace, recyklace), ale i definovat limity jejich využívání a podmínky, za kterých je jejich vliv na životní prostředí minimální nebo nejlépe pozitivní, jak je často deklarováno. Proto by měly být biodegradabilní plasty zařazeny do zvláštní kategorie plastového odpadu. Následně je nutné vypracovat strategie na snadné a ekonomicky výhodné metody jejich fyzikální recyklace, která je v současné době komplikovaná (například při recyklaci PHB dochází k jeho degradaci) [43]. Není možné spoléhat pouze na jejich kompostování, které v současné době není ekonomické, může vést ke kontaminaci půdy mikrobioplasty a tudíž nedává příliš smysl [181]. A neměla by se ani opomíjet osvěta laické veřejnosti, jak tyto produkty využívat, a hlavně jak správně zacházet s bioplastovým odpadem.

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Přílohy

Příloha A

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Review

A critical review of the overlooked challenge of determining micro-bioplastics in soil

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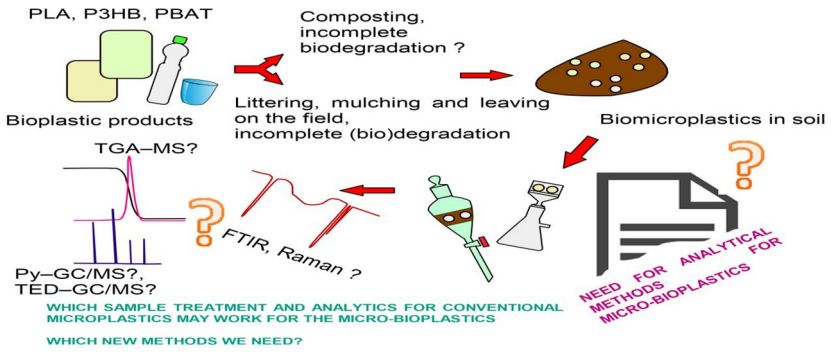
HIGHLIGHTS

- Biodegradable plastics may turn into micro-bioplastics with unknown life-time.
- There are no extraction and analytical techniques for micro-bioplastics in soil.
- Methods of micro-bioplastics analysis are inspired by conventional microplastics.
- Pyrolysis is among the most promising approaches for micro-bioplastics analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

Currently, non-biodegradable oil-based plastics are gradually being replaced by bio-based biodegradable plastics to prevent the formation of microplastics. For biodegradable materials to decompose completely, however, they require specific conditions that are rarely met in ecosystems. Paradoxically, this may lead to the fast production of microplastics from biodegradable materials, i.e. micro-bioplastics. Until recently, the scientific focus has been solely on the estimation of conventional microplastics. As a result, there is a lack of analytical methods for determining the amount of micro-bioplastics in soil. In this review, we address this problem by summarising sample pre-treatments and analytical techniques suitable for the determination of conventional microplastics, which serve as inspiration for the determination of micro-bioplastics from polyhydroxybutyrates, polylactic acid and polybutylene adipate terephthalate in soil. The analytical techniques include both pyrolysis-based techniques, i.e. thermoanalytical and non-thermoanalytical approaches including sample pre-separation and respective detection limits. We conclude that due to the incomplete knowledge of the production rate of micro-bioplastics, fate, sorption properties and toxicity, it is necessary to develop and validate a rapid and suitable method for their determination. Indeed, the use of thermoanalytical approaches seems to be the most promising strategy. Furthermore, we suggest how the development and analysis of micro-bioplastics should be addressed in future research.

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Contents

1. Introduction

In 2016, 335 million tons of plastic materials were produced worldwide (PlasticsEurope, 2017). This was mainly due to their resistance to corrosive chemicals, hydrolysis, microorganisms, heat and light; their low density; and their useful mechanical properties (Bagheri et al., 2017). Some of these advantages can turn into disadvantages in terms of processing waste and environmental pollution, however. For example, only 9% of all plastics produced in the last 65 years was recycled, while 12% was incinerated in waste incinerators and the remaining 79% was landfilled, i.e. stored in the environment (Geyer et al., 2017). Current studies estimate that five trillion pieces of plastics with a total mass of 250 million tons are floating in oceans across the world and that an additional eight million tons are added each year (Andrady, 2011; Guo and Wang, 2019).

Concentrations of microplastics in soil are more difficult to estimate because of complications caused by a multitude of soil biotic and abiotic processes, the interaction of particles with various components of soil, strong matrix effects and challenging extraction methods. A limited number of studies have focused on the determination of soil microplastic concentrations (Rillig, 2012), however, some estimates suggest that microplastic concentrations in terrestrial ecosystems could be 4–23 times higher than in aquatic ecosystems (de Souza Machado et al., 2018). Several authors have estimated that the microplastic content in soils can reach from $5.5 \cdot 10^{-3}$ wt% in protected Swiss floodplain soils (Scheurer and Bigalke, 2018) to 6.7 wt% in Australian urban/industrial area soils (Fuller and Gautam, 2016).

Conventional (oil-based) plastics are supposed to degrade very slowly in environmental conditions (Ward et al., 2019), and their gradual decomposition leads to the formation of mesoplastics (longest dimension 1 to 10 mm), microplastics (1 to 1000 μm) and nanoplastics (1 to 1000 nm) (Hartmann et al., 2018). In general, microplastics are categorised as either primary or secondary (Wong et al., 2020). Primary microplastics are made intentionally and used in cosmetics, cleaning products and abrasive mixtures, whereas secondary microplastics are formed by abrasion or disintegration of larger plastic objects or particles (Laskar and Kumar, 2019).

Once microplastics enter the soil, they become part of complex soil organic matter and mineral substituents, which further increases its persistence (Bläsing and Amelung, 2018). As a result, the particles accumulate in all ecosystems, raising concerns about their negative side effects (Thompson, 2004). The particles are dangerous because of their ability to adsorb and concentrate hazardous chemicals from water, such as pharmaceuticals or pesticides, and thereby serve as carriers of these compounds to organisms (Guo et al., 2019). When reaching certain concentrations, microplastics in aquatic ecosystems can cause chronic reproductive toxicity in microorganisms, higher mortality, food intake problems, endocrine disruption and oxidative stress; immune and nervous system disorders have also been observed (Jaikumar et al., 2019; Bosker et al., 2019; Lu et al., 2016). In general, as the radius of the particles decreases, their active surface increases, which can be assumed to cause greater biological activity and adsorption capacity of nanoplastics. Plastic nanoparticles can also penetrate the cell wall, thereby transporting the adsorbed substances directly to the cells (Ferreira et al., 2019). Information regarding the difference between conventional and biodegradable microplastics is very limited, however, some studies have shown that biodegradable microplastics are better sorbents (Gong et al., 2019; Zuo et al., 2019) and affect natural environments (Green et al., 2016; Chen et al., 2020).

In January 2018, the European Commission responded to the problems of recycling and disposing of plastics by confirming its continued promotion of recycling and identifying the possible benefits and risks of using biodegradable plastics (Haider et al., 2018). Bioplastics are defined according to the source of their material or how long they take

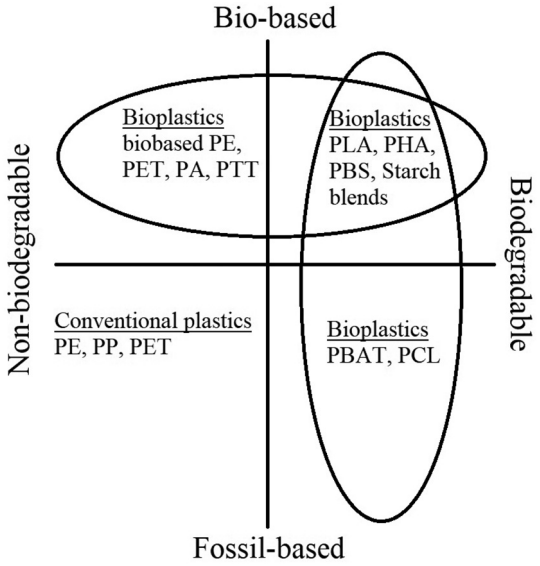

Fig. 1. Types of bioplastics and some examples (Ashter, 2016).

to biodegrade, for example, bioplastics made from plants or animals are called biobased bioplastics. However, not all biobased bioplastics are biodegradable and not all biodegradable plastics are biobased (Ashter, 2016) (see Fig. 1). The biodegradable bioplastics are supposed to undergo significant microbial biodegradation in a short period of time (according to biodegradation standards this should be 180 days) under favourable conditions (temperature, pH, humidity, etc.). The decomposition of these plastics is accompanied by the formation of water, methane and/or CO_2 and biomass. In this review, we focus on problems with the determination of biodegradable bioplastics.

One of the first disciplines to use bioplastics was medicine, which has used biodegradable plastics since the late 1980s as a specific active substance carrier, a replacement for human tissues and as surgical implants (Van de Velde and Kiekens, 2002). More recently, bioplastics have also gained great popularity in 3D printing (Ferreira et al., 2017). As materials, biodegradable plastics are promising alternatives to conventional, mostly non-biodegradable, plastics (Haider et al., 2018). This has led to them being used for food packaging, mulch films, geotextiles, waste bags, cosmetics and clothing (Van de Velde and Kiekens, 2002). In 2017, 2.05 million tons of biobased plastics were produced worldwide, of which 43.2% were biodegradable namely bioplastics (polybutylene adipate terephthalate (PBAT), polybutylene succinate (PBS), polylactic acid (PLA), polyhydroxybutyrates (PHA), starch-, pectin-, chitosan-based blends/composites (Bertolino et al., 2020; Cavallaro et al., 2019) and other biodegradable bioplastics) (Fig. 2). By 2022, this is expected to increase to 2.44 million tons (Agnihotri et al., 2019).

The complete biodegradation of bioplastics occurs only under very specific conditions, the most important being suitable temperature and pH, optimum humidity, a rich environment for microorganisms and sufficient nutrients (Bastioli, 2005). The most suitable environments for biodegradation are therefore compost (temperature, fungi, bacteria) > soil (fungi, bacteria) > freshwater (bacteria) > marine water (dilute bacteria). In fact, only industrial compost meets the optimal and controllable conditions for biodegradation, and in natural ecosystems, biodegradation conditions are often not feasible, leading to a significant slowdown in biodegradation. One of the first and most important steps of degradation is biofragmentation, which is dependent on the type and number of enzymes. Enzymes are produced intracellularly, and relatively specific conditions are required for their excretion.

Paradoxically, if the conditions are not met, microplastics are produced much faster in the case of biodegradable plastics than in conventional oil-based plastics (Sintim et al., 2019). Multiple biodegradation studies have been conducted and only a few achieved complete biodegradation (Emadian et al., 2017). Therefore, it is very likely that most of the unbiodegraded material is fragmented into microplastics or nanoplastics. As there are no analytical techniques for their determination, the presence of PHB biomicroplastics can only be proven by microscopy (Shruti and Kutralam-Muniasamy, 2019), and unlike conventional plastics (Rillig, 2012), the fate of these microplastics (Bläsing and Amelung, 2018) is not studied or discussed in any way.

With growing production and use of bioplastics, this topic clearly needs to be studied further. The first step in deciding the presence and impact of microplastics in the environment is their unambiguous identification and quantification. Although there are currently no analytical methods for the determination of biodegradable microplastics in water, soil or compost (Crawford and Quinn, 2017), it is possible to be inspired, at least methodically, by the research dealing with the analysis of conventional microplastics.

In this review, we focus on three types of biodegradable bioplastics: PBAT, PLA and PHB. PLA and PBAT were selected due their wide use and the controversy regarding their biodegradability and compostability (Tsuji and Suzuyoshi, 2002; Karamanlioglu et al., 2017; Tabasi and Ajji, 2015), while PHB was chosen due its promising potential as food-waste material (Obruca et al., 2010). The number of papers related to selected bioplastics is steadily increasing (Fig. 3). This review does not deal with bioplastics from starch and modified starches (even though they represent about 20% of all bioplastics) because PHB, PBAT and PLA represent bioplastics with a higher potential for microplastics formation according to their life-cycle assessment results as they can be made from waste and their production does not compete with food.

1.1. Biodegradation and biodegradation tests

The International Union for Pure and Applied Chemistry defines biodegradable polymers as polymers 'that are susceptible to degradation by biological activity, with the degradation accompanied by a lowering of its mass' (Vert et al., 2012). As this definition can refer to both water-soluble and biodegradable polymers, however, some other organisations also require the complete mineralisation of the plastics to carbon dioxide, water and biomass when defining biodegradable plastics (CEN/TR 15351:2006) (Haider et al., 2018; Horie et al., 2004). This extra definition has also been adopted by various national and international organisations, such as the International Organisation for Standardisation, to standardise biodegradability tests in fresh water, seawater, soil and compost, such as EN ISO 14851:1999, EN ISO 17556, ASTM D 5338 or ASTM D 6400.

Biodegradation has four stages. The first stage is biodeterioration, where the surface of the material is covered with a biofilm that causes fragmentation into smaller particles. During the second stage, the debris

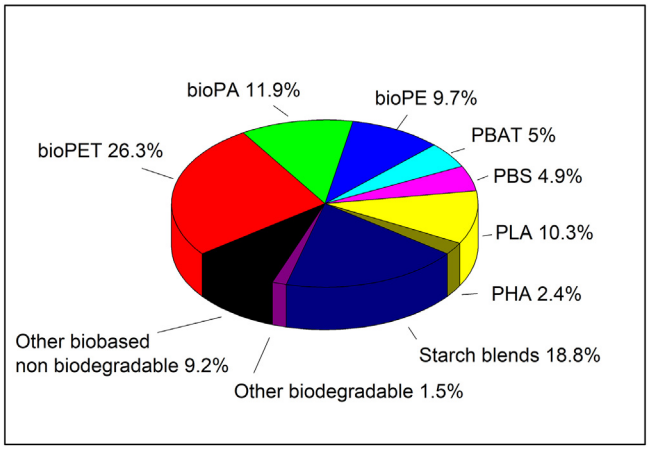

Fig. 2. Worldwide production of biobased plastics in 2017. Modified from Agnihotri et al. (2019).

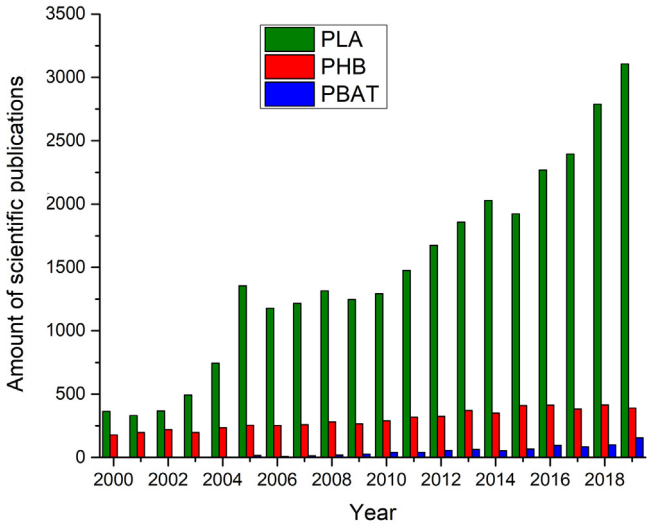

Fig. 3. The number of works related to P3BH, PLA and PBAT from 2000 to 2019.

is depolymerised by extracellular enzymes from microorganisms in the biofilm. In the third stage, the resulting small molecules are transported into the cells of the microorganism, which is called bio-assimilation. Then, in the final stage, mineralisation occurs, producing water, nitrogen and carbon dioxide for aerobic biodegradation and methane for anaerobic biodegradation (Bagheri et al., 2017; Haider et al., 2018).

The simplest way to measure biodegradation is by observing the sample mass loss during the biodegradation test (Chinaglia et al., 2018). This experiment is only suitable for larger pieces of plastics or foils, which means that as these pieces break down into smaller particles, inaccurate results are obtained (Emadian et al., 2017). For this reason, more advanced methods have been developed. For example, tests for aerobic biodegradation are based on the measurement of microbial oxygen consumption (biological oxygen demand), released carbon dioxide or total organic carbon (Emadian et al., 2017; Harrison et al., 2018). In contrast, tests for anaerobic degradation are based on the measurement of methane production (Ryan et al., 2017; Bátori et al., 2018). As a rule, these experiments are conducted under controlled conditions in artificial or well-defined natural test media.

The biodegradability of polymer products should be guaranteed, when a polymer obtains a certificate, e.g. "Vincotte OK biodegradable". The certification is based on a series of tests of biodegradation, disintegration and ecotoxicological safety (Harrison et al., 2018). Nonetheless, these tests do not include seasonal variations or extent of polymer degradation. In addition, the testing environments tend to be defined for a specific purpose. For this reason, aerobic and anaerobic biodegradation in wastewater, freshwater ecosystems and marine ecosystems are tested separately. Distortion of results may also occur due to different approaches to inoculum, insufficient reproducibility of results and a lack of ways to test in uncontrolled environments (Harrison et al., 2018). As a result, the rate of biodegradation is highly dependent on various parameters and also certified materials may not be biodegradable an open environment (Harrison et al., 2018). Indeed, under unfavourable conditions, the biodegradation of bioplastics can take several decades to produce microplastics similar to conventional plastics (Bagheri et al., 2017; Harrison et al., 2018).

1.2. Biodegradable polymers used in agriculture

Plastic mulching has become a global agricultural practice, therefore, the risk of plastic residue remaining in the soil and likely breaking down into microplastics is enhanced in agricultural soils (Steinmetz et al., 2016). 'Plasticulture', as it is known, includes films, foils, nonwoven fabrics for mulching, rods and tubes for tunnel structures, anti-hail and shading nets, seedling pots, stings and clips for tying plants and biodegradable seed covers and discs. About 60% of crops would not thrive without the use of plasticulture and because of this, 1 ha of agricultural soil may leave 80–200 kg of plastics after one season (Siwek et al., 2019). The benefits of mulch use include soil humidity reduction and water retention, which reduces the potential development of fungal diseases as well as lowering the potential of hypoxia in plant roots (Siwek et al., 2019).

Concerns about plastic waste in agriculture have broadened the focus of plasticulture to include biodegradable plastic mulching, which is now gaining popularity. As a result, the current trend is to replace conventional plastics with biodegradable plastics in agriculture (Shruti and Kutralam-Muniasamy, 2019). The latter's biodegradability is rarely 100%, however (Kasirajan and Ngouajio, 2012) (or such biodegradability success is simply uncertain, often due to seasonal changes in temperature and humidity), and they are suspected of contributing to the microplastic issue, especially as they can exhibit the same or similar issues as conventional microplastics (ecotoxicological effects, sorption of persistent contaminants, etc.). Therefore, the possibility of biodegradable plastics forming microplastics in agricultural fields and the environment is presumed (Sintim et al., 2019).

PLA, PHB and PBAT are among the biodegradable materials used in plasticulture. PHB consists of biodegradable and biocompatible polymers that are synthesised by bacteria as energy storage polymers (Obruca et al., 2014; Obruca et al., 2010). PHB can be completely biodegraded in all ecosystems, however, through industrial composting may be biodegraded only up to 78% (Hermann et al., 2011). The most common representative of PHB is poly-3-hydroxybutyrate (P3HB), which is produced by gram-negative and gram-positive bacteria (*Pseudomonas sp.*, *Bacillus sp.*, *Methylobacterium sp.*) under nutrient-deficient stress conditions (Ansari et al., 2016). P3HB is a hydrophobic material that exhibits increased crystallinity, a high melting point and high fragility, which complicates its processing and limits its potential use on an industrial scale (Obruca et al., 2014).

The mechanical properties of P3HB can be improved by adding other polymers (e.g. polylactic acid) to form copolymers (with 3-hydroxyvaleric acid) or by adding plasticisers (Ansari et al., 2016; Obruca et al., 2014). As these additives can significantly slow down the biodegradation of the final product (Bagheri et al., 2017), biodegradation enhancers such as starch are added to these blends. Another factor that makes it difficult to use PHB is its economic demands on production, but cost reduction can be achieved by producing P3HB from waste materials. A very good source of carbon for P3HB synthesising bacteria is rapeseed oil, which also has a high yield. Therefore, in theory, more than 1 g of PHA can be obtained per gram of oil (Obruca et al., 2010). Other promising waste materials include products from the sugar industry and hydrolysate coffee grounds (Obruca et al., 2014).

The second biodegradable polymer of interest is PBAT. PBAT is susceptible to biodegradation via aerobic bacteria and fungi and is suitable for industrial and home composting. According to various studies, the extent of its biodegradation can reach values between 65 and 90 wt% of C degradation depending on the industrial composting conditions. Its largest producer is the Germany-based company BASF (about 75 kt *p.a.*), which trades under the names Ecoflex® and Ecovio® and produces it as a blend with polylactic acid (PLA). PBAT is a synthetic polyester copolymer produced by melting polycondensation from 1,4-butanediol and adipic acid, employing tetrabutoxytitanium as the transesterification catalyst (Zhao et al., 2010; Koltzenburg et al., 2014).

PBAT is claimed to be non-toxic and non-ecotoxic and is industrially compostable in several weeks (n.d.) according to EN 13432 or ASTM 6400 (Siegenthaler et al., 2012). It can also be blended with starches (Shi et al., 2011) and PHAs (Hutníková and Fričová, 2016). It is used for the production of shopping bags, mulching foils for tillage into the soil and various horticulture products such as seed/fertilizer tapes, plant pots and erosion control nets (Siegenthaler et al., 2012) Therefore, there is potential for PBAT particles to be present in soil. Foils can also be blended with PBAT and nitrile rubber for increased toughness (Fernandes et al., 2017), which may put micro rubber particles into the soil. In terms of the medical field, the use of PBAT is very rare and is not predicted to increase.

Another widely used biodegradable polymer is polylactic acid (PLA), which is a biobased aliphatic polyester. It can be biodegraded under aerobic as well as anaerobic conditions by both bacteria and fungi (C degradation between 55 and 95%), but not generally through home composting. In contrast to biodegradation in organisms, non-enzymatic degradation of this biopolymer occurs in the environment (Shimao, 2001). As PLA can be absorbed by human and animal bodies, its use in medical implants is significantly increasing, and it is also very popular in 3D printing (Ferreira et al., 2017; Ezeh and Susmel, 2018). Due to the ease of processing it and its general yield strength and low gas permeability compared to other polymers (biodegradable as well as oil-based), much attention has been paid to this material in the past 20 years (Hamad et al., 2018).

Despite the advantages of PLA, however, it is almost always possible to find better and cheaper oil-based plastics. The price for PLA

pellets currently starts at $500 per tonne (Fahim et al., 2019), but costs can be reduced by adding cheaper biomass-based additives or other agricultural products, such as sawdust or starches. This not only reduces production costs but also speeds up the biodegradation of this plastic material, which otherwise biodegrades very slowly (Wan et al., 2019). Adding some of these materials to PLA is not always in line with sustainable development goals, however, as there may be competition for land as a systemic resource for agricultural food production (Karan et al., 2019).

2. Methods

We performed a literature search on Clarivate Analytics, Web of Science and Google Scholar that focused on the three biodegradable polymers most likely to be used for agriculture (P3HB, PLA, PBAT and their blends), as well as their utilisation, biodegradability and the possible analytical methods leading to their determination in various substrates, possibly in a soil matrix. We did not filter by publication date, and both the abbreviations and complete names of the polymers were searched together with the terms 'microplastics', 'analysis', 'determination', 'assay' and 'characterization'. To obtain an overview of any existing analytical techniques for these polymers, we also included some characterisation methods that are connected to the biopolymers' technology, production and material analysis. The material characterisation and biotechnological analysis were highly specified, however. For comparison, we reviewed analytical methods that have been used in the determination of microplastics from conventional polymers.

3. Determination of conventional microplastics

Man-made microplastics have been introduced into all parts of the environment, including animals (He et al., 2018; Rainieri and Barranco, 2019; Sun et al., 2019; Prata et al., 2019), and due to their harmful effects on living organisms (Jaikumar et al., 2019), quantitative and qualitative methods for their determination are being developed (Guo et al., 2019; Bosker et al., 2019; Elert et al., 2017). This makes it possible to assess the degree of primary and secondary microplastic contamination in different sites and the ability of sewage treatment plants to retain microplastics (Sun et al., 2019).

3.1. Extraction and purification

The matrix containing microplastics often consists of organic and inorganic mixtures (He et al., 2018). The easiest and cheapest way to remove larger particles from the sample is by sieving (He et al., 2018; Sun et al., 2019; Bläsing and Amelung, 2018). If the sample contains excess water, it can be removed by drying, filtration or evaporation. It is also possible to utilise different densities from the rest of the matrix and use density fractionation to separate the microplastics from the sample. Salt solutions are used for this purpose (NaCl, $CaCl_2$, $ZnCl_2$, NaI) (He et al., 2018; Sun et al., 2019; Silva et al., 2018). For the extraction of low-density plastics such as polyethylene (PE) and polypropylene (PP), only distilled water may be used in combination with sonication (Zhang et al., 2018).

Liu et al. (2018) used a saturated solution of NaCl with a density of 1.19 $g \cdot cm^{-3}$ to extract higher density plastics (such as polyethersulfone [PES]), whereas for the extraction of polyvinyl chloride (PVC) and polyethylene terephthalate (PET), Scheurer and Bigalke (2018) used a saturated solution of $CaCl_2$. Due to its higher density, this latter method also has greater efficiency. The disadvantage of this method is the interaction of calcium ions with the organic soil component, resulting in aggregates that complicate further analysis. Imhof et al. (2012) and Liebezeit and Dubaish (2012) developed a separation method for extraction using a zinc chloride solution with a density of 1.6 to 1.7 $g \cdot cm^{-3}$, while Dekiff et al. (2014) and Van Cauwenberghe et al. (2013) used a solution of NaI with a density of up to 1.8 $g \cdot cm^{-3}$ for extraction. The use of NaI and $ZnCl_2$ solutions are very effective due to their high densities, but they are more expensive, generate waste and are more dangerous than using NaCl or $CaCl_2$.

Organic compounds in the sample that could adversely affect the assay can be removed by decomposition (He et al., 2018). As conventional plastics are mostly resistant to an aggressive chemical environment, strong oxidising agents, bases and acids can be used to decompose the organic component of the sample. Indeed, decomposition by catalytic wet oxidation is very common (He et al., 2018; Sun et al., 2019; Silva et al., 2018). Hydrogen peroxide, sodium hypochlorite and Fenton's reagent or solutions of alkaline hydroxides and strong acids are mostly used for this method (Sun et al., 2019; Hurley et al., 2018). This type of degradation is not suitable for a bulk sample, however, as the entire process would take too long. The use of concentrated nitric acid is also not suitable for pH-sensitive plastics such as polystyrene (PS) or nylon (Erni-Cassola et al., 2017).

Hydrogen peroxide decomposition uses a 30% solution of peroxide, but the method is very slow and takes up to seven days to complete. By using Fenton's reagent instead of hydrogen peroxide, the degradation time can be shortened significantly (to 10 min or less) without destroying the microplastics in the sample (Tagg et al., 2017). A mixture of technical enzymes, such as lipases, amylases, proteinases, chitinases and cellulases, can also be used for decomposition (Sun et al., 2019; Silva et al., 2018). Cole et al. (2015) used the enzyme Proteinase-K for this purpose, achieving decomposition higher than 97% (for comparison, 72.1 ± 9.2% decomposition was achieved with the 2 M HCl and 91.3 ± 0.4% was achieved with 10 M NaOH at 60 °C). Meanwhile, Löder et al. (2017) developed a multistep decomposition method combining multiple enzymes (protease, chitinase, cellulase), a surfactant (sodium dodecyl sulfate) and hydrogen peroxide with an efficiency of 98.3 ± 0.1%.

3.2. Qualitative and semi-quantitative determination of conventional microplastics

Qualitative methods for the determination of microplastics can distinguish them from the matrix and other particles (e.g. cellulose, sand and glass) and identify the type of polymer, whereas semi-quantitative methods can determine, for example, the number of microplastics particles per volume or matrix weight or the proportion of microplastic areas occupied by a microscopic substrate. The most commonly used method is visual identification by microscope, which is a quick and simple process that makes it possible to determine the shape, texture and surface type of a particle. Particles smaller than 100 μm cannot be identified in this way, however (Song et al., 2015; Shim et al., 2017). Furthermore, this method may result in the false-positive identification of microplastics or vice versa as they may not be distinguishable from other matrix components (He et al., 2018; Silva et al., 2018).

It is also possible to make a mistake in particle counting when using a Petri dish divided into sectors (Carr et al., 2016). To confirm the composition of the microplastics and its differentiation from other particles, this method must be combined with Fourier transform infrared (FTIR) or Raman spectroscopy. For example, using FTIR Browne et al. (2011) identified polyamides, PES, PE, PP and PVC microplastics in effluent from sewage treatment plants, marine sediments and water from washing machines. Lares et al. (2018) used FTIR and Raman microscopy and identified PES, PE, PA and PP microplastic particles and fibres in sewage sludge and lake water. Further refinement of matrix analyses can be achieved using an electron microscope.

FTIR should be used for the qualitative determination of microplastics larger than 10 μm and Raman spectroscopy can be used to characterise particles larger than 1 μm. Polymers sensitive to photodegradation, such as PVC, are harder to determine using this method, however. Instead, PVC particles can be identified by means of attenuated total reflectance (ATR)–FTIR (Käppler et al., 2018). FTIR

and Raman spectroscopy are complementary and should be selected according to the structure of the microplastics expected in the sample. The FTIR method is used to determine microplastics with a structure where the vibrations of atoms in a molecule change its dipole moment, while Raman spectroscopy can determine microplastics with a structure of molecules where the vibrations of atoms change their polarizability (He et al., 2018).

For quicker determination of microplastics in German sediments, Primpke et al. (2017) used automatised focal-plane-array FTIR microscopy, where each array element works as an independent IR sensor. This allows the measurement of wide fields together with in-house automated data processing and a spectral database comparison. Renner et al. (2017) used ATR–FTIR automated peak detection software to analyse microplastic standards in reference samples, while Serranti et al. (2018) characterised marine microplastics using hyperspectral imaging that consists of a short-wavelength infrared spectrograph and lens with automated data evaluation from various software, a database comparison and principal component analysis statistical evaluation. Piehl et al. (2018) tested agricultural soil for plastic debris using sampling, sieving, filtration and ATR–FTIR and found 0.3 ± 0.4 particles·ha^{-1}. They had to omit all particles smaller than 1 mm, however, due to challenges with the sample pre-treatment.

Destructive methods are increasingly being used for the qualitative analysis of microplastics in various environments, including pyrolysis gas chromatography with mass spectrometry detection (Py–GC/MS), desorption-GC/MS and liquid chromatography (LC). For example, Dümichen et al. (2015 and 2017) used GC/MS for the thermodesorption of PP, PE and PS in fermentation residues taken from a biogas plant and PE, PS and PET in river water. This method is advantageous due to the speed of analysis and low sample consumption (20 mg) but involves high demands on sample homogenisation (Dümichen et al., 2017). To make vibrational-spectrometric microscopy methods quicker and more robust, Hufnagl et al. (2019) used a chemometric approach with a combination of spectral descriptors, such as random decision forest (RDF) classifiers, together with FTIR microscopy with a focal plane array (FPA) detector. In this preliminary study, the authors were able to identify PE, PP, PMMA (polymethyl methacrylate), PS and PAN (polyacrylonitrile) particles in the artificially microplastic-fortified samples of freshwater plankton (Hufnagl et al., 2019).

3.3. Quantitative determination of conventional microplastics

Quantitative determination of microplastics (especially in soil and other complex solid or colloidal matrices) is currently a major analytical challenge. The complexity of the samples makes analysis difficult because of possible interferences with the analyte creating a false-positive or negative signal (David et al., 2019). A quantitative determination is one that can distinguish between plastics and matrices or other materials, gives information about the type of plastics (at least approximately) and indicates the concentration of the microplastics in the chemical sense (i.e. by weight or volume).

A promising quantitative method for the determination of microplastics is Py–GC/MS, where the sample is pyrolyzed in an inert atmosphere and the resulting gaseous products are captured in a solvent or passed (most commonly by capillary) directly to the GC–MS. The advantage is that multiple plastics can be determined in a mixture and the sample can be analysed without any pre-treatment. The disadvantage is that it does not provide information on the number, size and shape of the microplastics (He et al., 2018). Fries et al. (2013) used a Py–GC/MS method to identify sediment from the island of Norderney, where due to strong tidal phenomena, a high accumulation of PE, PS and PVC sediments occur. They also identified additives contained therein (Fries et al., 2013). Nuelle et al. (2014) also used this method to determine PP, PET and PVC in sea sediments. The known methods of quantitative determination of microplastics in solid matrices are summarised in Table 1.

4. Determination of P3HB, PLA and PBAT in various matrices

Introducing biodegradable plastics products into the market could be connected with the formation of microplastics in a short time due to their imperfect or slow biodegradation (Sintim et al., 2019). Although the environmental persistence of biodegradable microplastics should be shorter than that of conventional plastics, they may have similar negative properties (Haider et al., 2018). Methods for the qualitative and quantitative determination of such microplastic particles are not available, which is in stark contrast to the number of methods available for bulk bioplastic materials. These tests would be useful for assessing environmental contamination and for verifying biodegradability tests as it is currently not possible to satisfactorily analyse the test medium for any residues of these materials once the test is over.

Only some of the methods used in conventional microplastics can be used to extract bioplastics from soil or other solid matrices. These are mainly physical processes such as filtration, sieving or sedimentation fractionation (Sun et al., 2019). Aggressive chemicals cannot be used to decompose the organic constituents of the matrix due to the poor resistance of microplastics. One of the alternatives seems to be the dissolution of bioplastics in chloroform (Krishnan et al., 2017). As other organic substances can be co-extracted (mainly humic fractions), however, the extract must be thoroughly purified.

Extraction using ionic liquids, which are more specific for dissolution, could be a promising alternative. Two different strategies can be used. The first is the dissolution of the biomass and the release of microplastics into a solution where they can be easily filtered out (Kobayashi et al., 2015; Lee and Yang, 2016; Dubey et al., 2017), with Kobayashi et al. (2015) indicating recovery of up to 98%. Only samples with a specific matrix can be analysed, e.g. matrices formed by bodies of microorganisms, and it is not yet clear if these methods will work for soil-type matrices. Additionally, the large consumption of ionic liquid has relatively low yields and little solvent recycling. The second strategy involves dissolving a suitable PHA bioplastics in ionic liquid and precipitating it again after filtering out the remaining biomass, as described in the US007763715B2 patent (Hecht et al., 2010).

4.1. P3HB

To determine P3HB (density 1.18–1.26 g·cm^{-3}) using the separation method, it must be depolymerised and determined as a monomer, i.e. hydroxybutyric acid. One possibility is the use of hydrosulfuric methanolysis. This method is not quantitative, however, as by-products such as crotonic acid and propene derivatives may be formed. The yields of this method are between 30 and 40% (Grubelnik et al., 2008; Comeau et al., 1988). Another possibility is cleavage with tetraethoxytitanate and trifluoroacetic acid ethyl ester (TFA). This method is suitable for further GC analysis, whereby the TFA chain may be lost and derivatisation is required (Grubelnik et al., 2008). The most suitable method for P3HB depolymerisation is to use reduction lithium aluminium hydride depolymerisation. The resulting monomer can then be determined using high-performance liquid chromatography with mass spectrometric detection and electrospray ionisation (HPLC–ESI/MS; detection limits range from 4.2 wt% in 10 μL aliquot from 2 mg of polymer) (Grubelnik et al., 2008).

Methods for determining intercellular P3HB in activated sludge cells can also be used to determine P3HB microplastics. These methods are based on the propanolytic or hydrolytic depolymerisation of plastics and the determination of the resulting products by ion chromatography (semi-quantitative, detection limits not stated) (Hesselmann et al., 1999). The determination of extraneous P3HB microplastics in activated sludge complicates the occurrence of natural P3HB that is a part of the cells of the microorganisms in the sludge. In fact, P3HB can form up to 10 times the percentage of the cell weight of microorganisms (Li et al., 2019).

Table 1
Summarised quantitative methods for detection of microplastics in solid matrices.

Microplastic type	Matrix	Pre-separation	Method	Detection results	Reference
Thermoanalytical methods					
PE	Sandy topsoil	None	TED–GC/MS	10,000 ppm (evaluable) 2500 ppm (detectable)	(Dümichen et al., 2015)
PS, PP, tire wear as SBR	Highway street runoff sludge	None	TED–GC/MS	0.2; 0.44 and 0.23 μg LOD respectively	(Dümichen et al., 2017)
PET	Model loamy sand	None	TGA–MS	600–2500 ppm LOD 17,200–184,000 ppm LOQ	(David et al., 2018)
PE, PP, PET, PS, PA6, PVC, PC, PMMA	Fish tissue	Enzymatic & chemical digestion + density separation	CP Py–GC/MS	0.4 to 290 μg (found evaluable)	(Fischer and Scholz-Böttcher, 2017)
PS	Tidal sediment	Chemical digestion + density separation	MF Py–GC/MS	LOD = 3 ng; LOQ = 16 ng	(Fischer and Scholz-Böttcher, 2019)
PE, PP, PS	Model loamy sand	Dissolution in TCB	Filament Py–GC/MS	1–86 ppm method LODs	(Steinmetz et al., 2020)
PET, PS, PVC	Model loamy sand	None	TGA + modeling	*Preliminary modeled*: PET: 3300 ppm LOD, 11500 ppm LOQ; PS: 9100 ppm LOD, 30800 ppm LOD; PVC: 22200 ppm LOD.	(David et al., 2019)
Non-thermoanalytical methods					
PE, PP	Cosmetic products	Extraction, dissolution	HTGPC (high-temperature gel-permeation chromatography)	For conc. 700–3700 ppm RSD 2.3–13.4% and recovery 92–96%	(Hintersteiner et al., 2015)
PC, PET	Landfill sludge	Alkali hydrolysis heating depolymerization	LC/MS/MS of depolymerization products	LOD 0.00832 ppm PC LOD 0.053 ppm PET	(Wang et al., 2017)
PS, PET	Model loamy sand	Extraction by solvents	RP–LC	Recovery >99% PS, >80% PET	(Elert et al., 2017)
PS, PVC	Sea sediments	Not mentioned	TGA–FTIR	LOQ 0.050 mg (PVC), 0.025 mg (PS)	Yu et al., 2019
Several types	Floodplain soil	Density separation, oxidative digestion	FTIR	As low as 5 ppm conc., LOD not specified	Scheurer and Bigalke, 2018

P3HB in bacterial biomass can be quantified using FTIR spectrometry and subsequent statistical data processing using partial least squares regression and further modeling (Kansiz et al., 2000). Arcos-Hernandez et al. (2010) developed an FTIR method to determine PHB in mixed cell cultures in concentrations from 0.011 *w*/w, and the R^2 was between 0.911 and 0.985 (see Isak et al., 2016 for further information), while Samek et al. (2016) employed Raman spectrometry to quantify the P3HB content in *Cupriavidus necator* H16 media with the lowest calibration point being 1.395 μmol·L^{-1}. It should be noted that the latter method only worked for amorphous P3HB. These two publications did not state any limits of detection in their methods, however, the methods were optimised for the determination of P3HB in cells and it is not clear if they would work for the determination of P3HB in soil.

Extracted P3HB can also be dissolved in chloroform when sulfuric acid is added. Crotonic acid is formed, the solution turns brown and the P3HB content can be determined spectrophotometrically (Krishnan et al., 2017). Detection limits were not stated, but the amount of P3HB in the sample should have been between 5 and 50 μg (Law and Slepecky, 1961). For the determination of P3HB in *Synechocystis sp.*, it is possible to use a commercial set that uses enzymatic depolymerisation, with the resulting solution analysed spectrophotometrically (Zilliges and Damrow, 2017). Very efficient extraction is needed to analyse micro-bioplastics in soil as the organic matter can form turbidity or substances with a similar absorption spectrum in the solution.

Karr et al. (1983) used HPLC/UV–VIS to determine P3HB in a *Rhizobium japonicum* culture after complicated digestion with sodium hypochlorite and digestion and depolymerisation to crotonic acid following extraction. The lowest concentration observed was 140 nmol·L^{-1}, and further detection limits were not stated. In contrast, Elhottová et al. (2000) employed GC/MS–MS to determine derivatised products of methanolysis of glycolipid extracts/isolates to determine the P3HB content in sandy loam soil, wastewater treatment sludge and *Bacillus megaterium* CC M 1464 culture. The lowest observed concentration was 1.16 nmol·L^{-1}, and further detection parameters were not published. Monteil-Rivera et al. (2007) used both methanolysis and acid hydrolysis of P3HB to obtain depolymerisation products and hydroxyalkeonic acids and then analysed them via SPME–GC/FID, with LODs from 1.6 to 53 mg·L^{-1}, LOQs of 5.5–175 mg·L^{-1}, recovery of 98–100% and RSDs of 2–8 (Monteil-Rivera et al., 2007). Similarly, Werker et al. (2008) used GC/FID after acid alcoholysis and hexane extraction to determine the P3HB content in activated sludge from various laboratory-scale bioreactors treating wastewater, with the lowest detectable concentration of 14 μmol of 3-hydroxybutyric acid/butyl ester corresponding to 1.2 mg of P3HB.

Torri et al. (2014) utilised offline Py–GC/FID to determine 2-alkenoic acids in bacterial biomass with a LOD of 100 μg (1 wt% of P3HB in biomass) and RSD ≤ 15%, stating that such a method was comparable to methods using methanolysis. Abbondanzi et al. (2017) further developed and reworked this method to enable even quicker and easier in-vial-thermolysis suitable for P3HB amounts of 0.5–2.5 mg (10–50% of P3HB in mixed microbial culture samples) detected by GC/MS. Similarly, Velasco Alvarez et al. (2017) performed a gas chromatography isotope dilution mass spectrometry (GC/IDMS) analysis of P3HB in *Escherichia coli* microbial biomass (after propanolysis and derivatisation) with ^{13}C–P3HB internal standard, with a LOD of 0.01 μg·g^{-1} (or 0.11 nmol·L^{-1}).

Another possibility is the method based on sample thermal decomposition followed by an analysis of gaseous products, which allows direct determination of P3HB in the matrix, similar to conventional microplastics (Nuelle et al., 2014; Yu et al., 2019; David et al., 2018). In the case of P3HB, which has low resistance to thermal degradation, chain cleavage occurs leading to a decrease in molecular weight (Fig. 4). The product of thermal degradation is crotonic acid with a molecular weight of 86.09 g/mol and its oligomers.

4.2. PLA

The mechanism of PLA (density 1.24 g·cm^{-3}) thermal degradation is more complicated and is attributed to random cleavage of the backbone

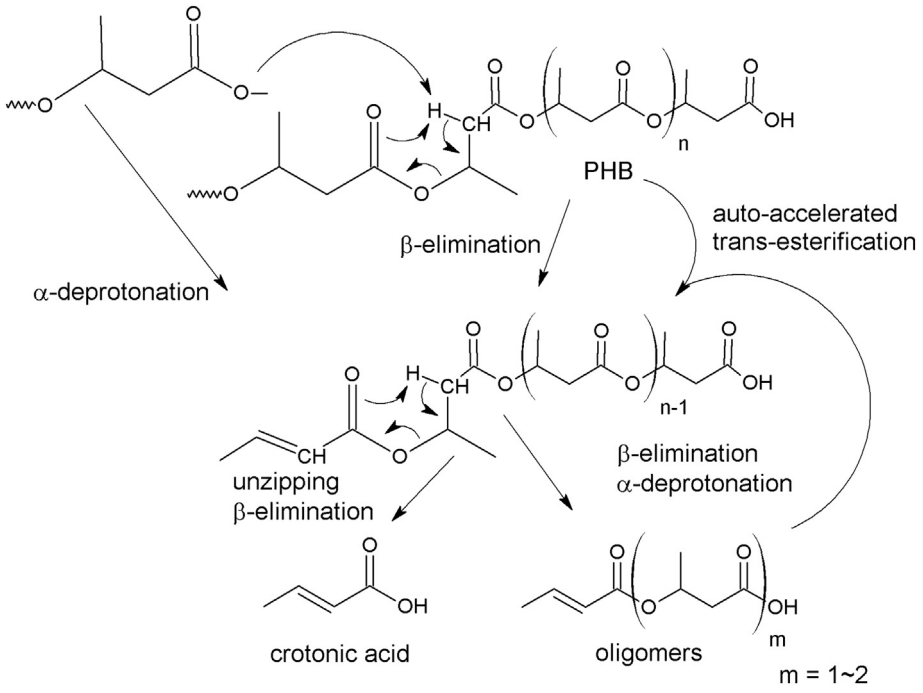

Fig. 4. Mechanism of P3HB thermal degradation (Bugnicourt et al., 2015).

(Hyon et al., 1998), depolymerisation (Gupta and Deshmukh, 1982) and transesterification reactions (Signori et al., 2009). Primary pyrolysis products are carbon monoxide, carbon dioxide, methyl ketene, acetaldehyde, water and cyclic oligomers. The secondary products are methane and butanedione, which are probably products of acetaldehyde degradation. Degradation is also affected by reactive end groups, catalyst residues, unreacted monomers and other impurities. In this case, the use of pyrolysis is more problematic. Pyrolysis can be used only if degradation products are not the same as matrix degradation products. The sample would then have to be pre-treated, which could lead to further problems.

For the determination of PLA as a film without a matrix, Arrieta et al. (2013) developed a sequential pyrolysis method (using Py–GC/MS) with detection limits starting at 0.1 μg according to an analyte, an R^2 from 0.841 to 0.985 and an RSD from 4.68 to 2.65. Moreover, by using the sophisticated hyphenated techniques used in food nanoanalysis, such as ultraperformance liquid chromatography-quadrupole/time-of-flight mass spectrometry (UPLC–QTOF–MS) or ultraperformance liquid chromatography-ion mobility spectroscopy-quadrupole time-of-flight mass spectrometry (UPLC–IMS–QTOF–MS), they were able to analyse various cyclic and linear oligomers of PLA (masses from 257 to 1175) in food simulants (solutions of ethanol and acetic acid) in concentrations around thousands ng/g (Ubeda et al., 2019). Moreover, atmospheric solid analysis probe-ion mobility mass spectrometry (ASAP–IM–MS) enables qualitative differentiation between PLA, PBS and PE in PLA–PBS and PLA–PE blends after the melting of the sample and thermal desorption of the analyte (Barrère et al., 2014).

4.3. PBAT

There have been many material characterisation works published regarding PBAT, but no analytical ones (e.g. how to quantify PBAT within a matrix). Some authors have tried to distinguish PBAT (density 1.25 $g{\cdot}cm^{-3}$) in blends from other polymers (mainly PLA) qualitatively, which can be achieved by differential scanning calorimetry (DSC), thermogravimetry (TG), Fourier transform infrared spectroscopy (FTIR) and elemental analysis (EA) (Zhao et al., 2010; Weng et al., 2013). Similar results obtained by Sikorska et al. (2017) confirmed these results. PBAT can be distinguished from P3HB in blends by utilising ^{1}H MAS NMR and ^{13}C CP/MAS NMR (Hutníková and Fričová, 2016) and Rizzarelli et al. (2016) were able to identify PE qualitatively from PBAT and PLA in PE/PBAT/PLA blends utilising TGA and Py–GC/MS (as well as quantifying PE in terms of 1–10 wt% in the blends). Chen et al. (2016) determined PBAT in blends with PLA and polypropylene carbonate (PPC) qualitatively via MIR (mid-infrared) and Raman spectrometry. They also quantitatively employed NIR (near-infrared) and Raman spectrometry following chemometrical data evaluation in tenths of wt%. R^2 was determined to be between 0.399 and 0.999 and the root mean square error of prediction (RMSEP) values were between 2.54% and 3.58% (Chen et al., 2016).

Recently, Duemichen et al. (2014) performed pyrolysis of polybutylene terephthalate (PBT) via TGA–FTIR, TGA–MS and TED–GC/MS. The question is whether the pyrolysis products characteristic for PBT, i.e. benzoic acid (m/z = 105/122), but-3-enyl benzoate (m/z = 105/77), biphenyl (m/z = 154/76), propyl 4-methyl benzoate (m/z = 119/136) and terephthalic acid di(but-3-enyl)ester (m/z = 203/149) are useful also for determination of PBTA.

5. Directions for future studies

The following research questions regarding the formation of microplastics from biodegradable plastics need to be investigated. This can be achieved by looking at the possibility of applying the existing analytical methods for conventional microplastics to microplastics from biodegradable plastics and developing tailored analytical methods to track the concentrations of microplastics from biodegradable plastics in soil. Then, it can be decided if biodegradable plastics that are left to biodegrade via industrial or household composting and those used in agriculture or littered present a future environmental issue.

Research question 1: Is it possible that rapidly formed microbioplastics also decompose more rapidly than conventional microplastics?

Research question 2: Is the use of mulch foils and composting bioplastic products and packaging another source of microplastics in the environment, especially in soil?

Research question 3: Is the impact of micro-bioplastics on the environment comparable to the known effect of conventional microplastics?

Research question 4: In what ways is it possible to identify micro-bioplastics in the soil and to differentiate them from conventional microplastics or even from each other? Are the analytical methods for determining the content of bioplastics or conventional plastics suitable for this?

6. Conclusions

Products made from biodegradable bioplastics are beginning to replace products made from conventional plastics due to pressure from legislation, organisations and customers. Consumers may also mistakenly believe that these materials are not a threat to the environment, which can lead to increased littering. For example, studies have shown that many people do not know how to handle bioplastic waste and can unintentionally harm the environment with it (Taufik et al., 2020). According to Jevons' paradox (a phenomenon where precautionary savings will generate more consumption), biobased plastics may also be consumed more and thus the production of (bio)plastic waste will rise (Alcott, 2005). This is a material for which there is currently no recycling infrastructure.

Composting is a very common method of bioplastic disposal, but the compost formed may contain many micro-bioplastic particles resulting from incomplete biodegradation, through which the soil may be contaminated. This has led to some composting plants separating bioplastics from biowaste, meaning they end up in incinerators or landfills together with conventional plastics (Barrett, 2019). It is not entirely clear if this waste will be responsible for a massive microparticle formation with high sorption capacity or chronic toxicity to living organisms. Therefore, a reliable and rapid method for the determination of these particles in all environmental situations, especially in soil, must be developed.

As is evident from this review, methods for determining micro-bioplastics could be inspired by the approaches already developed for conventional plastics, however, bioplastics have specificities that need to be considered when choosing the appropriate method. This is mainly due to their chemical instability, which makes it impossible to use aggressive chemicals when decomposing the matrix. This significantly complicates the analysis and requires methods that do not involve any pre-treatment of the sample. Also, the natural occurrence of the analysed polymer or its monomers may interfere with the analytical signal that the micro-bioplastic contamination gives.

Until now, it has been unknown which extraction methods and analytical methods should be developed or re-adopted for the determination of bio-microplastics in solid environmental matrices, particularly in soil.

Based on the reviewed techniques, it can be concluded that the best methods for the analysis of conventional microplastics are the methods based on pyrolysis and thermal analysis (TGA–MS, TGA and chemometrics, Py–GC/MS and TED–GC/MS). For extraction, only mild techniques can be used so as not to deteriorate and exclude the analyte. As for a Py–GC/MS with the injection of a liquid sample to the pyrolyzer, suitable solvents have to be found to dissolve the maximal amount of the biopolymer but a minimal amount of the matrix.

From the known characterisation methods for the biopolymers, for P3HB, the selective depolymerisation and determination using HPLC–ESI/MS according to Grubelnik et al. (2008) is promising. Another option is methanolysis and acid hydrolysis of P3HB to hydroxyalkeonic acids and subsequent analysis via SPME–GC/FID (Monteil-Rivera et al., 2007). For PLA, so far, only UPLC–IMS–QTOF–MS seems to be convenient (Ubeda et al., 2019), but this is not accessible to most labs.

As for analysis of PBAT, no analytical techniques are yet known, except for the material-characterisation ones. Here, the results of analysis of identification of PBAT and PLA in PE/PBAT/PLA blends utilising TGA and Py–GC/MS as published by Rizzarelli et al. (2016) might be a good starting point. Also, applicable seem to be use of pyrolysis products of polybutylene terephthalate (PBT) published by Duemichen et al. (2014).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Příloha B

molecules

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Article

A Simple Method for Quantification of Polyhydroxybutyrate and Polylactic Acid Micro-Bioplastics in Soils by Evolved Gas Analysis

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Abstract: Conventional plastics are being slowly replaced by biodegradable ones to prevent plastic pollution. However, in the natural environment, the biodegradation of plastics is usually slow or incomplete due to unfavorable conditions and leads to faster micro-bioplastic formation. Many analytical methods were developed to determine microplastics, but micro-bioplastics are still overlooked. This work presents a simple method for determining poly-3-hydroxybutyrate and polylactic acid micro-bioplastics in soil based on the thermogravimetry–mass spectrometry analysis of low molecular gases evolved during pyrolysis. For the method development, model soils containing different soil organic carbon contents were spiked with micro-bioplastics. Specific gaseous pyrolysis products of the analytes were identified, while the ratio of their amounts appeared to be constant above the level of detection of the suggested method. The constant ratio was explained as a lower soil influence on the evolution of the gaseous product, and it was suggested as an additional identification parameter. The advantages of the presented method are no sample pretreatment, presumably no need for an internal standard, low temperature needed for the transfer of gaseous products and the possibility of using its principles with other, cheaper detectors. The method can find application in the verification of biodegradation tests and in the monitoring of soils after the application of biodegradable products.

Keywords: microplastics; biodegradable plastics; micro-bioplastics; soil; evolved gas analysis

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1. Introduction

Due to the steadily increasing usage of plastic materials, microplastic particles (microplastics, MPs) of 1–1000 μm in dimension are entering all environmental compartments [1–5]. The most affected environmental compartment is soil, where the microplastic concentrations were estimated to be up to 23 times higher than in aquatic ecosystems [6]. A major source of microplastics in soil is agriculture, where they are formed due to the deterioration of mulch foils, tunnels, holders and other plastic structures [7]. Microplastics can also enter soil via the application of compost or wastewater sludge [8–11]. Another source of MPs in soil is the abrasion of particles from tires (rubber particles) and subsequent atmospheric deposition [12,13].

For this reason, there is growing concern about the environmental impact of microplastics on the soil biota. For example, it was found that poly(ethylene) (PE) microplastics in soil negatively affect spermatogenesis in earthworms [14], and that springtail mobility in soils is reduced by microbead and microfiber contamination [15]. Moreover, negative effects of MPs were also observed on plant root growth [16,17]. Soil microbiota is also

negatively affected by MPs [18], either by the inactivation of soil enzymes [19] or by the enrichment of certain strains on the surface of microplastics, which shifts soil functional properties [20]. These effects are complex and not yet fully understood.

The effects on the abiotic components of soil are not understood either [21]. MPs, when present in soil, may reduce the water stability of soil aggregates and change the bulk density [22] or soil density in the rhizosphere [20]; they may also reduce >30 µm soil pore volume [23] or cause soil drying and cracking [24]. Furthermore, all microplastics discursively increase total soil organic carbon, thus biasing soil analysis results [25].

Bioplastics were introduced to prevent overall plastic contamination. Bioplastics can be degraded in the environment by micro-organisms to water and carbon dioxide in aerobic degradation or to methane, carbon dioxide and water in anaerobic degradation [26]. For quick and complete degradation, suitable conditions are required (count of micro-organisms, appropriate pH, temperature, humidity, soil particle size, etc.), which are rarely met in natural environments. Thus, frequently, only the accelerated breakdown into smaller particles of bio-microplastic particles (BMPs) occurs [27,28]. The source of BMPs in soil may then be agriculture through the imperfect biodegradation of slow-release bio-based or -coated fertilizers, mulching foils and the application of contaminated compost [29–31]. Soil contamination can also occur through consumers' improper management of bioplastic waste, such as littering or home composting [32].

Currently, most of the world's scientists focus on the effects of conventional microplastics on the environment. However, BMP effects on different ecosystems are overlooked, and there is no information on whether replacing conventional plastics with biodegradable ones has more benefits than drawbacks [33]. Ecotoxicological tests on *Daphnia magna*, lettuce and tomato plants showed that BMPs have similar or even worse negative environmental effects than conventional microplastics [34–36]. However, on the adsorption of pollutants onto the surface of BMPs and conventional microplastics, i.e., on whether BMPs have comparable or even worse properties than conventional microplastics, very few studies have been published so far [37].

Not all the available assays developed to determine conventional microplastics can be used to determine BMPs in soil. Aggressive reagents that could irreversibly alter the properties and BMP content of the sample should be avoided. Grubelni et al. developed a method to analyze poly(hydroxybutyrate) (PHB) MPs in soil, using depolymerization followed by the determination of the crotonic acid and propene products using liquid chromatography coupled with mass spectrometry (LC/MS) [38]. Low recovery (30–40%) and the requirement of a pure sample make this method unsuitable for environmental analyses. Arcos-Hernandez et al. used Fourier transform infrared spectrometry (FTIR) to determine the PHB content in bacterial cells [39], and Krishnan et al. extracted PHB into chloroform, with PHB being decomposed upon the addition of sulfuric acid, yielding brown crotonic acid, which was subsequently determined spectrophotometrically [40]. Both methods require sample pretreatment, complicating the case of bioplastics in soil. Pyrolysis-based techniques are a promising way to avoid any manipulation of the samples. The pyrolysis products of both PHB and PLA bioplastics are well defined [41–43]. Pyrolysis coupled with gas chromatography and mass spectrometry (Py-GC/MS) was used to characterize the composition of polyhydroxyalkanoates produced by bacteria, but it was never applied to determine bioplastics in soil [44].

Analytical approaches for the determination of conventional microplastics are inspirations for the determination of BMPs [27]. For microplastic determination, the studied soil is usually dried [45]; high drying temperatures are not recommended, because they cause changes in the structural properties of the polymers [46]. Since the soil matrix contains large amounts of interfering organic matter, sample purification and analyte extraction are necessary for most of the methods [47]. The simplest and cheapest sample pretreatment method is sieving, which can remove larger particles from the analyzed soil [48]. Suitable density fractionations with water/saturated salt solutions are used to separate microplastics from the solid matrix [47,49,50]. The organic component that these processes cannot

remove must then be decomposed, but such decomposition may be performed only if the analytes are resistant to the digestion agent [51]. Catalytic wet oxidations with various reagents are generally used for this purpose [52].

A suitable analytical method for determining microplastics in soil must apply to MP sizes and to microplastics with different compositions, properties, shapes, ages and additive contents [53]. Destructive and non-destructive methods can be used to quantify microplastics in soil. Thermal analysis combined with mass spectrometry (MS) is a typical representative of the former. In this technique, the sample is thermally decomposed without air, and the gaseous products of this reaction are analyzed by MS (pyrolysis directly coupled with gas chromatography with mass spectrometric detection (Py-GC/MS)) [54]. E. Käppler et al. used Py-GC/MS to determine PE, PS, poly(ethylene terephtalate) (PET) and PP microplastics and microfibers in river sediments [55]. Thermogravimetry (TG) can also be used for pyrolysis purposes, as shown by David et al., to determine PET microplastics in soil [56]. The second approach is thermoextractive desorption followed by GC/MS. Here, thermal degradation is carried out in an inert atmosphere; then, its products pass through a solid-phase adsorption bar and are followingly thermally desorbed into the GC/MS detector. This method is suitable for larger sample volumes and can separate the degradation products evolved from labile soil organic matter molecules. Dümichen et al. determined PP, PE and PS microplastics in residues from fermentation tanks and PE, PS and PET microplastics in river sediments by thermal desorption gas chromatography with mass detection (GC/MS) [57,58], while Goßmann et al. used this technique to determine microplastics resulting from tire abrasion [59].

Microplastics in soil can also be extracted with a suitable solvent and then determined in solution by means of high-performance liquid chromatography (HPLC) or quantitative proton nuclear magnetic resonance (q-^{1}H-NMR). To quantify PET and polycarbonates, Wang et al. used alkaline depolymerization followed by liquid chromatography with mass spectrometric detection LC/MS [60]. The disadvantage of this technique is incomplete depolymerization, which leads to high uncertainty in the analyses. Nelson et al. extracted the micro-bioplastics formed from poly(butylene adipate-co-terephthalate) (PBAT) mulch foil into deuterated chloroform using various methods and then determined them by q-^{1}H-NMR [61]. According to the authors, this analysis is simple, does not require any sample pretreatment and is suitable for the fast and precise determination of microplastics in soil.

The most used non-destructive methods for the determination of microplastics in soil are vibrational spectroscopy techniques. Their advantage is the possibility of determining the number and shape of the particles, though they are limited by the needs of a blank and of drying the sample [53]. Fourier transform infrared with attenuated total reflection (FTIR-ATR) is the most used method to determine microplastics in soil, since it allows one to analyze larger particles and does not require any special sample pretreatment [54]. Raman spectrometry can be used as a complementary method to FTIR. The advantage of this technique is that it can analyze samples containing water, which is a strong interferent in FTIR. Nevertheless, interference occurs due to the inorganic and organic components contained in soil. Therefore, it is necessary to remove the interfering components of the sample using the pretreatment methods mentioned above, which means that neither of the two methods is suitable for the determination of micro-bioplastics in soil [53].

This work aims to develop a simple, rapid, robust, solvent-free, scalable and cheap method that would be suitable for detecting and quantifying PHB and PLA micro-bioplastics in soil, which would be suitable for the assessment of the contamination of agricultural soils and the verification of biodegradation experiments. The method of choice is thermogravimetry coupled with mass spectrometry (TG-MS), which was already demonstrated to determine conventional microplastics with no special sample pretreatment [56].

2. Materials and Methods

2.1. Materials Used for Experiments

Two plastics with the greatest future potential, PHB and poly(lactic acid) (PLA), were used to develop this method. PHB was in powder form (most particles between 64 and 125 µm) from Y1000P (TianAn Biologic Materials, Ningbo City, China). Ingeo 4060D PLA from NatureWorks (Minnetonka, MN, USA) was obtained in granules. To obtain microplastics, the granules were cooled with liquid nitrogen and ground with a shear mill to particles smaller than 1 mm [62].

2.2. Soils Used for Experiments

In total, five soils were used. The first soil was LUFA 2.2 soil (LUFA, Speyer, Germany) (see Table 1 for properties). Next, two soils collected in Siberia in 2010 were used for analyses with PHB (Table 1). Last, soils from the vicinity of the villages of Šaratice and Postoupky in South Moravian Region (Czech Republic) were used for PLA analyses (Table 1). After being received, all soils were air-dried and sieved through a 2 mm sieve.

Table 1. Properties of soils used for experiments.

Soil	Soil Type	C_{org} (%)	Sampling Location
P84	Luvic cambisol	0.59 ± 0.24	49°31′81.36″ N 17°35′88.31″ E
P185	Phaeozem	1.47 ± 0.69	49°11′74.64″ N 16°80′35.13″ E
LUFA 2.2	Loamy sand (lS)	1.77 ± 0.56	n.a.
HS5	Protocalcic chernozem	4.88 ± 0.05	55°33′12.96″ N 84°08′06.60″ E
HS45	Albic Luvisol	6.70 ± 0.25	56°51′33.43″ N 83°04′26.71″ E

2.3. Preparation of Calibration Mixtures

Calibration mixtures of soil and MBPs were prepared directly in 85 µL alumina TGA crucibles (NETZSCH, Selb, Germany) for better homogenization, using Mettler AE240 analytical balances. A total of 40 mg of soil was always mixed with MBPs to achieve the desired wt% concentration. For PHB, a concentration series of calibration mixtures was prepared in LUFA with PHB content from 0.19 to 3.04%; in HS5 soil, from 0.9 to 2.95%; and in soil HS45, from 0.41 to 3.08%. For PLA, a concentration series of calibration mixtures was prepared in LUFA with PLA content from 0.09 to 5.00%; in P84 soil, from 0.21 to 5.30%; and in P185 soil from 0.05 to 5.09%.

2.4. Evolved Gas Analysis System

The analysis was performed using a Netzsch Jupiter STA 449 F1 thermal analyzer (NETZSCH, Selb, Germany) connected by a 3 m long heated capillary with an internal diameter of 60 µm to an Agilent 5977B MS MSD (Agilent Technologies, Santa Clara, CA, USA). The ionization source used for the experiments was electron impact ionization, and the data were recorded in selected ion monitoring mode. The gaseous products went directly into the MS. Each calibration mixture was measured only once due to the impossibility of preparing an identical mixture in the pan. Instead, multiple calibration mixtures were created to generate calibration curves. The measurements were carried out in an inert argon atmosphere with a 50 mL/min flow rate. The capillary connecting the TG and MS systems was heated to 120 °C to avoid the frequent problem of high molecular-degradation-product sorption and desorption on the capillary (no need to use an internal standard), to achieve the most economical and transferable conditions and to use older and less heated capillaries for the measurements. The PHB measurements were carried out at a

10 K/min heating rate from 36 °C to 500 °C. The PLA measurements were also performed at a 10 K/min heating rate from 36 °C to 560 °C.

2.5. Evolved Gas Analysis System

First, pure bioplastics were measured to determine specific pyrolysis products that enabled them to be detected. To determine the m/z of PHB degradation products (for degradation products and MS fragments, see Figure S1, Supplementary Materials), 2.90 mg of pure PHB was measured first, then 45.51 mg of pure soil was measured as a blank; finally, soil containing 2.95% PHB (containing 1.31 mg of PHB) was measured. To select the suitable m/z for PHB detection, the blank signal had to be negligible, and the peak maxima for pure PHB, PHB-contaminated soil and PHB mass loss from thermogravimetry (in which case there was a small lag because of the delay in the transfer capillary) had to correspond with each other. These conditions were met for m/z 68 (Figure 1a), which corresponds to the crotonic acid fragment, and m/z 86, which corresponds to crotonic acid (Figure 1b).

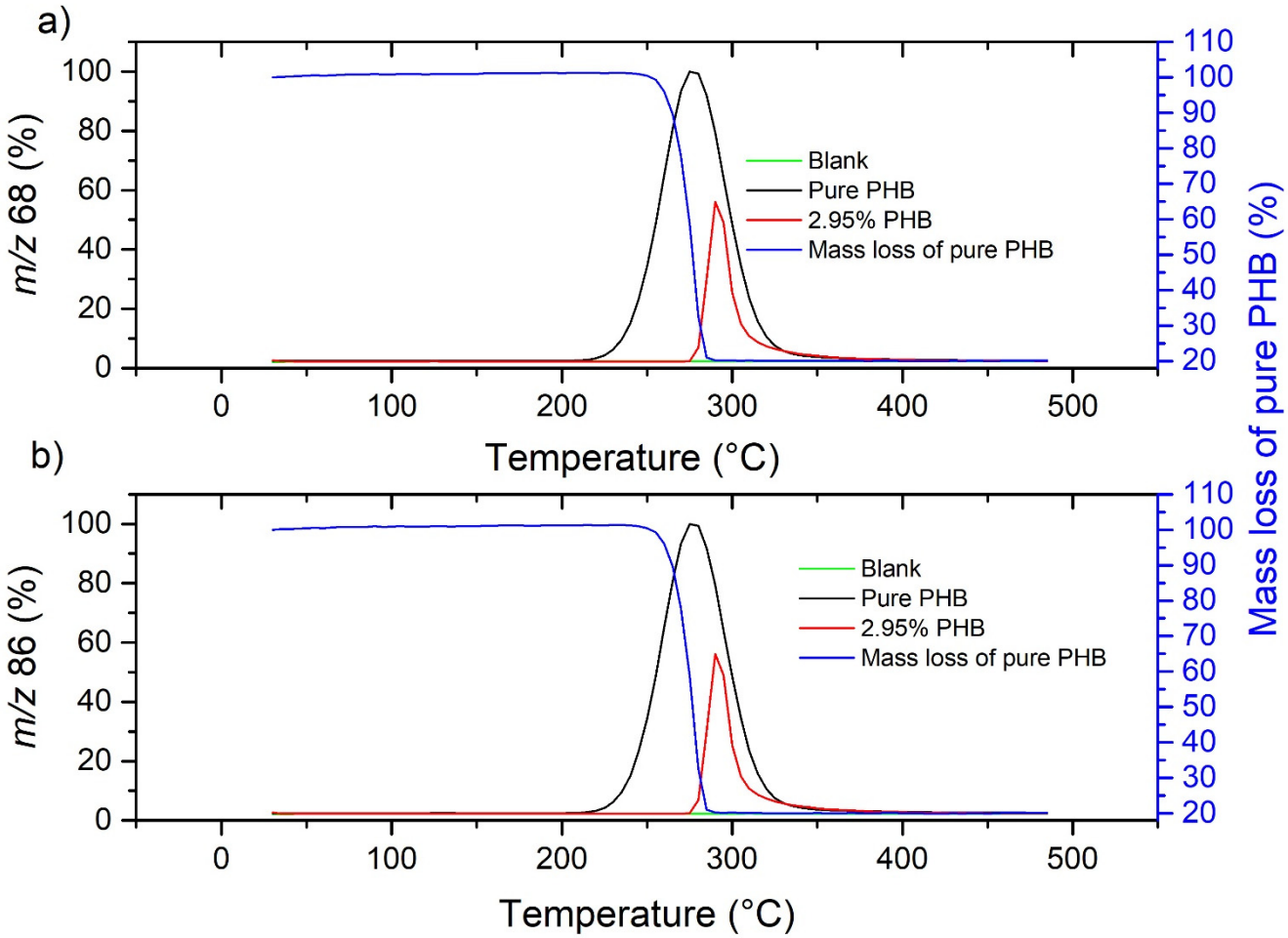

Figure 1. Plots of the peaks at m/z 68 (**a**) and 86 (**b**) of blank, pure PHB and HS5 soil containing 2.95% PHB suitable for PHB detection.

To determine the m/z of PLA degradation products (for degradation products and MS fragments, see Figure S2, Supplementary Materials), 35.14 mg of pure PLA was first measured, then 39.32 mg of pure soil was measured as a blank; finally, soil containing 5.30% PLA (containing 2.21 mg of PLA) was measured. In addition, to select the appropriate m/z for PLA detection, the blank signal had to be negligible and the peak maxima for pure PLA, PLA-contaminated soil and PLA mass loss from thermogravimetry had to correspond with each other (in this case, there was a small lag because of the delay in the transfer capillary). These conditions were met by m/z 29 (Figure 2) and m/z 43, which are acetaldehyde

fragments. m/z 44 was not used for detection, even though it is an abundant ion for acetaldehyde because it is commonly evolved during pyrolysis of other microplastics and organic compounds. The monomer (m/z 56) and oligomers (m/z 100 and 128) of PLA observed by Arrieta et al. were not detected, probably due to condensation or sorption of these pyrolysis products in the capillary, as described previously by Dümichen et al. and Schindler et al. [63–65].

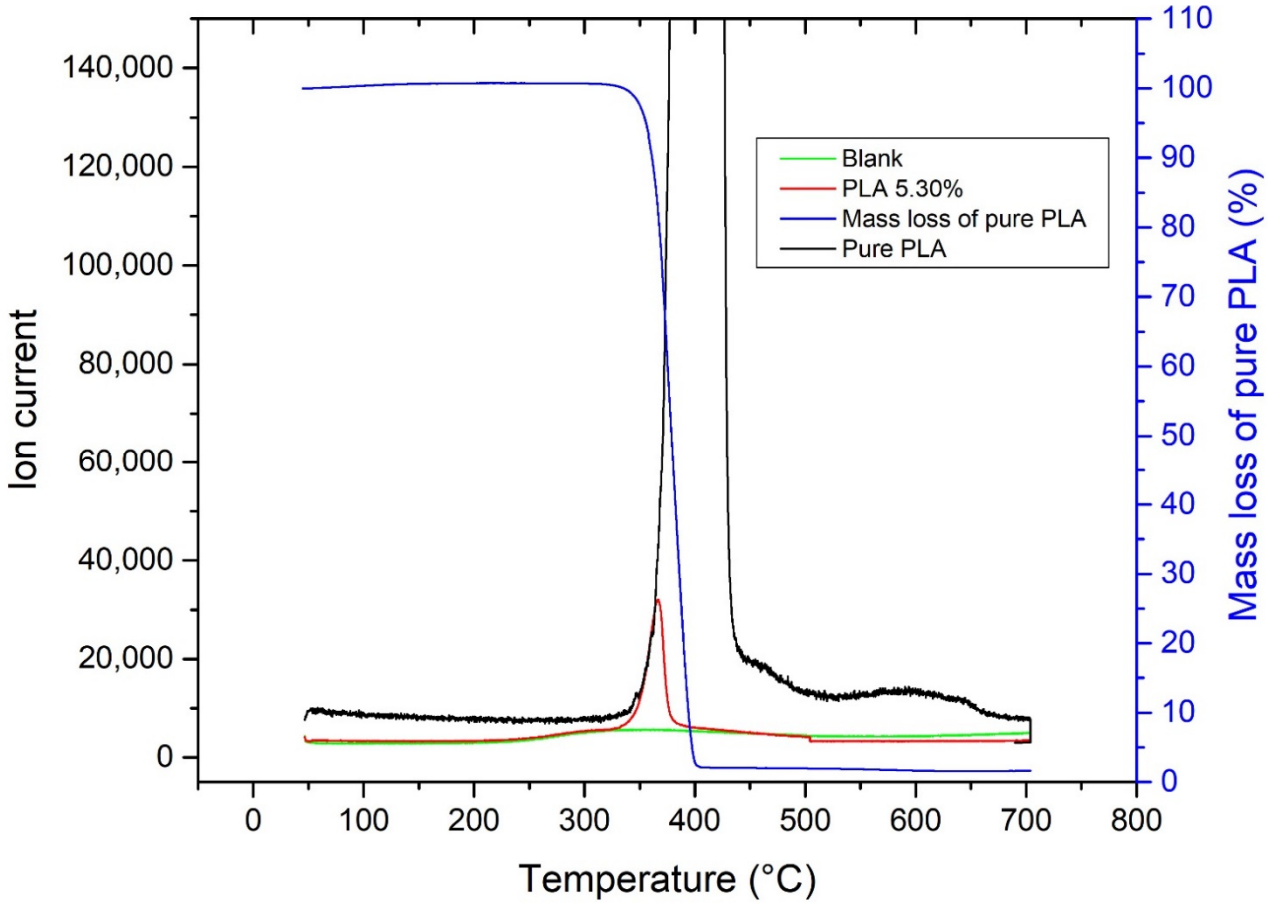

Figure 2. The plot of the peaks at m/z 29 of blank, pure PLA and P185 soil containing 5.30% PLA suitable for PLA detection.

To avoid the condensation and sorption of the degradation gaseous products in the capillary, the measurements were repeated with 21.24 mg of pure PLA with a capillary transfer temperature of 280 °C. In this experiment, all m/z missing in the previous experiment were detected.

2.6. Evolved Gas Analysis System

The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to German standard DIN 32645 using the R package envalysis v0.3.3 (code publicly available from https://doi.org/cn74, accessed on the 20 November 2021), which was used by [56,66].

3. Results and Discussion

3.1. Results Obtained at a Low Capillary Temperature

Figure 3 reports exemplary peaks of the soil and PLA calibration mixtures for P185 soil and m/z 29 and the peaks of the PHB-spiked soils for HS5 soil and m/z 86. This figure shows that the PLA-spiked-soil peaks were symmetrical, while the PHB-spiked-soil peaks tailed off to higher temperatures. This phenomenon was observed for all PHB-containing

soils; probably, this can also be related to the sorption of gaseous degradation products in the capillary. Thus, as shown by the results of the analyses, the soils investigated in this work (and it can be assumed that this is true for mineral soils in general) produced negligible amounts of gases interfering with the signal at lower m/z, which was stronger and easier to process.

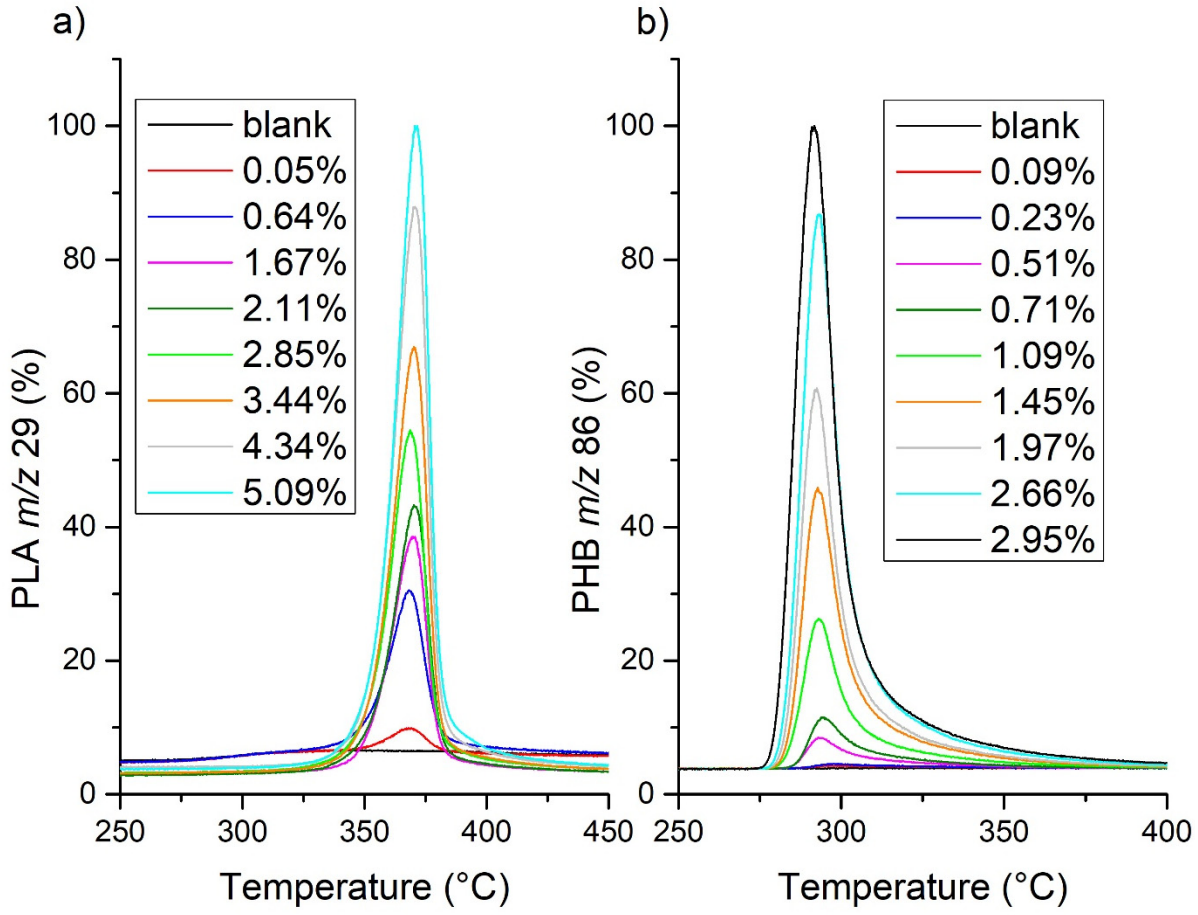

Figure 3. Example PLA (**a**) calibration mixture peaks for soil P185 and m/z 29 and PHB (**b**) calibration mixture peaks for soil HS5 and m/z 86.

The peaks obtained for PHB-spiked soils were integrated with the temperature interval from 265 to 380 °C, which was from 295 to 410 °C for PLA. Then, the intensities of all signals were normalized to the signal of the calibration mixture with the highest concentration. The resulting parameters of the calibration lines for PHB determination are given in Table 2, and a sample calibration line for HS5 soil and m/z 68 is shown in Figure 4 (other calibration lines are shown in Supporting Information Figures S3–S7). Similarly, the parameters of the calibration lines for PLA determination in soil are given in Table 3, and a sample calibration line for P185 soil and m/z 29 is shown in Figure 5 (other calibration lines are shown in Supporting Information Figures S8–S12).

In contrast to ref. [56], where LOQs of 18.40 and 51.00% were achieved without the use of an internal standard and with an internal standard of 1.72 and 6.53% for the soil PET analyses, significantly better results were achieved for PHB even without standardization to an internal standard, with HS45 soil and m/z 86 performing the worst, with an LOQ of 4.00%. However, for this soil, it was possible to use m/z 68, thus reducing the LOQ to 1.61%. The results for HS5 soil were comparable for both m/z 68 (1.56%) and m/z 86 (1.60%). For the determination of PHB in LUFA soil, the LOQs for both m/z were also found to be comparable, at 2.82% and 3.00%, respectively. In the case of determination of PLA in soil, the LOQs were higher, which may be because the selected m/z were not specific to PLA

alone but were also released to a lesser extent by the soils themselves. The best result was obtained for P84 soil and m/z 29, with an LOQ of 1.82%. In addition, a higher LOQ was found in LUFA soil for this MBP, ranging from 2.88 to 3.88%.

Table 2. Parameters of calibration lines for the determination of PHB micro-bioplastics in standard LUFA soil and two real soils by detection of degradation gas products with m/z 68 and 86.

Soil	*m/z*	Slope	Intercept	Adj. R^2	RSE (%)	LOD (%)	LOQ (%)
HS5	68	8166	−1439	0.9822	3.249	0.48	1.56
	86	9491	−2068	0.9811	3.372	0.49	1.60
HS45	68	5126	159	0.9897	0.224	0.52	1.61
	86	5773	250	0.9662	0.425	0.95	4.00
LUFA	68	9630	394	0.9775	0.888	0.75	2.82
	86	11,529	303	0.9761	0.921	0.77	3.00

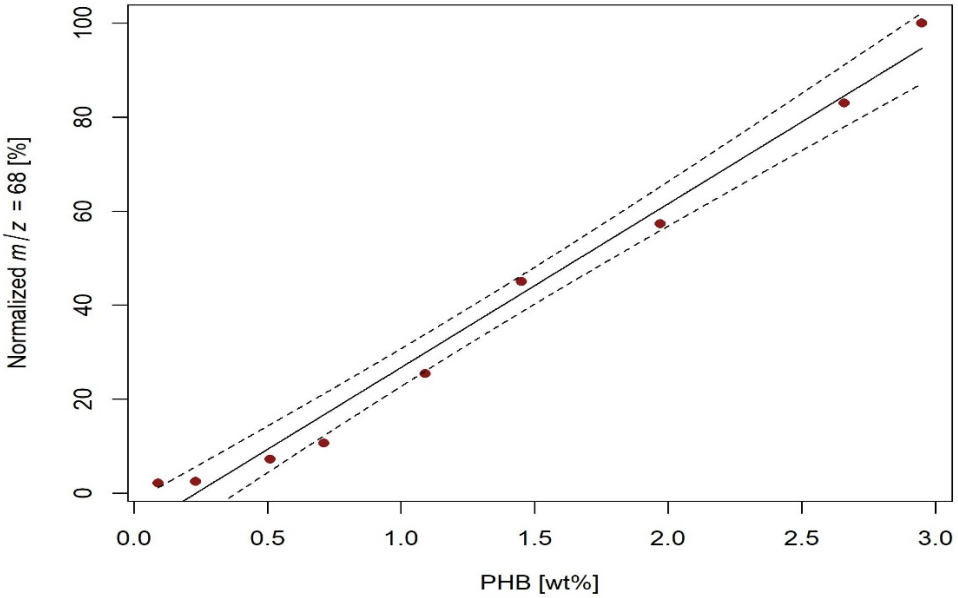

Figure 4. Calibration line for determination of PHB in HS5 soil by detection of degradation products with m/z 68; dashed line indicates 95% confidence interval.

Table 3. Parameters of calibration lines for the determination of PLA micro-bioplastics in standard LUFA soil and two real soils by detection of degradation gas products with m/z 29 and 43.

Soil	*m/z*	Slope	Intercept	Adj. R^2	RSE (%)	LOD (%)	LOQ (%)
P84	29	176,237	58,873	0.9808	1.977	0.80	2.55
	43	72,464	30,106	0.9913	1.104	0.57	1.82
P185	29	180,984	65,753	0.9809	5.457	0.91	2.88
	43	69,949	50,891	0.958	9.513	1.37	4.72
LUFA	29	188,508	−3302	0.9839	0.388	0.96	2.88
	43	71,715	35,492	0.975	0.428	1.20	3.76

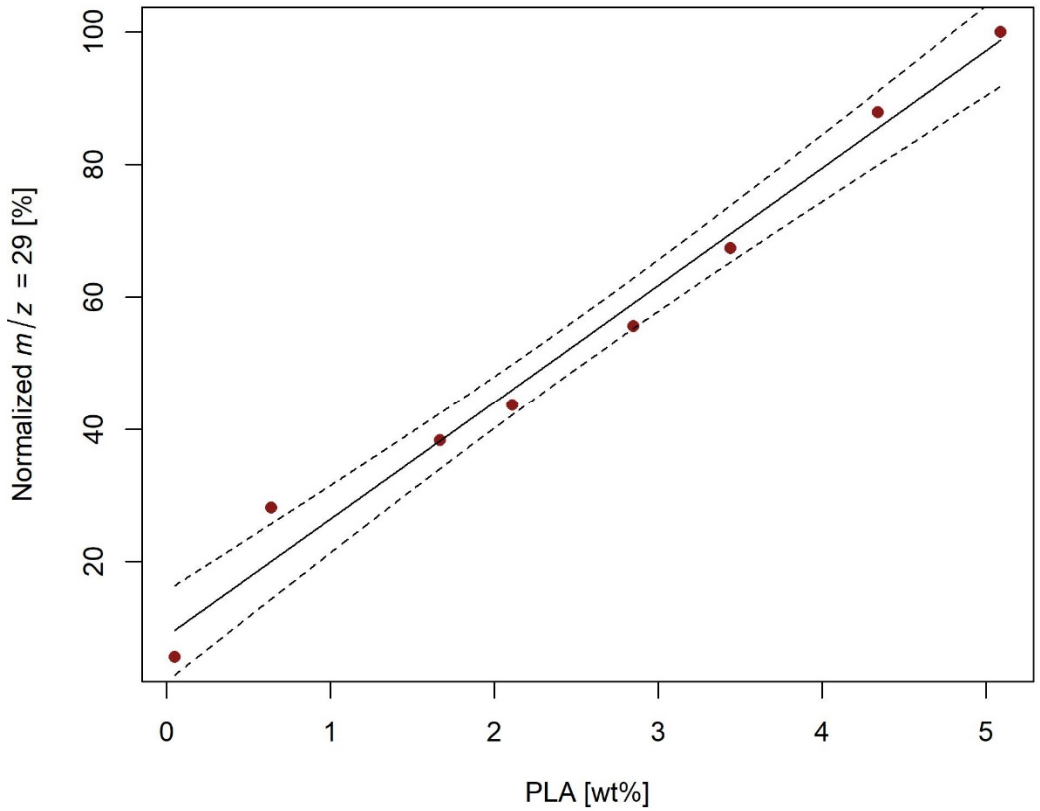

Figure 5. Calibration line for determination of PLA in P185 soil by detection of degradation products with m/z 29; dashed line indicates 95% confidence interval.

3.2. Influence of Soil on the Analysis of Micro-Bioplastics

As shown in the previous section, both PLA and PHB provide some typical fragments that can be used qualitatively and quantitatively to analyze these MBPs in soil. Thus, their presence can indicate the presence of MBPs in soil. However, it cannot be ruled out that these gaseous degradation products released during the thermal degradation of PLA (acetaldehyde) may also be released from the soil, and interference between soil and MBP signals may occur. The source of these gases is soil organic matter, from which ions with the same m/z as those of pure bioplastics may be released. Therefore, soils with a higher proportion of soil organic matter, which may overlay lower concentrations of micro-bioplastics, could become a problem. The solution to this potential problem is the observation, as the results of the previous chapter suggest, that the gaseous products from pure MBPs always leave in a constant stoichiometric ratio, unlike in soils where this ratio is different.

To demonstrate this phenomenon, the organic matter or organic carbon content of the tested soils had to be considered. The organic carbon in the studied soils ranged from 0.59 ± 0.24% for P84 soil to 6.70 ± 0.25% for HS45 soil (Table 1). To assess the effect of soil on PLA analyses, the ratio of the areas of all ion peaks at m/z 43 and m/z 29 was calculated and plotted versus concentration in a graph (Figure 6). This figure shows that, at low MBP/soil organic matter ratios, the signal was significantly affected by gases released from the soil. As the concentration (i.e., MBP/soil organic matter ratio) increased, the peak-area ratio was constant at concentrations from approximately 0.5 to 1%, which was correlated with the detection limit. In contrast, no relationships were observed between soil organic matter content and the blank peak-area ratio deviation from the peak-area ratio at high soil PLA concentrations.

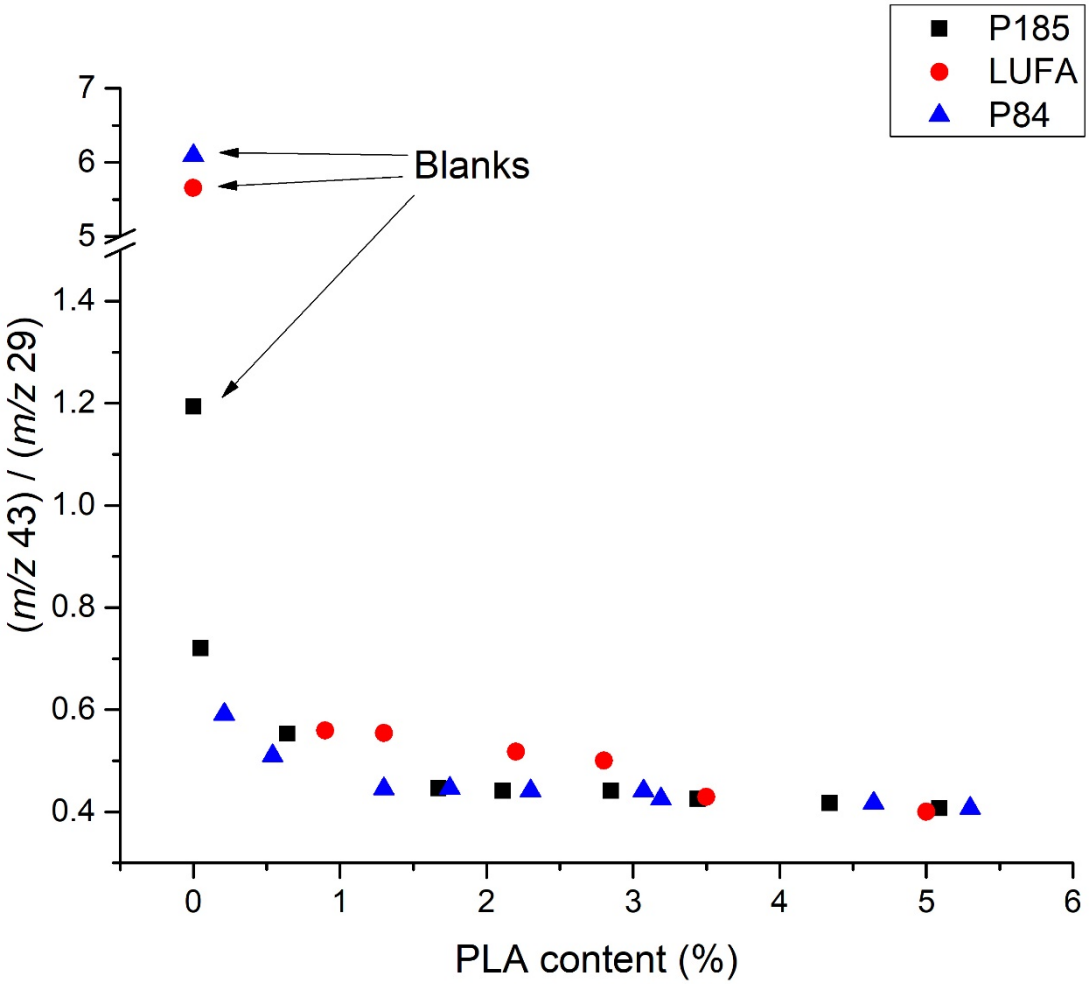

Figure 6. Ratios of PLA peaks of ions with m/z 43 and 29 in different soils as a function of MBP concentration.

A similar effect was observed for the PHB ion peak ratio at m/z 68 and 86 (Figure 7). This suggests that the composition of soil organic matter was more important than its concentration for the studied soils. We can also infer that the thermal decomposition of these soils results in a negligible release of detection gases for both bioplastics, which only affects the analyses below the detection limits; thus, all the selected ions can be used for the detection of these bioplastics.

Our results show that microplastics can be analyzed even by using lower molecular weights of pyrolysis gases using TG-MS. Furthermore, there was an attempt to keep the capillary at the lowest possible temperature, which minimized the possibility of adsorption of small molecules on the column. The analysis based on lower molecular weights increases the possibility to scale the analysis up. In other words, this approach can be used in analyses with other techniques equipped with cheaper detectors, such as the flame ionization detector (GC-FID). A major weakness of microplastic analyses of natural soils is the sampling of the soil, especially if it is contaminated with smaller plastic particles. Most methods are based on soil sampling, homogenization and subsequent quartering, thus reducing the sample volume. In the case of soil and similar substrates, such as sludge, this can lead to under- or overestimating the results. Therefore, a batch method is needed for some applications. The results reported here suggest the possibility of conducting the analyses in a large-scale pyrolizer (i.e., pyrolyzing large amounts of samples) connected to a simple detector, thus enabling the analysis of small molecules evolved from the contaminated soil to be conducted. In addition, the use of a constant ratio between typical gases avoids the use of internal standards, which can be problematic when using batch methods.

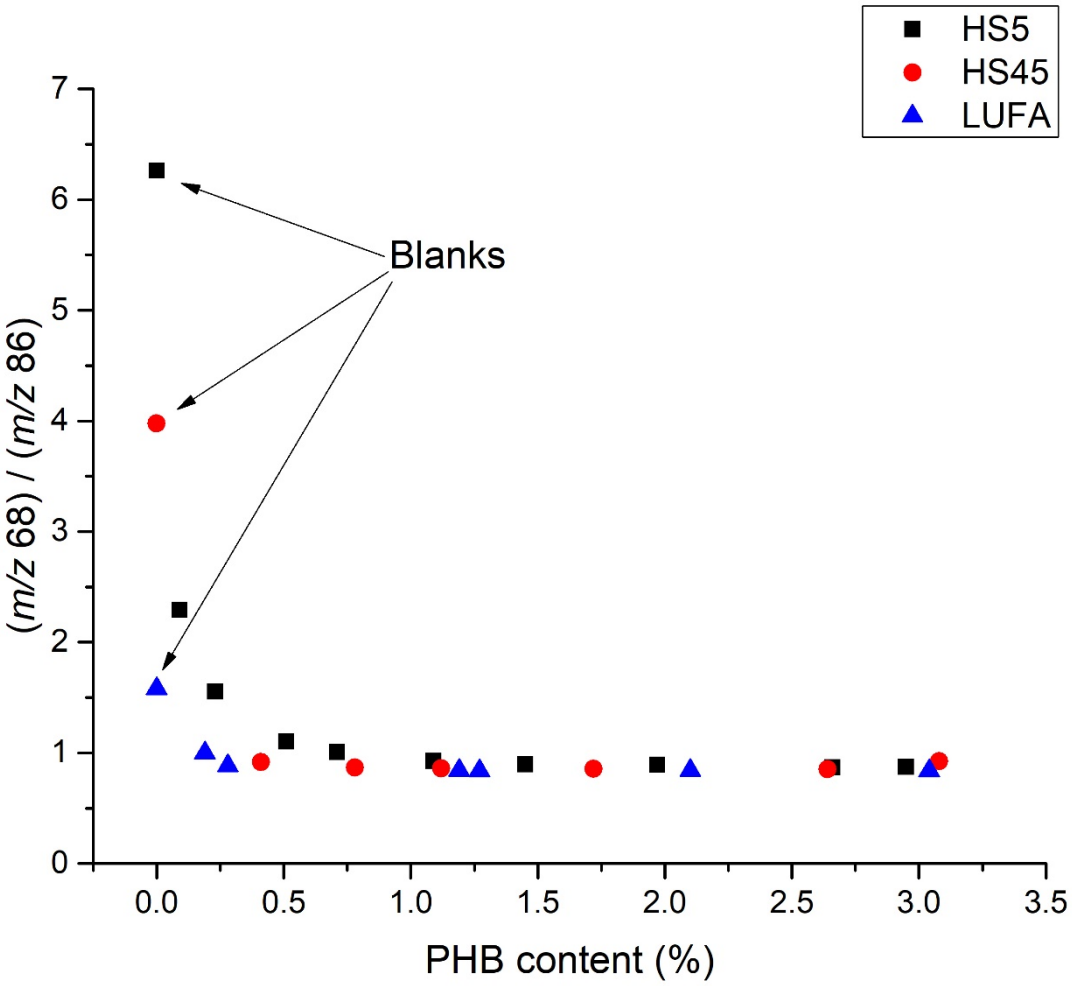

Figure 7. Ratios of PHB peaks of ions with m/z 68 and 86 in different soils as a function of MBP concentration.

3.3. Results Obtained at a High Capillary Temperature

To demonstrate the suitability and advantage of the proposed approach, i.e., low capillary temperature and low molecular weight, we also present the data obtained in the experiment with high capillary temperature. A higher capillary transfer temperature could indeed result in a lower LOQ for the determination of MBPs in soil. This could be due to a better detection of higher specific m/z, as the direct condensation and sorption of gaseous products in the capillary would not occur. Increasing the capillary temperature could also reduce peak tailing, as observed for PHB (Figure 3).

The results show that, although these m/z are specific to PLA and would be useful for samples containing higher amounts of organic matter, the signal at m/z 100 and 128 was not intense enough for the analysis of real samples (Figure 8). Although the signal at m/z 56 (Figure 9) from the PLA dimer was more intense, there was signal splitting, and the main peak was not separated from the other two peaks [67]. The last peak ended at a temperature higher than 550 °C, which increased the analysis time and was instrumentally more demanding. In addition, it extended the peaks over a larger temperature range, which is generally problematic for quantitative analyses. Moreover, even in this case, sorption onto the column cannot be excluded and further temperature increases could lead to secondary reactions. In addition, the upscaling of this analysis discussed in the previous chapter would be technologically problematic due to the need to maintain the equipment at the desired temperature.

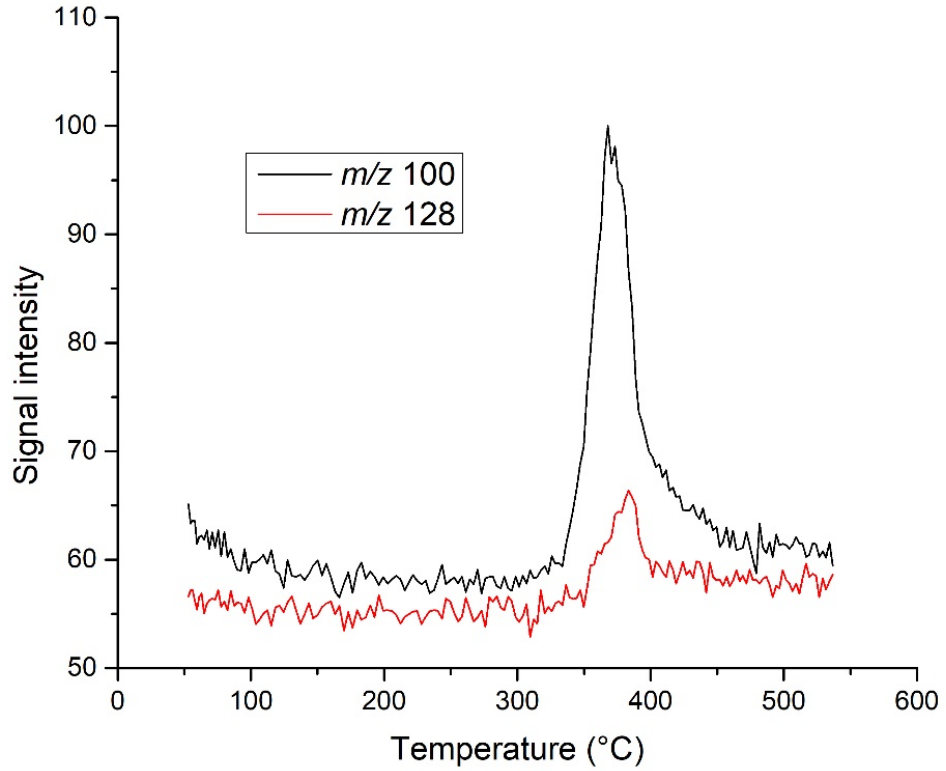

Figure 8. Comparison of peaks at m/z 100 and 128 obtained with the capillary heated at 280 °C.

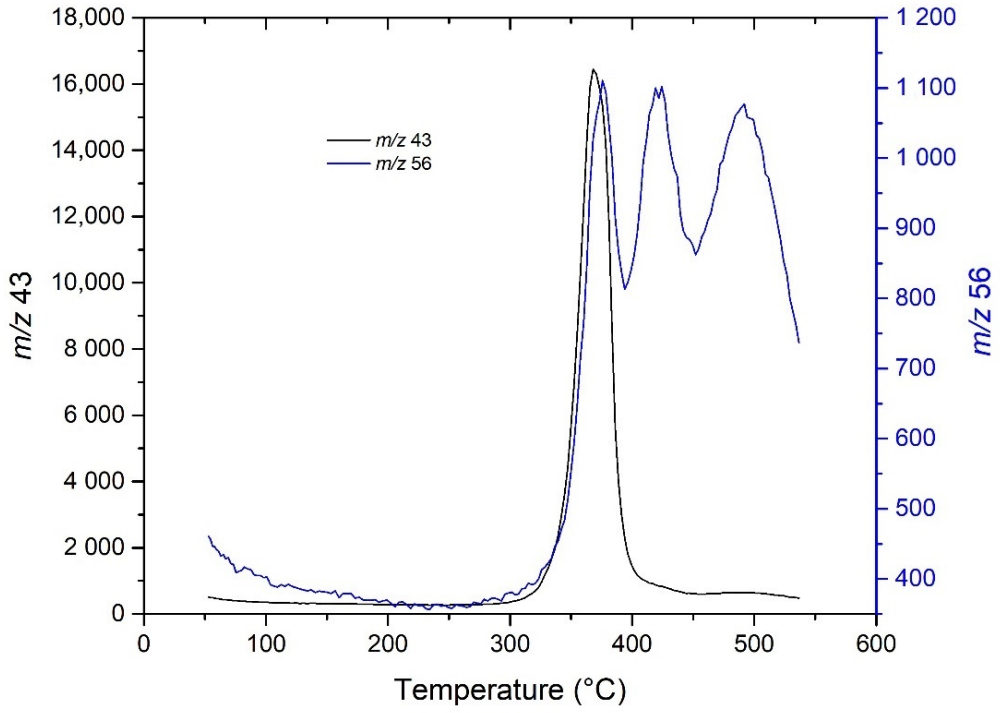

Figure 9. Peaks at m/z 56 obtained with the capillary heated to 280 °C. The peak of the signal at m/z 43 from the same experiment is also shown for comparison (n.b., the two peaks have different y-axes).

3.4. Other TG-MS Measurement Alternatives

Another alternative that could improve the LOD and LOQ determination of MBPs in soil would be using a system where the TG detector is coupled directly to the MS detector

without using a capillary [65]. Although this method requires minimal pretreatment of the sample prior to measurement, which is necessary for analyzing MBPs, its major drawback is the small amount of sample used for the analyses. Thus, this method needs to ensure thorough homogenization of real samples and higher replication of measurements to obtain representative results. It would also be problematic to analyze soils with larger bioplastic particles. This problem could be solved by using a system that can measure larger sample volumes.

The determination of both bioplastics in soils and other matrices (e.g., activated sludge and compost) rich in organic matter remains an analytical challenge, because the detection m/z for PLA can be strongly released from the matrix itself during analysis. For PHB, it would be necessary to distinguish anthropogenic PHB from natural PHB, which is primarily produced by bacteria in activated sludge as a carbon and energy storage polymer [68].

4. Conclusions

In this work, we present, for the first time, a method for the determination of PHB and PLA in soil using TG-MS. The method is inspired by the work of [56], where TG–MS were used for the quantitative analysis of PET microplastics in standard LUFA soil. This method uses a capillary heated to a lower temperature, and lower m/z are used for detection; therefore, it can be adapted to cheaper and simpler TG-MS or Py-MS. In this work, we show that similar or better results can be achieved in LUFA soil, and that the method can be used for analyses of real soils.

This work aims to develop a simple and, possibly, scalable method to analyze residual MBPs after biodegradation experiments. Since the recommended concentration of bioplastics in biodegradation experiments according to ISO 17556 is 0.1%, the LOD and the LOQ are not sufficient to detect residual bioplastics. The possible inclusion of this technique in the verification of biodegradation tests would need to be refined by using an internal standard, by analyzing a larger volume of the sample, or by using a larger number of samples in the biodegradation experiments. As bioplastics currently account for only a fraction of the world's plastic production, and thus have not yet entered the environment in large quantities, it is not possible to estimate the environmentally relevant concentrations; thus, it is not possible to assess the suitability of the method for analyzing real samples. On the other hand, the development of bioplastic fertilizers and bioplastic mulch films may lead to the accumulation of bioplastics in agricultural soils; thus, the number of bioplastics in soil could be detectable by this method [29,69].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27061898/s1, Figure S1: Degradation products and MS fragments of PHB. Figure S2: Degradation product and MS fragments of PLA. Figure S3: Calibration line for determination of PHB in HS5 soil by detection of degradation product with m/z 86, dashed line indicates 95% confidence interval. Figure S4: Calibration line for determination of PHB in HS45 soil by detection of degradation product with m/z 68, dashed line indicates 95% confidence interval. Figure S5: Calibration line for determination of PHB in HS45 soil by detection of degradation product with m/z 86, dashed line indicates 95% confidence interval. Figure S6: Calibration line for determination of PHB in LUFA soil by detection of degradation product with m/z 68, dashed line indicates 95% confidence interval. Figure S7: Calibration line for determination of PHB in LUFA soil by detection of degradation product with m/z 86, dashed line indicates 95% confidence interval. Figure S8: Calibration line for determination of PLA in P185 soil by detection of degradation product with m/z 43, dashed line indicates 95% confidence interval. Figure S9: Calibration line for determination of PLA in P84 soil by detection of degradation product with m/z 29, dashed line indicates 95% confidence interval. Figure S10: Calibration line for determination of PLA in P84 soil by detection of degradation product with m/z 43, dashed line indicates 95% confidence interval. Figure S11: Calibration line for determination of PLA in LUFA soil by detection of degradation product with m/z 29, dashed line indicates 95% confidence interval. Figure S12: Calibration line for determination of PLA in LUFA soil by detection of degradation product with m/z 43, dashed line indicates 95% confidence interval.

Author Contributions: Conceptualization, J.F., J.K. and J.D.; methodology, J.D. and J.F.; formal analysis, J.F., I.R. and J.D.; data curation, J.F. and P.P.; writing—original draft preparation, J.F., J.K. and J.D.; writing—review and editing, J.F., M.B., J.K. and J.D.; supervision, J.K. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are not available from the authors.

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molecules

MDPI

Supporting Information

A Simple Method for Quantification of Polyhydroxybutyrate and Polylactic Acid Micro-Bioplastics in Soils by Evolved Gas Analysis

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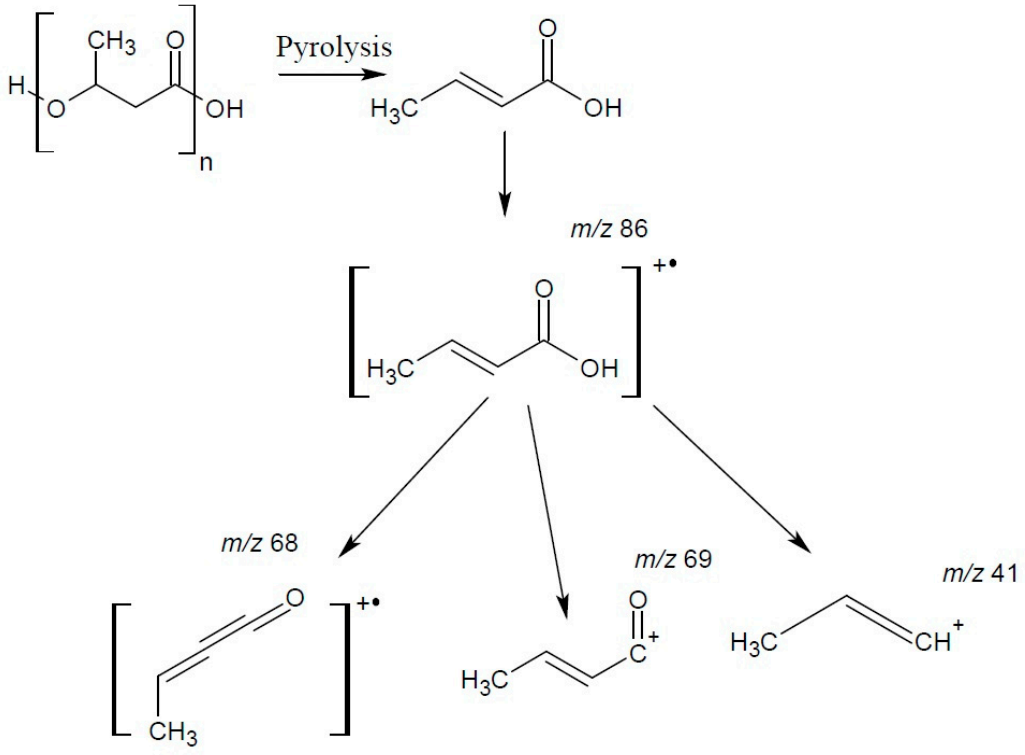

Figure S1. Degradation products and MS fragments of PHB.

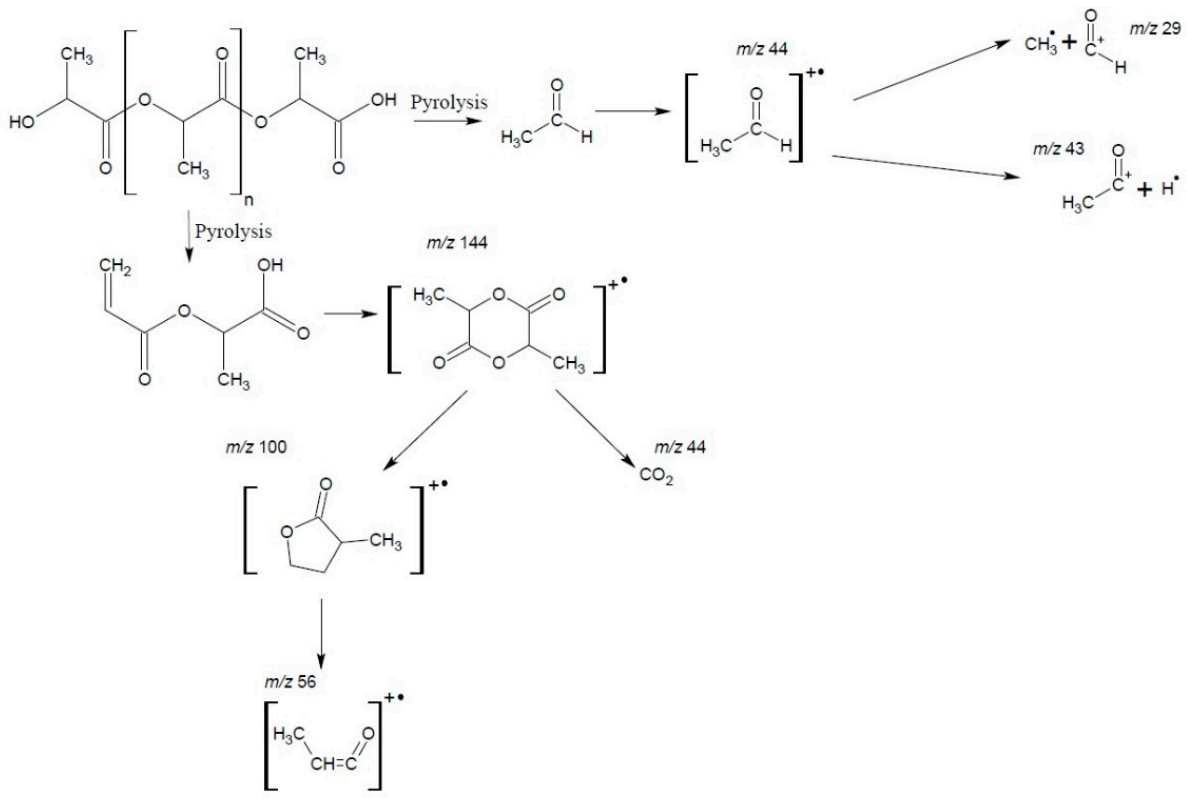

Figure S2. Degradation product and MS fragments of PLA.

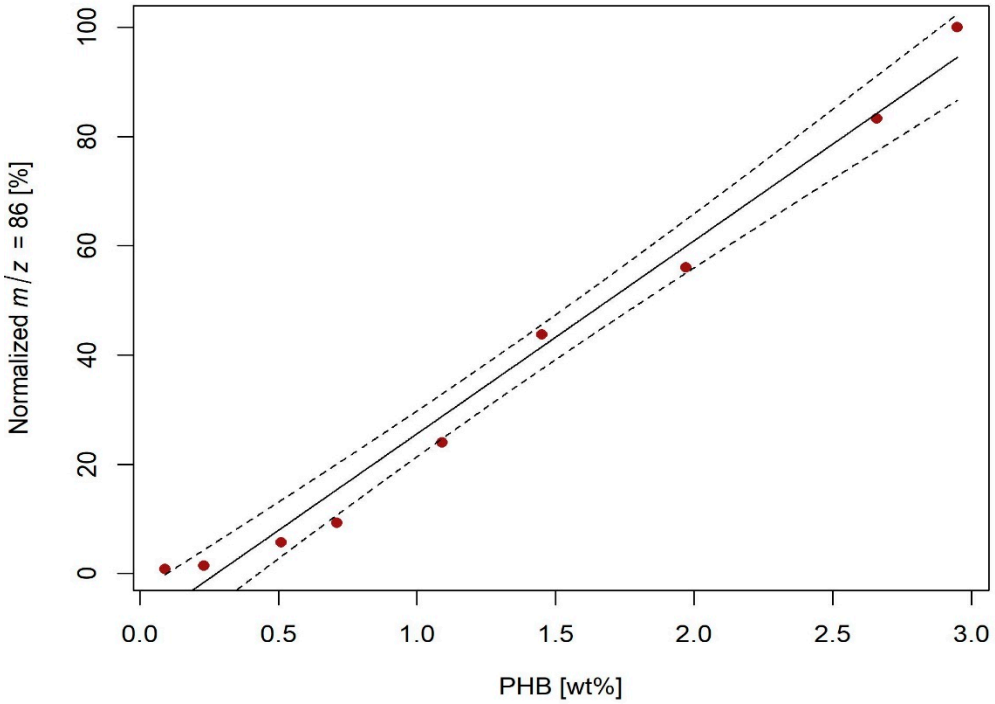

Figure S3. Calibration line for determination of PHB in HS5 soil by detection of degradation product with *m*/*z* 86, dashed line indicates 95% confidence interval.

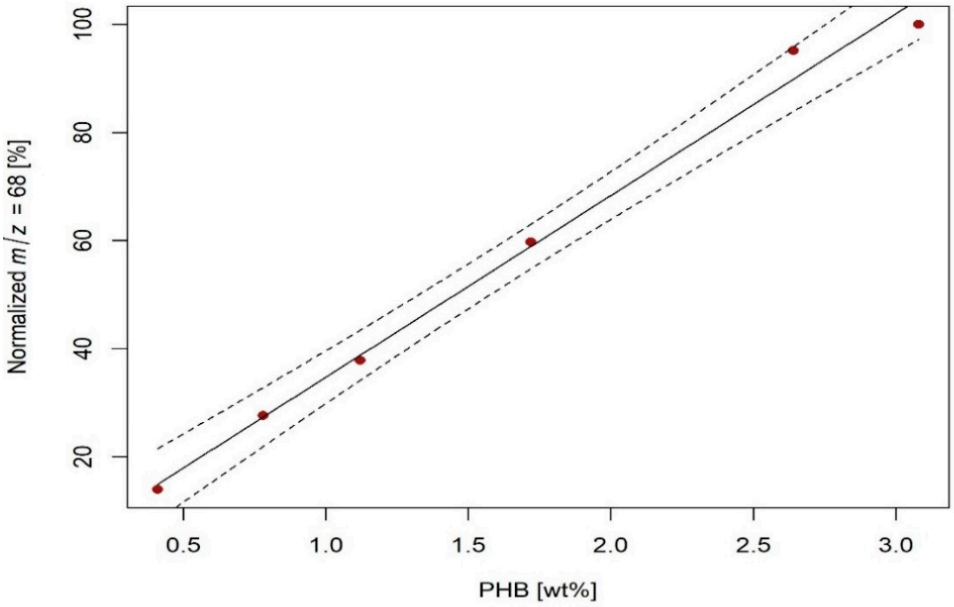

Figure S4. Calibration line for determination of PHB in HS45 soil by detection of degradation product with *m*/*z* 68, dashed line indicates 95% confidence interval.

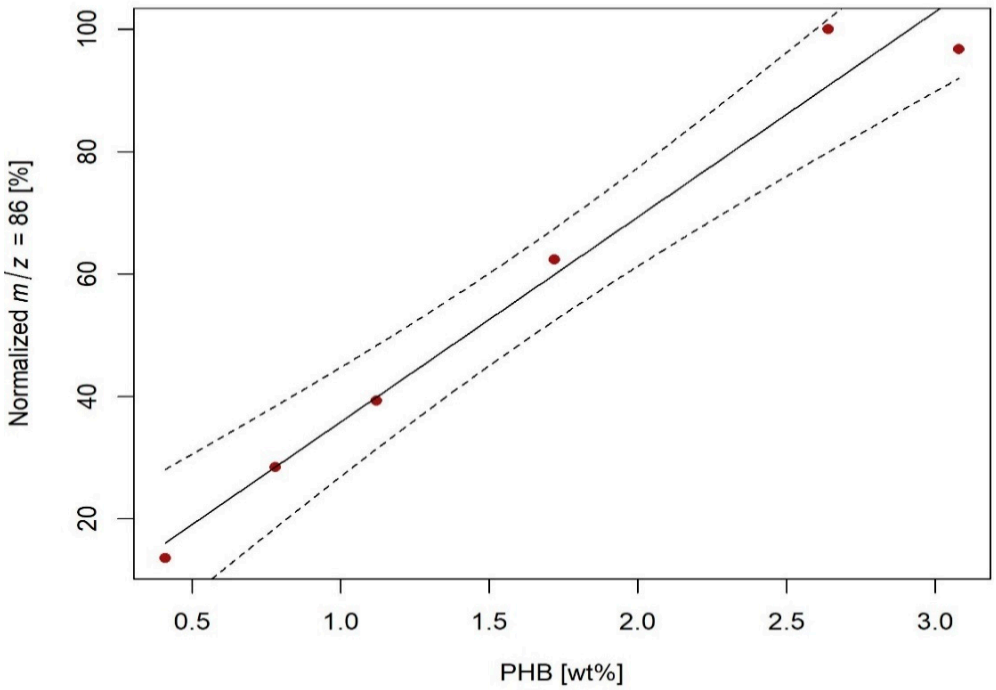

Figure S5. Calibration line for determination of PHB in HS45 soil by detection of degradation product with *m*/*z* 86, dashed line indicates 95% confidence interval.

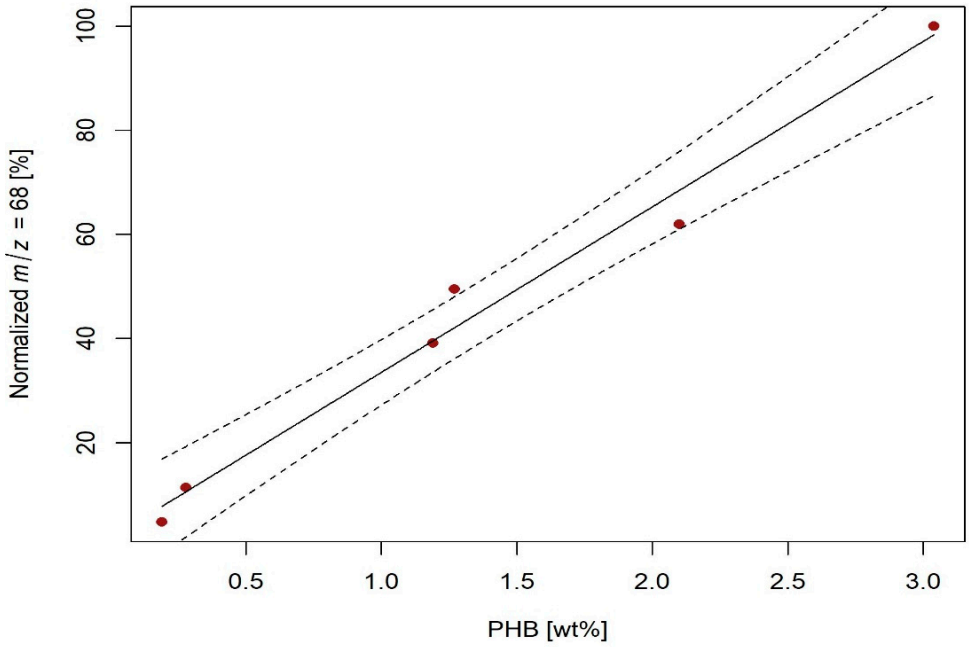

Figure S6. Calibration line for determination of PHB in LUFA soil by detection of degradation product with *m*/*z* 68, dashed line indicates 95% confidence interval.

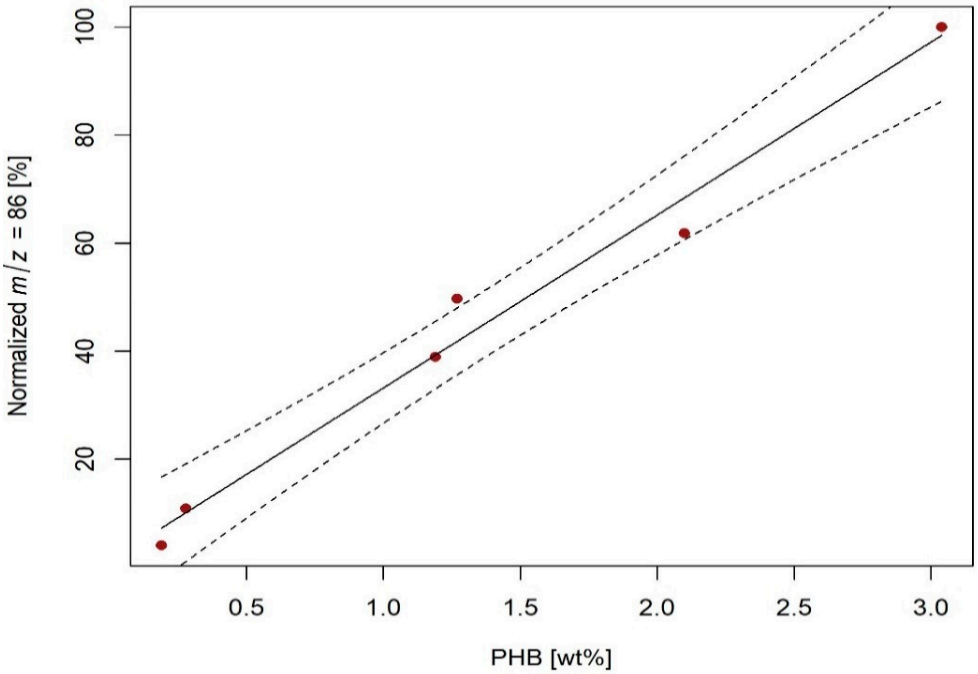

Figure S7. Calibration line for determination of PHB in LUFA soil by detection of degradation product with *m*/*z* 86, dashed line indicates 95% confidence interval.

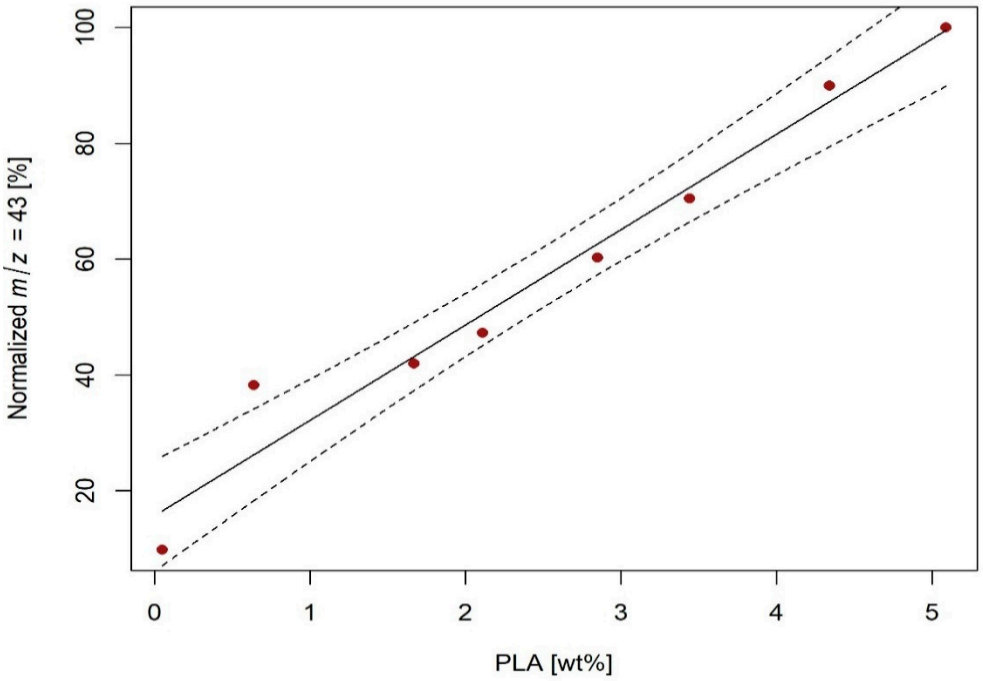

Figure S8. Calibration line for determination of PLA in P185 soil by detection of degradation product with *m*/*z* 43, dashed line indicates 95% confidence interval.

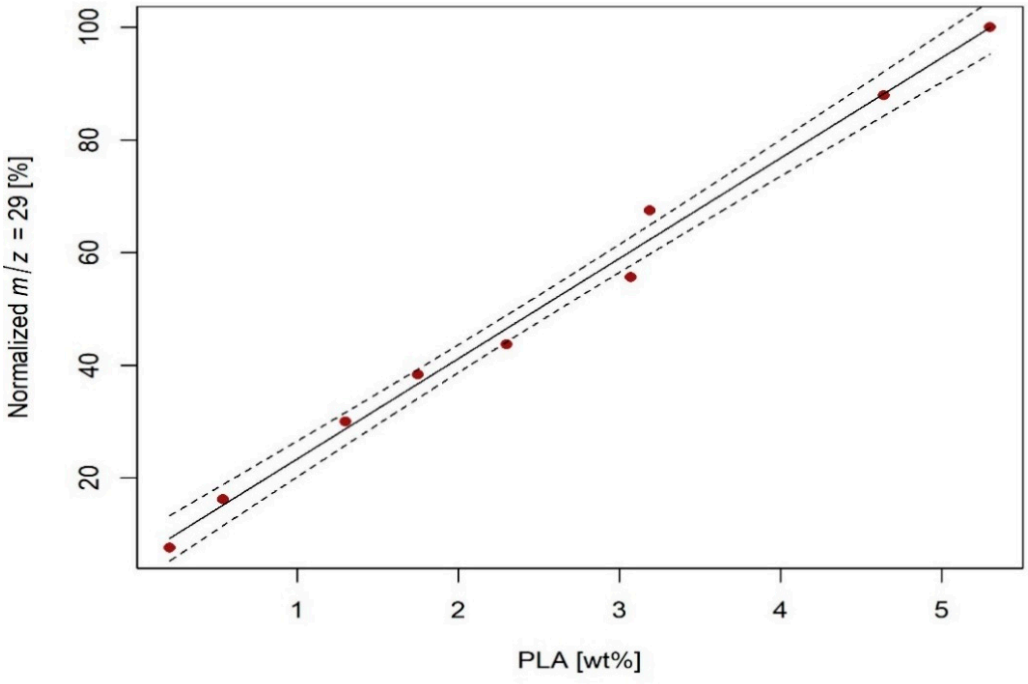

Figure S9. Calibration line for determination of PLA in P84 soil by detection of degradation product with *m*/*z* 29, dashed line indicates 95% confidence interval.

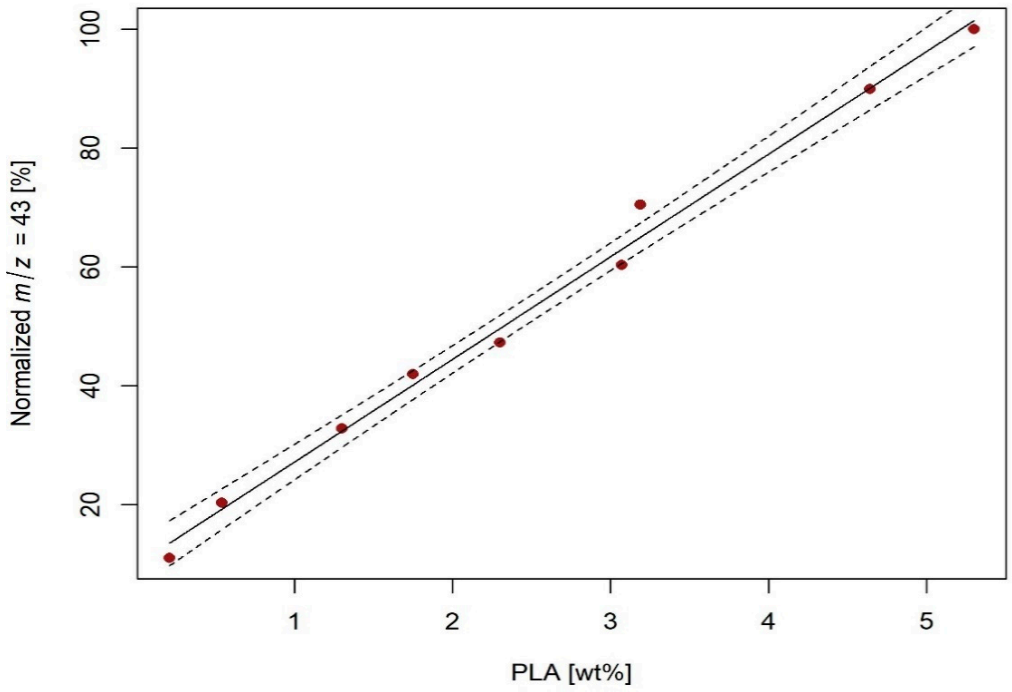

Figure S10. Calibration line for determination of PLA in P84 soil by detection of degradation product with *m*/*z* 43, dashed line indicates 95% confidence interval.

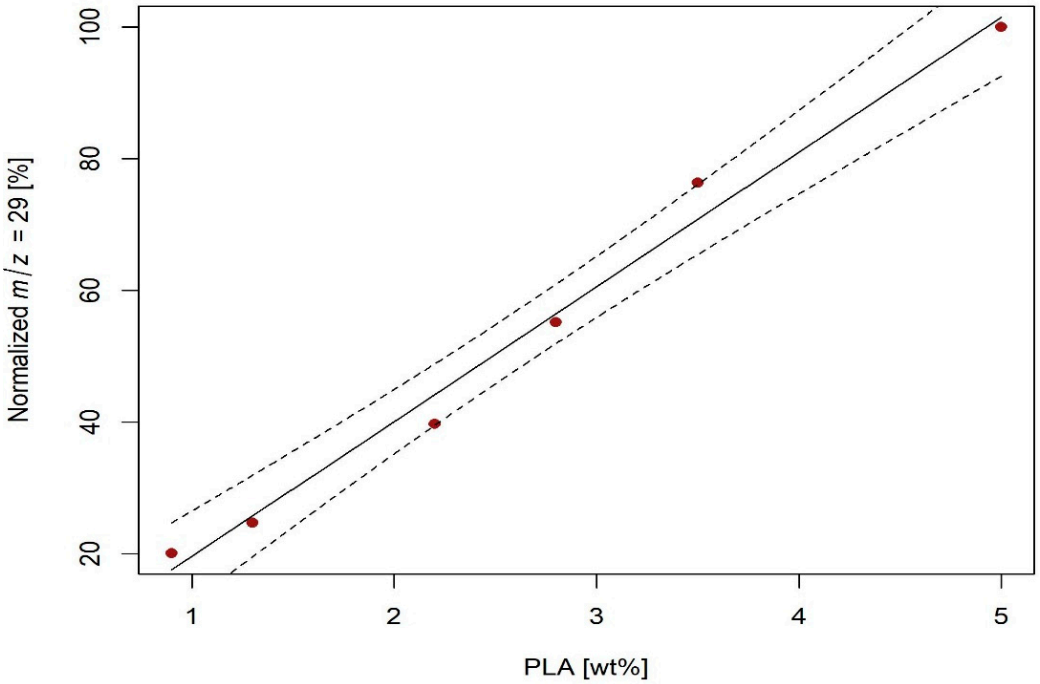

Figure S11. Calibration line for determination of PLA in LUFA soil by detection of degradation product with *m*/*z* 29, dashed line indicates 95% confidence interval.

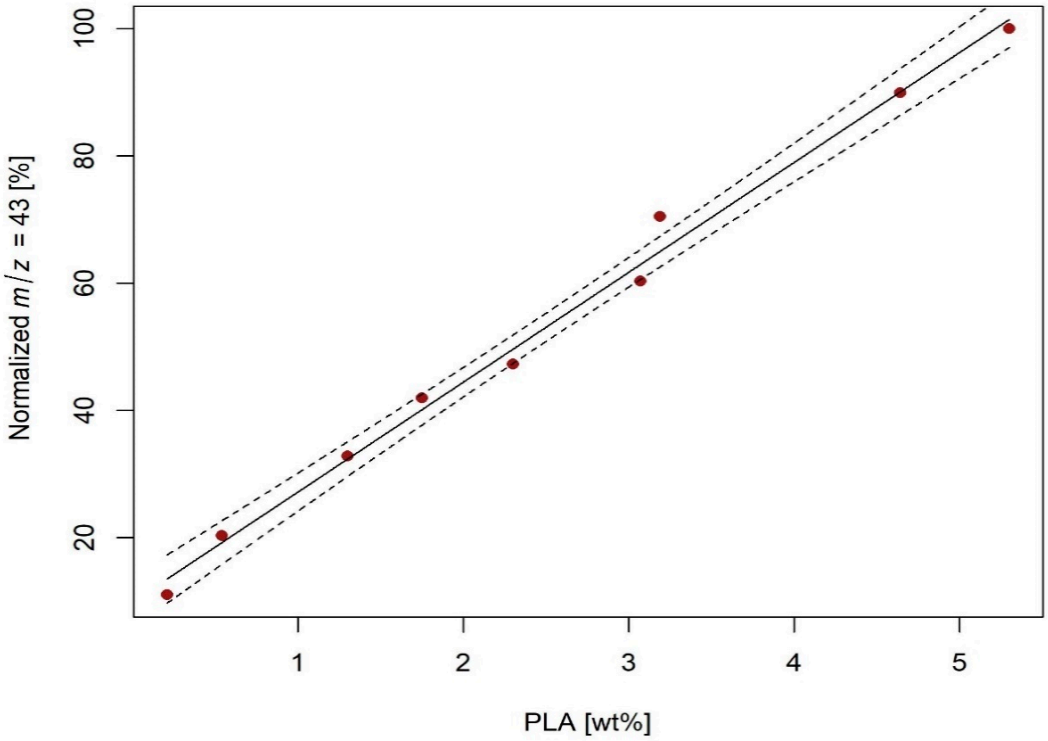

Figure S12. Calibration line for determination of PLA in LUFA soil by detection of degradation product with *m*/*z* 43, dashed line indicates 95% confidence interval.

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Influence of Poly-3-hydroxybutyrate Micro-Bioplastics and Polyethylene Terephthalate Microplastics on the Soil Organic Matter Structure and Soil Water Properties

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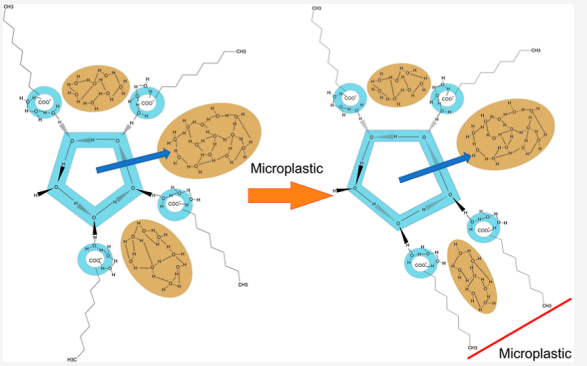
ABSTRACT: Adverse effects of microplastics on soil abiotic properties have been attributed to changes in the soil structure. Notably, however, the effects on the supramolecular structure of soil organic matter (SOM) have been overlooked, despite their key role in most soil properties. This work accordingly investigated the influence of plastic residues at various concentrations on the SOM supramolecular structure and soil water properties. To model plastic residues of micro-bioplastics, spherical or spherical-like poly-3-hydroxybutyrate (PHB) was used, while polyethylene terephthalate (PET) was used as a model of conventional microplastics. The results suggest that both types of plastic residues affect SOM properties, including physical stability (represented by water molecule bridges), water binding (represented by decreased desorption enthalpy or faster desorption), and the stability of SOM aliphatic crystallites. The results further showed that the polyester-based microplastics and micro-bioplastics affected the SOM abiotic characteristics and that therefore the observed effects cannot be attributed solely to changes in the whole soil structure. Notably, similar adverse effects on SOM were observed for both tested plastic residues, although the effect of PHB was less pronounced compared to that of PET.



KEYWORDS: *PET microplastics, PHB micro-bioplastics, DSC, water, soil*

1. INTRODUCTION

Soil contamination by microplastics—plastic particles between 1 μm and 5000 μm in size—is one of the most widely discussed environmental issues today.[1,2] The main source of primary microplastics in agricultural soils is sewage sludge fertilization[3] and fertilizer coating,[4] while secondary microplastics originate from eroded plastic mulching.[5] Other main sources include irrigation, flooding, littering, and atmospheric deposition.[6,7] Microplastics from nonbiodegradable polymers have been found in most soils, including critical arable and urban soils.[8] Although the decomposition rate of microplastics in soil is unknown, they are thought to be persistent.[2] Nevertheless, this persistence depends on the type of microplastics, the environmental conditions, and microbial adaptation, as suggested by the discovery of polyethylene terephthalate (PET)-degrading bacteria in the vicinity of a Japanese recycling facility.[4]

Soil is a particle-rich environment with a massive internal surface area, and microplastics can be viewed as a soil physical contaminant.[9] Therefore, many interactions with mineral and non-living organic particles can be expected. However, studies that focus on such interactions are scarce,[6] and little is known about the interactions themselves.[10] Recently, it was speculated that microplastics could interact with amphiphilic and hydrophobic compounds in soils and influence the stability of soil aggregates.[9] Similarly, Souza Machado et al.[11] found changes in soil aggregation, bulk density, water-holding capacity, and hydraulic conductivity caused by the presence of microplastics in concentrations between 0.05 and 2.00%. Zhang et al.[12] observed that polyester fibers at concentrations ranging from 0.1 to 3.0% affect the pore size distribution and aggregation of a soil, while the polyethylene film significantly increases the rate of soil water desorption and desiccation cracking behavior.[10] Additionally, other authors[13] found that plastic film residues caused significant changes in the physical properties of soil, such as the initial gravimetric water content, bulk density, total porosity, and soil water distribution. Wan et

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al.[10] observed that fragments of plastic films created channels for water movement, enhancing the desorption of water from soil, and speculated about the destruction of the soil's structural integrity. Other studies have shown the limiting effect of microplastics on seed germination as they block seed pores, which reduces water penetration[14] and inhibits plant growth.[15] These observations suggest that microplastics are relevant long-term anthropogenic stressors.[11]

One strategy to avoid secondary microplastics is to replace nonbiodegradable plastics (usually petroleum-based) with biodegradable ones (preferably those manufactured from the waste of renewable feedstock) whose fragments may be completely biodegraded.[16] The biodegradation comprises several phases, such as biodeterioration and biofragmentation,[17−19] during which the plastic is fragmented into small pieces. The following stage is bioassimilation,[20] in which the low-molecular-weight products that are formed during the previous stage are transported to cells. The final phase is mineralization,[20] which results in low-molecular-weight products. Needless to say, this biodegradation is accompanied by an increase in the count of biodegrading microorganisms and therefore also in the production of biomass. The biodegradation processes depend on the environmental conditions, such as the temperature, moisture, nutrient supply, aeration, and number of biodegrading microorganisms.[21−25]

However, the potential risks linked to the use of biodegradable plastics are rarely considered, although several related issues have already been reported including similar toxicity of additives from conventional plastics and biodegradable bioplastics (hereinafter referred to as bioplastics),[16] negative effects on wheat growth,[26] and enhanced C and nutrient turnover, which may potentially alter the soil's ecological functioning and biogeochemical cycling[27] Another risk associated with biodegradable plastics is the increased rate and ease of microplastic production compared to that of nonbiodegradable plastics. For example, the use of biodegradable plastic mulch films would lead to the formation of microbioplastics in larger quantities.[26] The optimal biodegradation conditions are rarely met, and biodegradable plastics can biodeteriorate and quickly form micro-bioplastics.[24,28] In the related literature, biodegradation tests were not always conclusive in clearly distinguishing between fragmentation and biodegradation,[29] and incomplete degradation at the end of the experiment was sometimes suggested. One example of slow biodegradation under controlled laboratory conditions involved the over 6 weeks biodegradation of aliphatic−aromatic polyester polybutylene adipate terephthalate (PBAT) in agricultural soils, which brought only small changes in the material.[30] Other authors observed about 50% of mass loss among monofilaments composed of PBAT in standardized sandy soil, with a minor change in molecular weight after 22 months.[30] Importantly, the formation of residues is not reflected in the respective norms for testing of biodegradation (e.g., ISO 17556 and ASTM D5988), which require only partial degradation from 6 months to 2 years. Therefore, the replacement of nonbiodegradable plastics by biodegradable bioplastics can represent a solution for the persistence of microplastics in soil, but from a short- and medium-term perspective, the influence of micro-bioplastics on soil should be investigated. An issue can also arise when using composts as soil amendments, as even under degradation favorable conditions for full-scale composting taking 18 weeks,[31] an incomplete degradation of polylactic acid/polyhydroxyalkanoate (PLA/PHA) and PBAT films was observed.

Polyhydroxybutyrates (PHBs) are among the most currently investigated biodegradable polymers. PHB is formed as an intracellular bacterial reserve of carbon and energy and serves as an ion sink in various Gram-positive and Gram-negative bacteria in response to reduced nutrients.[32] The biodegradation of PHB occurs in aerobic and anaerobic microbially active environments, and its rate strongly depends on the moisture level, nutrient supply, temperature, and pH.[33] Although it is considered a fully biodegradable and environmentally biocompatible plastic, aquatic environment tests revealed the negative effects of PHB nanoplastics (75−200 nm at concentrations ranging from 25 to 100 mg/L) on the cellular growth and physiological parameters of organisms of different trophic levels.[34] To the best of our knowledge, PHB's toxicity to soil biota has not yet been reported. PHB content in the soil is expected to increase in the future as it is a polymer that is applicable, among others, to delivery systems for pesticides to soils,[35,36] coating of fertilizers,[36] and biodegradable mulching.[37]

In light of the above review, there is a lack of literature studies on the effect of microplastics and micro-bioplastics on abiotic soil components. Several effects of microplastics on soil properties and water in soil have been observed,[9,10] which the authors explained as related to the changes to the physical properties of investigated soils. We hypothesize that this only partially explains the effects as it overlooks the possible effect of microplastics on the supramolecular structure of soil organic matter (SOM). The structure of SOM contains moieties with various polarities that interact with extraneous materials in a way that changes their conformation, inducing changes in SOM properties as a result. In mineral soils, the SOM content is low, as is the subsequent effect. In organic soils, however, these changes in the structure may influence the soil abiotic processes more significantly.

This study aims to clarify the effect of microplastics and micro-bioplastics on the properties of SOM by analyzing the effects on organic soil, which is a model for SOM. This approach can amplify both possible changes and their detectability, which would be difficult in mineral soils with low SOM content. As model bioplastics was used poly-3-hydroxybutyrate (PHB), which is a poorly wettable polymer,[38] and its effect on SOM under arid conditions with a limited water supply is hard to predict. Therefore, its influence was studied in soil that had been dried under different conditions to obtain soils with various types of water configurations (i.e., structures that were formed by water molecules) that were typical for arid conditions. As a microplastics counterpart, PET of similar wettability was used, which also belongs to the group of polyesters and represents one of the most widely distributed microplastics in soils.[39]

2. EXPERIMENTAL SECTION

2.1. Experiment Preparation. A sapric histosol from Totes Moor (Fuhrberg, Germany) was used as a model of SOM due to its high organic matter content. The properties of this sapric histosol (hereinafter referred to as SOM) have been described in prior studies.[40,41] Briefly, it consists of 93.2 ± 0.2% of organic matter, 52% organic carbon content, 123 $mmol_c\ kg^{-1}$ cation exchange capacity, and a pH of 2.7 (determined in 0.01 M $CaCl_2$). Air-dried SOM was ground in an agate mortar and sieved through a 125 μm sieve. Later in the text, we only use the term "SOM".

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Two grams of sieved SOM was mixed with 11 mL of suspension containing PET or PHB particles (<63 μm, the size distributions of the obtained fractions are reported in the Supporting Information as Figures S1 and S2) and carefully homogenized by vortex stirring for 10 min. PHB (Y1000P) was obtained from TianAn Biologic Materials Co., Ltd. (Ningbo City, China).[42] The particles had spherical or spherical-like shapes (Figure S3). The contact angle of PHB is reported between 70[43] and ∼81°,[44] which makes it slightly hydrophobic. The PET particles were obtained as a residual material from mechanical recycling from PETKA CZ a.s. (Brno, Czech Republic), purified, and then sieved. The contact angle of PET varies between 66 and 81°,[45] that is, it is also poorly wettable compared to PHB. The final concentrations of PHB and PET in the SOM were 0% (uncontaminated SOM, i.e., blank), 0.1, 0.5, 1, 5, and 10% (w/w). These concentrations were chosen for the following reasons. First, we wanted to observe possible effects in a wide range of SOM-to-plastic ratios to reveal possible trends. Second, the ratio between, for example, SOM and PHB in 10% (w/w) contamination is roughly 10 to 1 (w/w) in organic soil, but in mineral soil, where the SOM content is lower (e.g., 1%), the same ratio is decreased to roughly 0.1 to 1; in other words, the 10% contamination in organic soil represents the effect of 0.1% in mineral soil. For these reasons, we selected higher ratios, which may be rare for organic soils (locally possible when using mulching from PHB) but are less rare in mineral soils, where comparable ratios can be reached through the use of coated fertilizers and delivery systems. The homogenized samples (SOM with PET or PHB) were dried in a desiccator at a controlled temperature (20 ± 1 °C) at three different relative humidity (RH) levels (43 ± 3, 76 ± 3, and 92 ± 3%) for 3 weeks to obtain SOM samples with three different water structure types. The RHs were maintained using saturated salt solutions: 43% RH using K_2CO_3, 76% using NaCl, and 92% using KNO_3 (PENTA s.r.o., Czech Republic).

2.2. Preparation of the Samples for Measurement. Approximately 5 mg of all microplastics-spiked sapric histosol samples were weighed in a Tzero aluminum pan and hermetically sealed using a Tzero press (both TA Instruments, New Castle, USA). Each sample was measured three times using a DSC Discovery 2500 equipped with an RCS90 cooling system (TA Instrument, New Castle, USA) to determine the parameters described in Sections 2.3, 2.4, and 2.5. In all cases, the purging gas was pure nitrogen (50 mL per min).

The experiments conducted as described in following sections were designed to observe the effect of contamination on various thermophysical properties, which are reflecting the SOM properties.

2.3. Stability of Water Molecule Bridges. The hermetically sealed pans containing samples were subjected to the following temperature regime: cooling from 20 to −50 °C at 10 °C per min, followed by an increase to 110 °C at 10 °C per min. These cycles were then repeated.

The representative differential scanning calorimetry (DSC) record of the samples containing 10% of PHB microplastics is reported in the Supporting Information (Figure S4). The analyses of records were performed using Trios software (TA Instruments, New Castle, USA). The record shows the development of heat flow with temperature with a step transition, which is typical for water molecule bridge (WaMB) breaks.[46] The temperature of the WaMB break in SOM was determined as an onset (T_{onset}) (Figure S4). This temperature disrupts the weakest WaMB, while a further temperature increase disrupts WaMBs of higher stability.[47]

2.4. Determination of Desorption Enthalpy and Water Content. Prior to the measurement, the hermetically sealed pans with samples were perforated, after which they were immediately placed into the DSC oven to be measured. The following temperature program was applied: cooling from 20 to −30 °C at 10 °C per min and heating to 185 °C at 7 °C per min. After the experiments were completed, the pan was weighed to determine the amount of evaporated water compared to the total water content.

An exemplary water desorption record from SOM containing 10% microplastics that was dried at 43% is reported in the Supporting Information (Figure S5), where a blue line indicates a cooling step, while a green line represents a heating step. The record shows an endothermal event between ∼0 and ∼120 °C, which corresponds to the desorption of water from SOM (hereinafter, we will use the term "desorption" to refer to this process). The desorption enthalpy of water was determined by integrating the peak using the Trios software. The record shows a peak temperature of 64 °C and an enthalpy of 1.144 J. The enthalpy was normalized with the weight of the water. As a result, the enthalpy was determined as joules per gram of evaporated water. The second endothermal peak in Figure S5 corresponds to the melting of PHB (the melting point of PHB is between 173.6 and 176 °C).[48−50]

2.5. Determination of Aliphatic Crystallite Content. The content of aliphatic crystallites was assessed from their melting enthalpy, which was retrieved from the melting phase during the second DSC heating run. During the second run, the overlapping WaMB transition was not present.[46] Therefore, the melting enthalpy was proportional to the aliphatic crystallite content. The exemplary record and melting enthalpy determination are presented in Figure S6.

3. RESULTS

3.1. Water Content in SOM after Contamination. The comparison of the water contents in SOM before and after contamination is reported in Figure 1. To express the deviations, the results are plotted as the difference between the water content in noncontaminated and contaminated SOMs. The noncontaminated sample represents a control measurement and is expressed as solid red reference line; the interval of the highest experimental error of all three blanks is marked with a dashed red line. In Figure 1, the difference between the water content in SOM in noncontaminated and contaminated SOMs is expressed as $\Delta m/m$.

The results showed that for 43, 76, and 92% RHs, the water contents in the SOM mass fraction for uncontaminated SOM in the PET experiments were 0.150 ± 0.002, 0.196 ± 0.004, and 0.265 ± 0.007 w/w, respectively. In the PHB experiments, the values were 0.152 ± 0.002, 0.185 ± 0.007, and 0.272 ± 0.006 w/w, respectively (Table S1).

At 43% RH, the samples with up to 5% microplastic contamination resulted in an insignificant change in the water content in the SOM except for the sample containing 0.1% of PET. At higher concentrations of microplastics, the water content decreased.

At 76% RH, the amount of water in the SOM slightly increased up to 5% and then decreased. However, except for the highest concentrations, this increase was predominantly minor and within the margin of experimental error.

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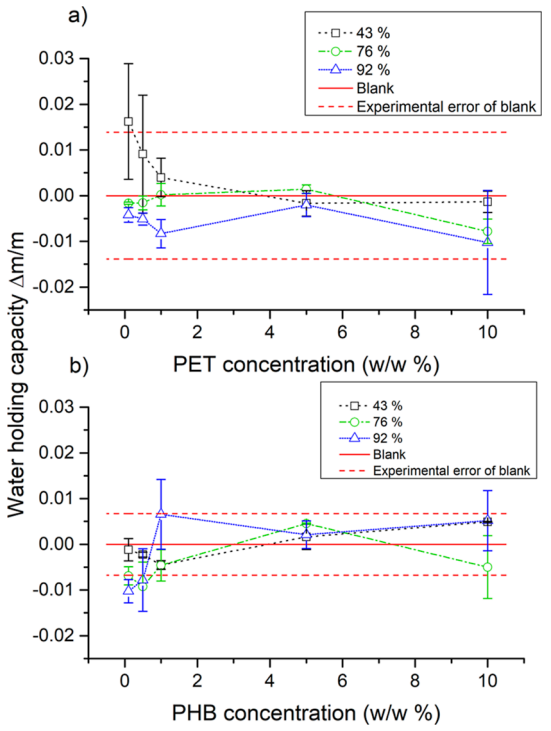

Figure 1. Water content in SOM dried at different RHs contaminated by different concentrations of PET (a) and PHB (b). The water content was normalized by SOM and microplastics or micro-bioplastics (n.b. differences in *Y* axes).

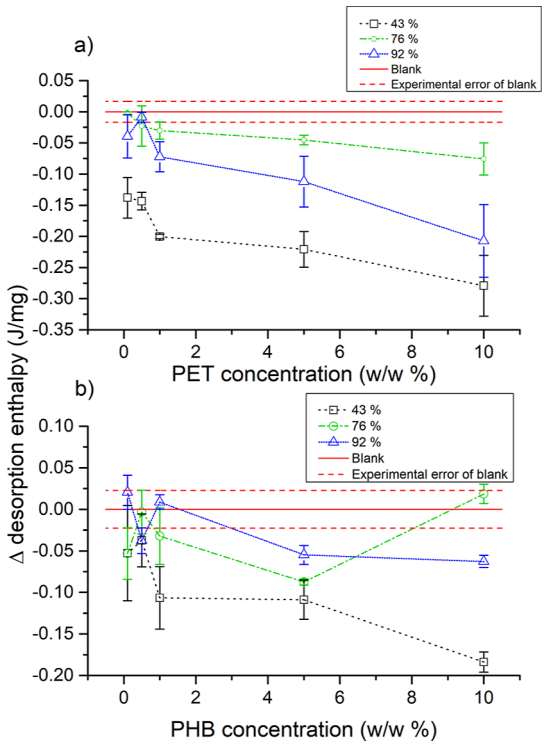

Figure 2. Desorption enthalpy of bound water related to pure water in SOM dried at different RHs contaminated by different concentrations of PET (a) and PHB (b). The measured heat was normalized by the mass of evaporated water.

At 92% RH, the water content in the PET-contaminated SOM was unchanged, except for the highest concentration. However, the PHB contamination led to a decrease in the water content of the 0.1 and 0.5% samples and an increase at higher PHB concentrations.

In addition, the amount of water in the SOM was normalized with the SOM mass content without PHB or PET to determine the effect of microplastics on the SOM alone (Table S2). The results suggest that the microplastics did not predominantly affect the water content (Figure S7).

3.2. Desorption Enthalpy—Water Binding Strength. The comparison of desorption enthalpy of water from SOM before and after contamination is reported in Figure 2. To express the deviations, the results are plotted as the difference between the desorption enthalpy of the water from non-contaminated and contaminated SOMs. The noncontaminated SOM is expressed as a solid red reference line; the interval of the highest experimental error of all three blanks is marked with a dashed red line. In Figure 2, the difference between the desorption enthalpy from noncontaminated and contaminated SOMs is expressed as Δ evaporation enthalpy.

For 43, 76, and 92% RHs, the desorption enthalpy values for uncontaminated SOM in the PET experiment were 2.26 ± 0.01, 2.03 ± 0.01, and 2.12 ± 2 J per mg_{H_2O}, respectively. In the PHB experiment, the values were 2.02 ± 0.02, 1.854 ± 0.007, and 1.827 ± 0.008 J per mg_{H_2O}, respectively (Table S3).

The general trend for all RH values was that an increasing concentration of microplastics and micro-bioplastics decreased the desorption enthalpy, which reflects the water binding strength (Figure 2). Exceptions could be observed for lower concentrations of microplastics and micro-bioplastics (up to 1%). The effect of decreasing the enthalpy was more pronounced for PET than for PHB; for example, the enthalpy with a contamination of 1% led to a decrease in the enthalpy of 43, 76, and 92% RH of −0.2 ± 0.004, −0.03 ± 0.01, and −0.07 ± 0.02 J per mg_{H_2O} for PET and −0.06 ± 0.04, −0.03 ± 0.03, and 0.009 ± 0.009 J per mg_{H_2O} for PHB, respectively.

3.3. Stability of WaMBs—Structural Stability of SOM. Figure 3 compares the T_{onset} temperatures of WaMB disruption in SOM before and after contamination. To express the deviations, the results are plotted as the difference between the T_{onset} temperatures of WaMB for noncontaminated and contaminated SOM. The noncontaminated SOM values are expressed as a solid red reference line; the interval of the highest experimental error of all three blanks is marked with a dashed red line. In Figure 3, the difference between the WaMB temperatures of noncontaminated and contaminated SOMs is expressed as ΔT_{onset}.

In the PET experiments, for 43, 76, and 92% RHs, the T_{onset} temperatures of WaMB were 47.2 ± 0.2, 47.4 ± 0.02, and 45.9 ± 0.4 °C, respectively. In the PHB experiments, the T_{onset} temperatures were 49.8 ± 0.05, 48.7 ± 0.07, and 46.3 ± 0.04 °C, respectively (Table S4).

No general trend was observed for WaMB temperatures. At 43% RH, for the 0.1−5% PHB, T_{onset} decreased and increased at 10% PHB. This is in contrast to the trend of PET-contaminated SOM, which showed a decrease.

At 76% RH, for PHB at 0.1−1% was observed an increase and a decrease between 5 and 10%. On the contrary, for PET, at a 0.5% contamination, T_{onset} increased.

At 92% RH, the 0.1−0.5% PHB contamination caused a decrease in T_{onset}, and an increase was observed at higher concentrations. The contamination by PET caused a decrease in T_{onset}.

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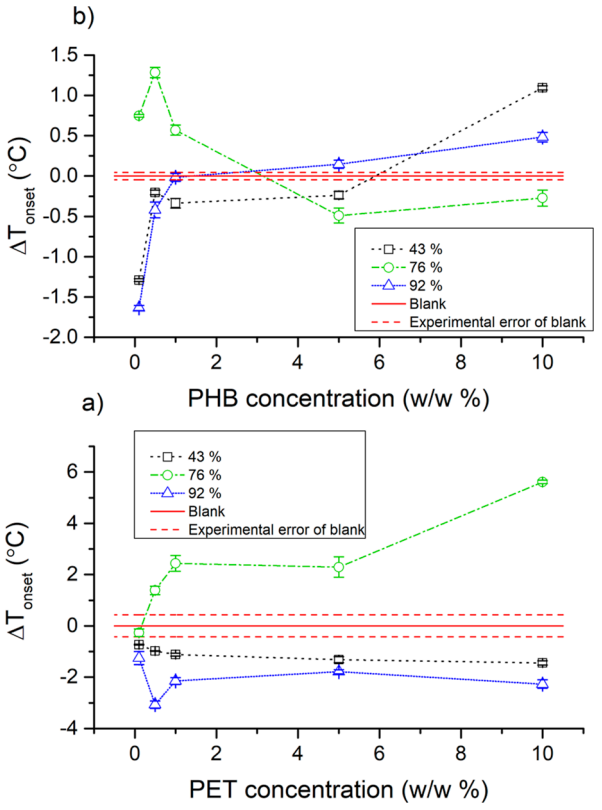

Figure 3. Stability of WaMBs (expressed as ΔTonset required for breaking the weakest WaMB from the uncontaminated SOM) related to uncontaminated SOM in SOM dried at different RHs contaminated by different concentrations of PET (a) and PHB (b).

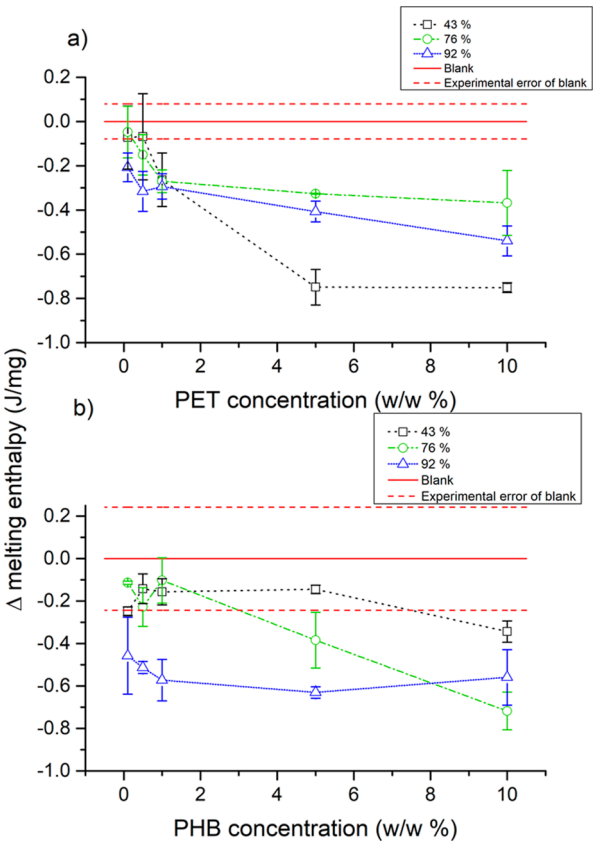

Figure 4. Melting enthalpy corresponding to the aliphatic crystallite content related to uncontaminated SOM in SOM dried at different RHs contaminated by different concentrations of PET (a) and PHB (b). The measured heat was normalized by SOM with microplastics.

3.4. Stability of Aliphatic Crystallites. Figure 4 compares the differences in the enthalpies before and after contamination, that is, between the noncontaminated and contaminated samples. The noncontaminated samples are expressed as a solid red reference line; the interval of the highest experimental error of all three blanks is marked with a dashed red line. In Figure 4 are the reported values obtained after normalization of the enthalpy using the whole mass of the SOM.

For 43, 76, and 92% RHs, the melting enthalpies of the crystallites for blank SOM in the PET experiment were 2.30 ± 0.08, 2.06 ± 0.03, and 2.44 ± 0.04 J per mg, respectively. In the PHB experiment, they were 2.60 ± 0.03, 2.66 ± 0.06, and 2.7 ± 0.2 J per mg, respectively (Table S5).

The results show that increasing the concentration of PET in SOM nonlinearly decreases the melting enthalpies of crystallites at all RHs. For PHB, the decrease was observed mainly at 92% RH; for 76% RH, the decrease was observed for 5 and 10% PHB concentrations; and at 43%, the decrease was observed only for the concentration of 10%.

The melting enthalpies of the crystallites in the SOM were also normalized using the SOM content without PHB or PET to determine the effect of microplastics solely on the SOM (Table S6). The results (Figure S8) clearly indicate that increasing the plastics content caused a decrease in melting enthalpy, indicating a decrease in the content of SOM aliphatic crystallites.

4. DISCUSSION

Comparing the effect of PHB micro-bioplastics and PET microplastics on SOM reveals similar effects on the water content (Figure 1), water binding strength (desorption enthalpy) (Figure 2), and aliphatic crystallite content (Figure 4). Differences caused by plastic spiking were observed for the structural stability (Figure 3).

4.1. Effects of Microplastics and Micro-Bioplastics on Properties of Water in SOM. Prior to any discussion about the effect of plastics on SOM, it is important to describe the changes in the water structure in SOM caused by changes in RH. According to previous studies,[47,51] when the vacuum-dried sapric histosol is exposed to an RH of below 46%, water is mainly adsorbed on the so-called primary sorption sites. These sites consist of polar functional groups and strongly bind water. An increase in the RH (from 35 to 43% in the commented case) results in further water sorption and a progressive increase in sorption site size. As a result, two or more adjacent sites can connect to form a WaMB. These WaMBs are thermally stable because they are formed between groups that are very close to each other. By further increasing the RH (~76%), weaker bridges are formed between the functional groups, which are more distant from each other. Simultaneously, within the WaMB, five-membered water clusters are formed. Such structured water clusters can enter the hydrophobic pores and interact with hydrophobic walls via van der Waals forces. As a result, water molecules accumulate

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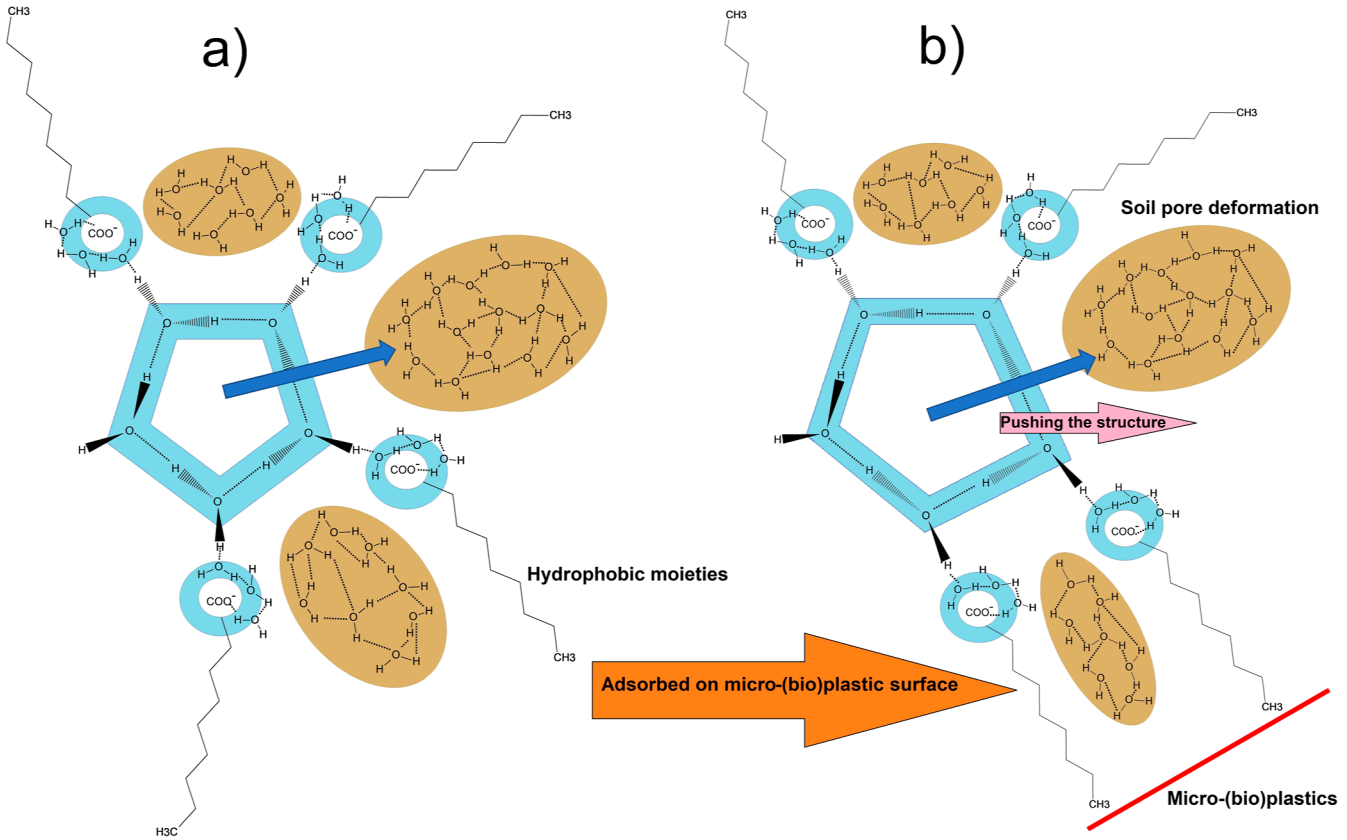

Figure 5. Explanation of the water state in SOM. The blue pentagram is the water pentamer, and in brown is the water in hydrophobic cavities (a) without (bio)microplastic and (b) deformation of the molecular structure caused by the (bio) microplastics (red line).

in the pore and form the water phase.[47] This situation is illustrated in Figure 5a of this work.

It should be noted that in contrast to the work[47] in which the SOM was dried in vacuum and then exposed to constant humidity, in this work, the samples were prepared by evaporating water from the sample after contamination. This is expected to result in effects that are associated with the re-conformation of SOM molecules during drying.

The PHB and PET microplastics had a conclusive effect on WaMB temperature (Figure 3) and desorption enthalpy (Figure 2) and no effect on the water content in SOM (Figure 1). These findings lead to the following hypotheses:

a Microplastics affect SOM pores by clogging and affecting the formation and stability of WaMBs and desorption enthalpy;

b Microplastics directly affect the hot spots where the WaMBs are formed;

c Microplastics affect the structure of SOM, which subsequently affects the hot spots where WaMBs are formed.

Hypothesis (a): This hypothesis is based on the notion that SOM is mostly a microporous system[52] and that moisture desorption from soil involves coupled heat and mass transfer. This whole process is influenced by the transport properties of liquid water, vapor, and heat, atmospheric evaporative demand, and vapor and heat exchanges between the soil and the environment.[53] Any disruption of this system influences the processes of desorption. The results reported in this work showed that the PHB and PET microplastics affected the SOM structure, which did not affect the water content (Figure 1 and Table S2 in the Supporting Information). However, they significantly decreased the desorption enthalpy (Figure 2). The decrease can be interpreted as a change in water−SOM interaction and faster water desorption (see Section 4.3). It is noteworthy that faster desorption (i.e., desiccation) was also possible to be observed visually when manipulating the SOM. The micropores form approximately 95−99% of the SOM surface area, with maximum restrictions of approximately 0.5 nm.[54] In this scenario, the particles would be adsorbed on the surface, which would influence the water desorption from SOM. However, the water content that was counted per SOM did not change (Figure S7). This scenario would accordingly not explain the decrease in desorption enthalpy.

Hypothesis (b): The WaMB is a cluster of water molecules formed between adjacent functional groups and can therefore be affected by RH or mechanically.[55] The size is estimated to be between 1.0 and 1.3 nm. It comprises 14 water molecules and can reduce the system's energy by 42−84 kJ/mol.[56] Therefore, a specific number of water molecules are required to form a WaMB.[57] More specifically, both an excess and a deficiency of water molecules can destabilize WaMBs.[58] In addition, the penetration of poorly wettable PHB and PET would not disrupt the hydrophilic WaMB hot spot as it would be energetically unfavorable. Therefore, the direct effect on the WaMB hotspot can also be excluded.

Hypothesis (c): The effect on the structure's rigidity depends on the drying atmosphere and, therefore, on the prevailing type of water in the SOM. In the sample that contained mainly adsorbed water, the rigidity of SOM increased alongside the PHB and PET concentration. By contrast, in the sample with more water molecules in the

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WaMBs in the form of clusters, the SOM's rigidity decreased as PHB and PET concentrations increased (Figure 3). As discussed above, PHB and PET are poorly wettable polymers. The SOM is a supramolecular mixture of various molecules,[59] which form domains of various polarities.[60] Additionally, the mixture includes amphiphilic and hydrophobic molecules and moieties, which can adsorb on the PHB surface. The high affinity of liposoluble compounds such as chlorobenzene (CB) and *o*-nitrochlorobenzene (*o*-NCB) to PHB were demonstrated in an earlier report.[61] Such a preferential sorption of certain SOM molecules caused the re-conformation of the SOM supramolecular structure, in which presumably hydrophobic moieties are directed toward the PHB surface, similarly to processes occurring in soil[62,63] or at the air/dissolved organic matter interphase.[61] Depending on the PHB concentration, the interaction between hydrophobic moieties and the PHB surface impacts the original organization of the WaMB hot spot and disrupts the configuration and porosity of the SOMs' polar domains. The stability of the bridges is related to the distance between the polar groups and the polarity of the surrounding molecules and possible contaminants.[64] Small amounts of either PHB or PET can affect the bridge by disrupting the WaMB's surroundings. The increasing concentrations gradually strengthen the bridge by pressing the bridges together, either mechanically or by inducing chemical changes in the vicinity of the WaMB, which is similar to the behavior of various chemicals.[64] The pushing effect is supported by Mikutta et al.,[55] who observed a shift in WaMB temperature in mineral soils due to the pushing effect of minerals on SOM. The situation with microplastics is illustrated in Figure 5b.

Hypothesis (c) can explain the behavior of water in contaminated SOM that has been dried under various RHs. It was observed that once lower concentrations of PHB microbioplastics were added, WaMBs were destabilized in SOM that was exposed to 43% RH; however, they were stabilized when the micro-bioplastic concentration was increased (Figure 3). Moreover, the desorption enthalpy gradually decreased (Figure 2), indicating that the presence of PHB influenced the structure in the way that water in the SOM is bound weaker. As the obtained desorption enthalpy represents the sum of all water interactions in the SOM, it is proportional to the interaction energy between SOM and water molecules plus the energy cost of water transport in the microporous SOM structure. Therefore, the reduction in desorption enthalpy is due to the disruption of the SOM structure. We speculate that the PHB size and interaction with SOM molecules may lead to the opening of the SOM pores, thus facilitating water desorption. This also explains the effect of PET microplastics, which decreased the WaMB strength at all concentrations.

The results contain some inconsistencies for SOM exposed to 76% RH, where the addition of a lower concentration of microplastics did not significantly affect the amount of water (Figure 3). We attribute this observation to the differences in the contact angles between both polymers, which affects both their affinity to SOM and their ability to disrupt the supramolecular structure of SOM. Also, the situation with WaMB at 43 and 92% RHs for PHB is peculiar (Figure 3). We conclude that the desorption enthalpy decreased (Figure 2), which is due to both the re-conformation of the structure and the effect on water retention. A similar explanation can be drawn for PET. the physical structure of SOM is corrupted, which decreases the WaMB stability. As the surface of PET is slightly less wettable (i.e., hydrophobic) than PHB, PET affects the WaMB hot spots more and the surrounding hydrophobic cavities less. As a result, the WaMB becomes less stable as the PET concentrations increase.

4.2. Influence on Aliphatic Crystallites. The extent to which crystalline aliphatic fractions interact with extraneous compounds or particles has not been well-described in the literature.[65] In the SOM under study, the aliphatic crystallites consist of the following two types of moieties: a fraction that comprises biopolymer residues that may originate from higher plants and a fraction that comprises microbiological fragments.[65] Their content in the studied sapric histosol, including both plant and microbial fractions, was estimated to be 2.8−7.6% of the total mass. The crystallites structure was altered by an interaction with monoaromatic[65] and some polycyclic aromatic hydrocarbons.[66] Despite the hydrophobic characteristic of the crystallites, their structure responds both to polar solvents, such as water and methanol, and less polar solvents, such as acetone and hexane.[65]

These results indicate that the addition of both PHB and PET to SOM led to a decrease in the melting enthalpy (Figure 4), which can be attributed to the decrease in aliphatic crystallite content. A partial weakening of the crystals, which would also decrease enthalpy, would be reflected in a decrease in the melting temperature of the crystallites, which was not observed. According to Hu et al.,[67] the diameter of a poly(methylene) region is estimated to be more than 3 times the crystallite thickness, that is, it exceeds 12 nm. Therefore, although its size is significantly lower than that of PHB or PET, it has comparable wettability (see the contact angles reported in the Experimental Section) and therefore hydrophobicity. The decrease in the crystallite content suggests that their interaction results in the decomposition of their crystalline structure. More specifically, the nanometer-sized crystallites, composed of a wide range of chemical structures,[68] have lower thermodynamic stability than fully developed and chemically pure crystallites. This is caused by their small size,[64] as well as the fact that interaction with the PHB or PET partially or fully spreads the molecules (adsorption) on their surface, which is similar to a melting process.

Due to their arrangement,[67] the aliphatic crystallites represent a biologically stable pool of SOM,[69] and their concentration in SOM increases over time due to selective enrichment.[70] On the contrary, the amorphous domains of aliphatic moieties are less refractory and biodegrade faster.[67] As suggested by our data, the PET microplastics and PHB micro-bioplastics destabilize a refractory pool of SOM, thereby contributing to faster SOM mineralization. We speculate that this may be a more serious problem for biodegradable PHB as its biodegradation relates to increased microorganisms, which can cause faster biodegradation of adsorbed aliphatic moieties. On the contrary, the refractory PET may protect the "melted" aliphatic moieties from mineralization by their mutual interaction, which in some cases increases the persistence of molecules against the degrading enzymes.

The aliphatic crystallites represent up to 10 percent of the total SOM,[46] and their environmental relevance is still not understood. As aforementioned, they are presumably biologically recalcitrant. However, the data obtained up to now indicate that they are relatively unstable upon interaction with various chemicals or particles; they can both "melt"[65] and "grow".[51,65] Their existence reflects one of the main SOM stabilization mechanisms,[71] but the current awareness on

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aliphatic crystallites does not allow for any prediction of their fate.

4.3. Environmental Relevance and Upscaling of the Results. The most affected parameters in SOM that has been contaminated by microplastics are desorption enthalpy (Figure 2) and WaMB transition (Figure 3). The data indicate that the intrusion of PHB microplastics into the SOM structure influences the original pore system and introduces less wettable—that is, relatively hydrophobic—surfaces on which water molecules self-organize to minimize contact. This is entropically unfavorable because it disrupts the existing water structure and imposes a new, more ordered structure on the surrounding water molecules.[72] As a result, the water configuration changes, which has an influence on its dynamics.

A mechanistic account of desorption enthalpy and its decrease as caused by microplastics should accordingly be discussed due to its environmental relevance. In almost all cases, except for 76% RH, a constant decrease in desorption enthalpy was observed when the PHB concentration was increased (Table S3). At 10% PHB, this decrease was approximately 10%. In other words, both PHB micro-bioplastics and PET microplastics support and accelerate the desorption of water from SOM in proportion to their increasing concentrations. This faster desorption is more pronounced in SOM that is dried at a lower RH, that is, both PHB micro-bioplastics and PET microplastics, and influences the amount of water adsorbed on polar sites. This finding points to a greater risk of soil water loss due to microplastic contamination in arid and semiarid areas or during dry periods.

Notably, the observed effects would be the strongest during the primary phase when introducing the microplastics and micro-bioplastics into the soil as the fate of plastics is later dictated by the activity of the microorganisms. During the later phases, the fate and effect of both microplastics would differ because depending on the conditions, PHB will fragment and biodegrade, while PET will become a more or less persistent part of the soil. However, these effects (including the influence of the particle's shape) are beyond the scope of this work and will be addressed in future studies.

As aforementioned, the effects of microplastics and micro-bioplastics on abiotic soil components are still not well-understood. Until now, the effects were explained mostly as the effect of small particles on soil aggregation,[9,10,73,] while some authors concluded that this effect is putatively mediated by soil biota.[74] Here, we focused on short-term effects and excluded the possible biotic effects by using sapric histosol with low microbial activity. After the introduction in SOM, in the short term, we observed interactions of selected microplastics and micro-bioplastics with SOM. This caused changes in the supramolecular conformation of SOM moieties and shifted its properties on the nano-level scale. Taking into account previous results,[74] we conclude that the short-term effects are mainly abiotic, while the long-term mechanism may include biotic processes influencing soil aggregation.

Notably, it is assumed that PHB will be used extensively in the future, meaning that its increasing input into the soil (mulch, delivery systems, coatings, or littering) can also be expected. Therefore, it is important to be aware of possible problems that may arise as a result of its use in agriculture or in other industries.[75] The aim of this work was not to frame PHB as a new contaminant but rather to underscore the observation that the potential problems that bioplastics may cause in soil should be comprehensively investigated rather than overlooked. Future studies should thus aim to develop recommendations as to how such problems with bioplastics in agriculture might be avoided as bioplastics currently seem to be the only way to address the soil microplastics problem.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c01970.

Size distribution of PET and PHB particles suspended in deionized water, SEM image of the PHB particle used for experiments, DSC record and determination of WaMB transition, exemplary record and determination of desorption enthalpy of water from SOM contaminated with 10 % of P3HB microplastics, exemplary record and determination of melting enthalpy of soil aliphatic crystallites from SOM contaminated with 10 % of P3HB microplastics, water content in SOM dried at different RHs contaminated by different concentrations of PET and PHB, melting enthalpy corresponding to the aliphatic crystallite content related to uncontaminated SOM in SOM dried at different RHs contaminated by different concentrations of PET and PHB, average of water content in SOM normalized with SOM and microplastics and with only SOM and the deviations from the blank sample, average desorption enthalpy of SOM water and the deviations from the blank sample, average T_{onset} temperature of WaMB and the deviations from the blank sample, and average soil aliphatic crystallite melting enthalpy normalized with SOM and microplastics and with SOM and the deviations from the blank sample (PDF)

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Notes

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SUPPORTING INFORMATION

Influence of poly-3-hydroxybutyrate micro-bioplastics and polyethylene terephthalate microplastics on soil organic matter structure and soil water properties

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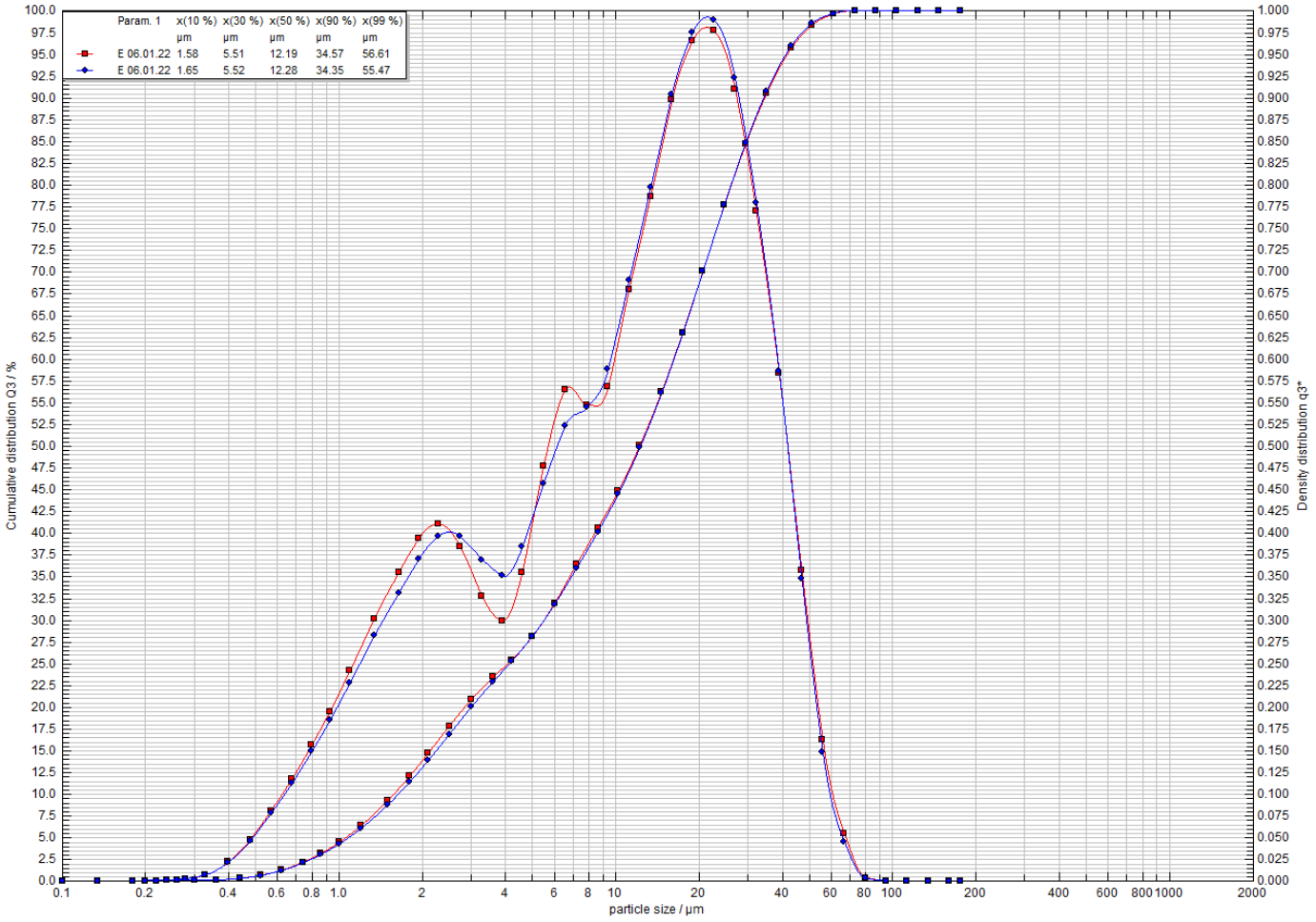

Figure S1: The repeated measurements of size distribution of PET particles suspended in deionized water after sonification. The suspension was measured with the dynamic light scattering system HELOS Particles Size Analysis System from Sympatec GmbH.

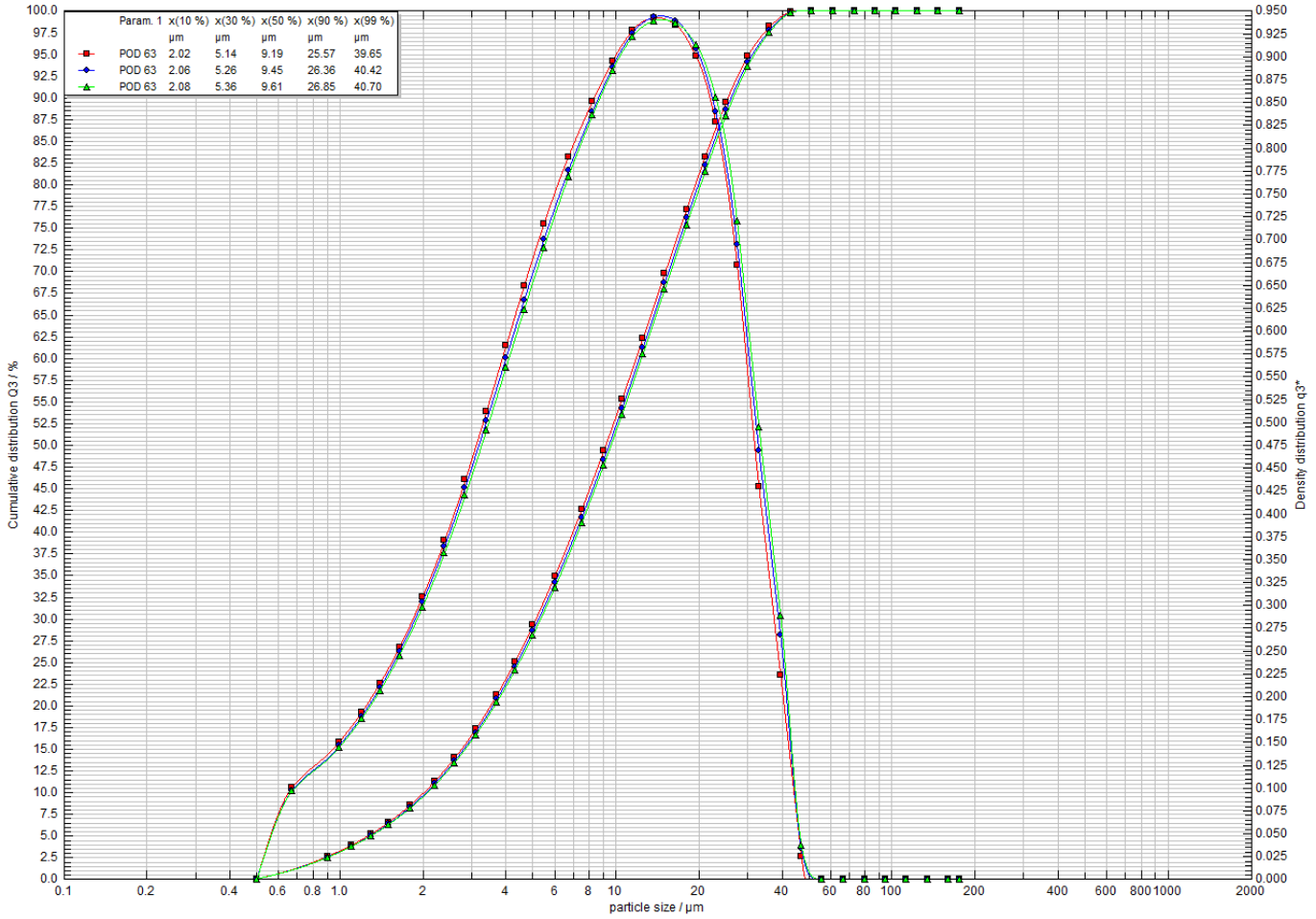

Figure S2: The repeated measurements of size distribution of PHB particles suspended in deionized water after sonification. The suspension was measured with the dynamic light scattering system HELOS Particles Size Analysis System from Sympatec GmbH.

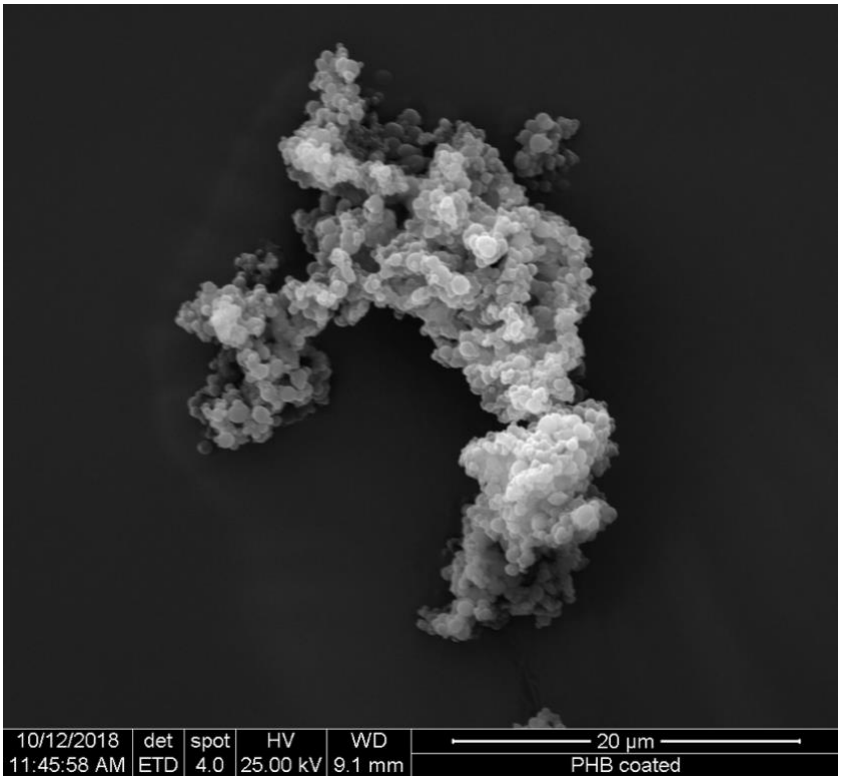

Figure S3: Scanning electron microscope image of polyhydroxybutyrate particle used for the experiments

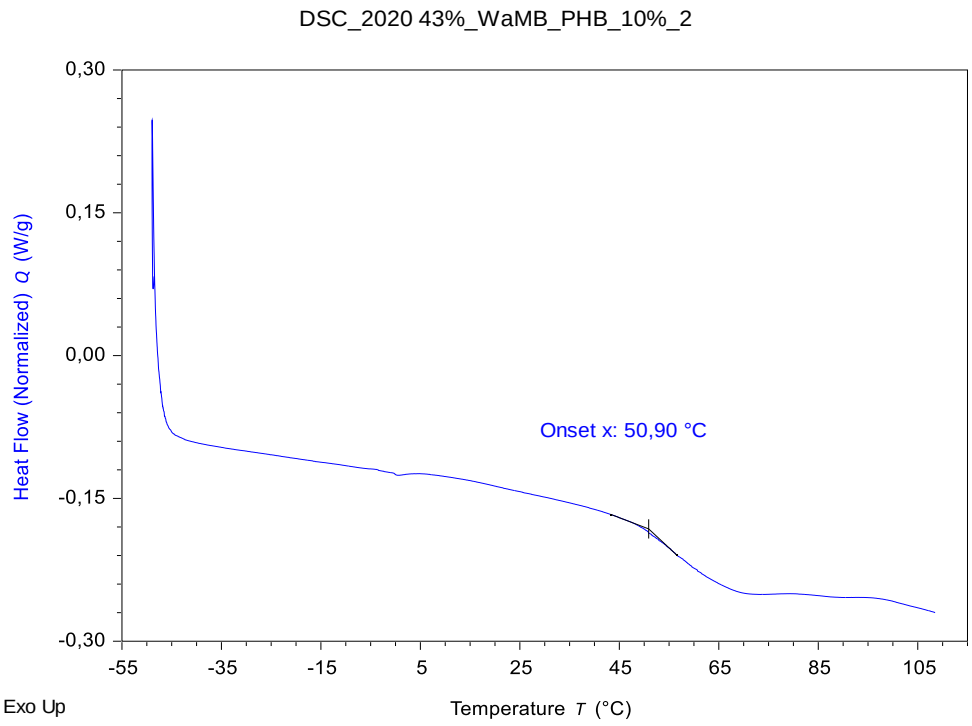

Figure S4: DSC record and determination of WaMB transition.

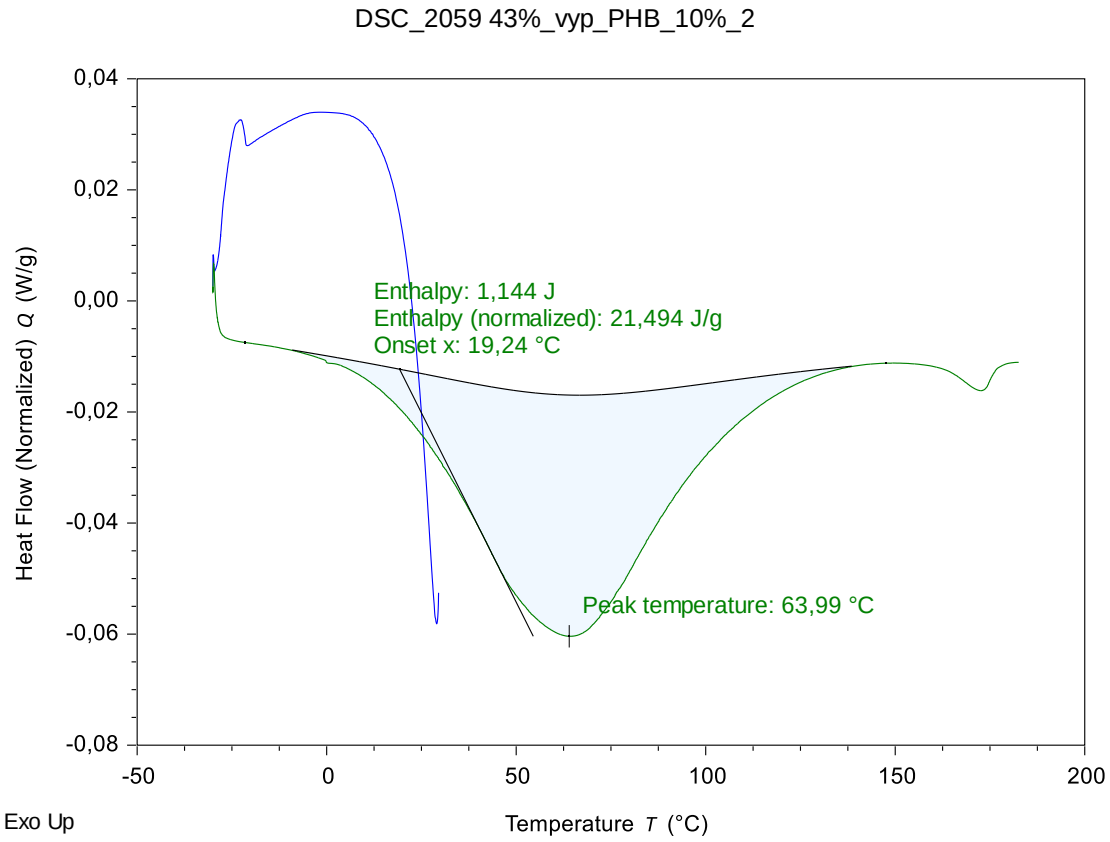

Figure S5: An exemplary record and determination of desorption enthalpy of water from SOM contaminated with 10 % of P3HB microplastics. Blue line stands for cooling from laboratory temperature to -30 °C and green line stands for heating to 185 °C.

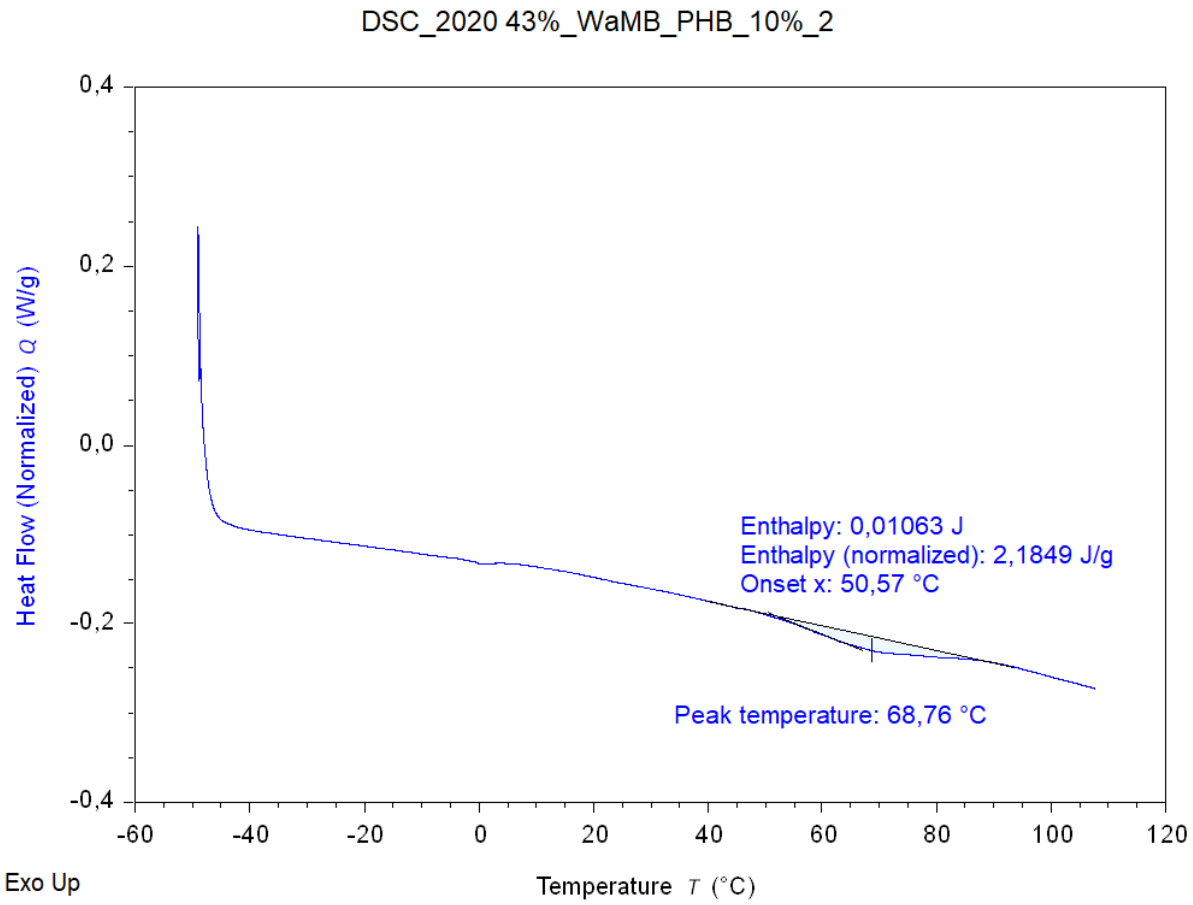

Figure S6: An exemplary record and determination of melting enthalpy of soil aliphatic crystallites from SOM contaminated with 10 % of P3HB microplastics.

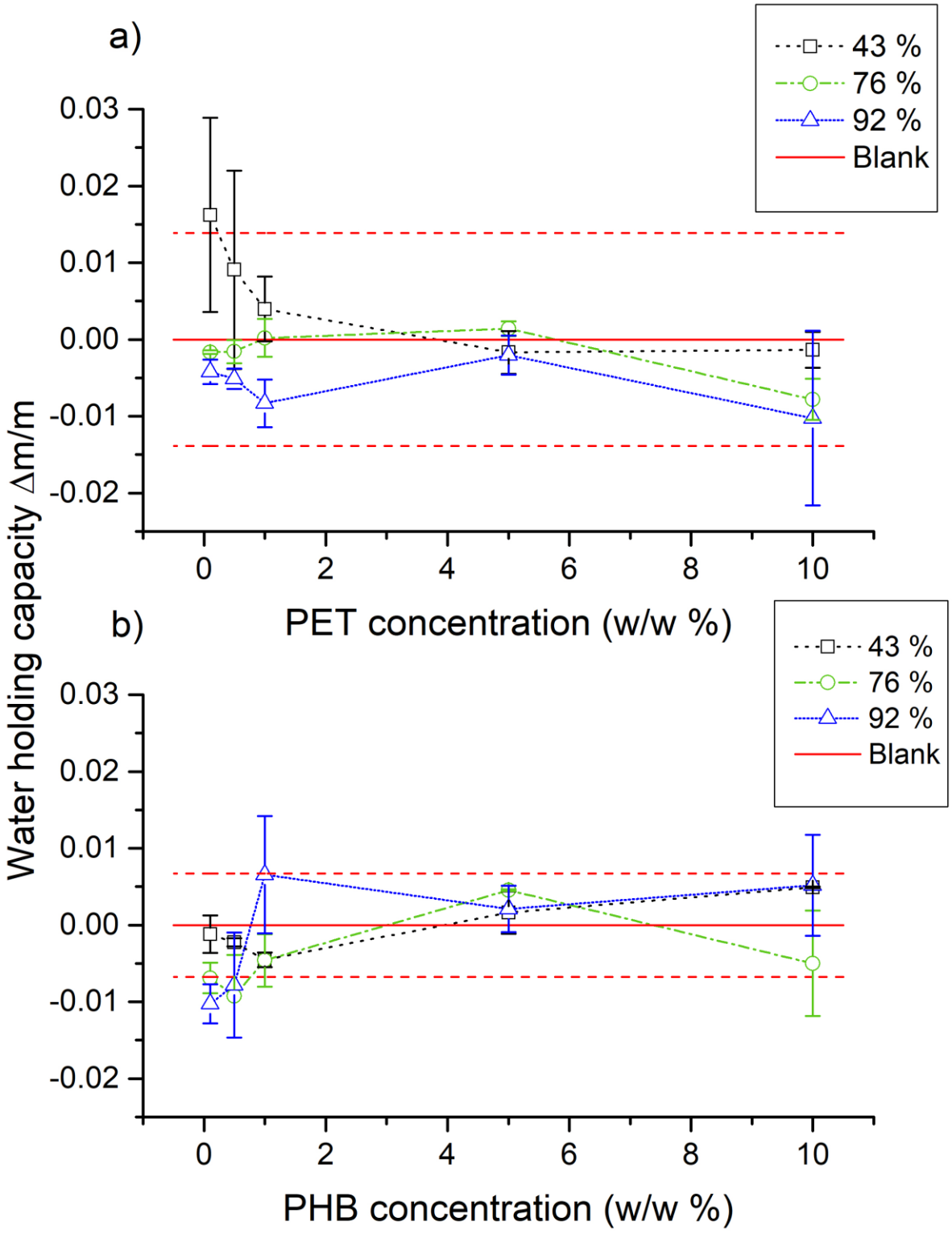

Figure S7: Water content in SOM dried at different relative humidities contaminated by different concentrations of PET (a) and PHB (b). The water content was normalized only by SOM.

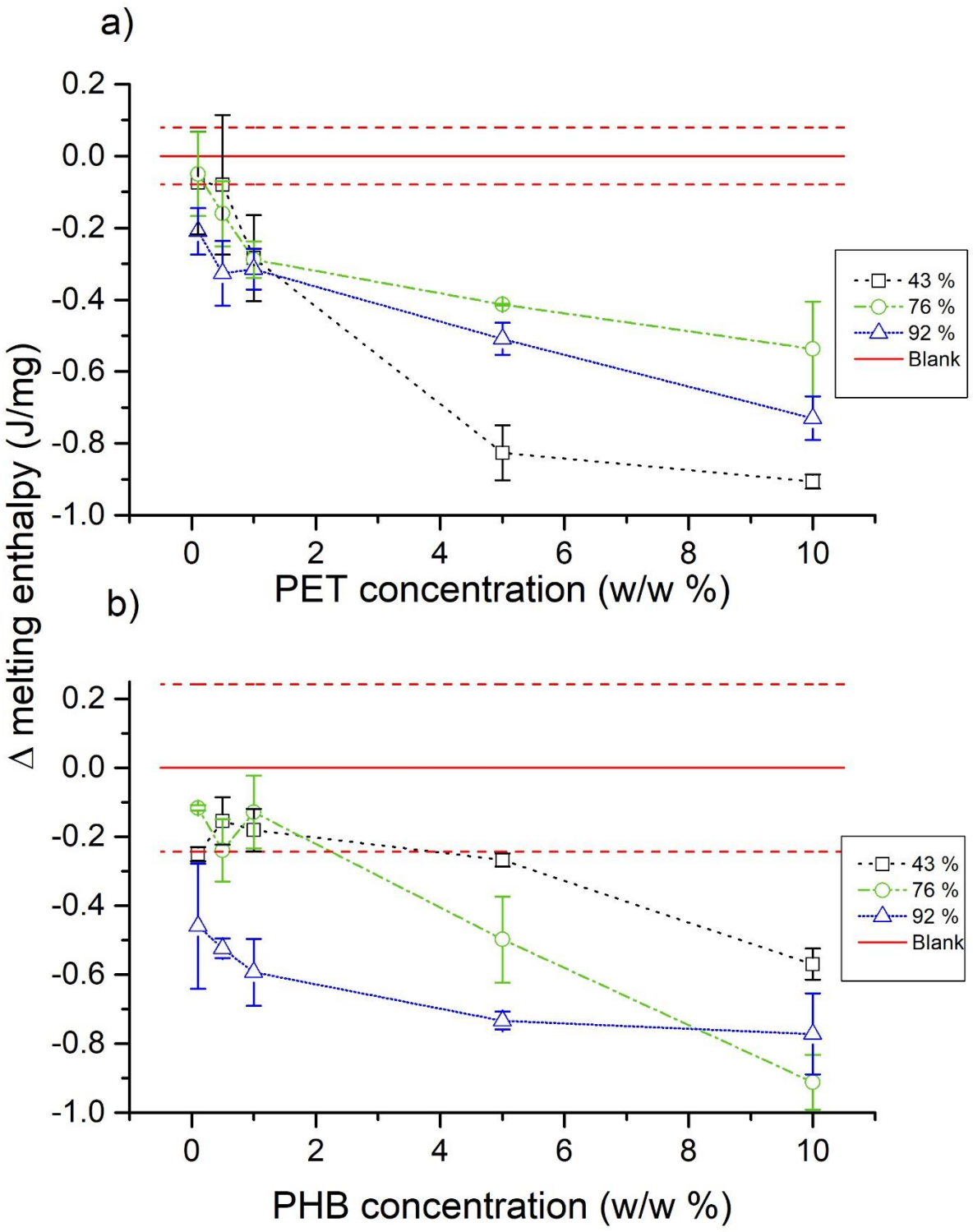

Figure S8: Melting enthalpy corresponding to the aliphatic crystallites content related to uncontaminated SOM in SOM dried at different relative humidities contaminated by different concentrations of PET (a) and PHB (b). The measured heat was normalized by SOM and added micro-plastics or micro-bioplastics (n.b. differences in Y axes).

Table S1: Average of the water content in SOM normalized with SOM and microplastics (m/m norm) and the deviations from blank sample (Δm/m norm)

PET						
	RH 43 %		RH 76 %		RH 92 %	
Concentration %	m/m norm	Δm/m norm	m/m norm	Δm/m norm	m/m norm	Δm/m norm
Blank	0.150	0.000	0.196	0.000	0.265	0.000
0.1	0.166	0.016	0.194	-0.002	0.270	0.005
0.5	0.159	0.009	0.194	-0.002	0.269	0.004
1.0	0.154	0.004	0.196	0.000	0.266	0.001
5.0	0.148	-0.002	0.197	0.001	0.272	0.007
10.0	0.149	-0.001	0.188	-0.008	0.264	-0.001
PHB						
	RH 43 %		RH 76 %		RH 92 %	
Concentration %	m/m norm	Δm/m norm	m/m norm	Δm/m norm	m/m norm	Δm/m norm
Blank	0.152	0.000	0.185	0.000	0.272	0.000
0.1	0.151	-0.001	0.178	-0.007	0.262	-0.010
0.5	0.150	-0.002	0.176	-0.009	0.264	-0.008
1.0	0.147	-0.005	0.180	-0.005	0.279	0.007
5.0	0.153	0.002	0.189	0.005	0.274	0.002
10.0	0.157	0.005	0.180	-0.005	0.277	0.005

Table S2: Average of the water content in SOM normalized only with SOM (m/m norm) and the deviations from blank sample (Δm/m norm)

PET						
	RH 43 %		RH 76 %		RH 92 %	
Concentration %	m/m norm	Δm/m norm	m/m norm	Δm/m norm	m/m norm	Δm/m norm
Blank	0.150	0.000	0.196	0.000	0.274	0.000
0.1	0.166	0.016	0.194	-0.002	0.269	0.004
0.5	0.158	0.008	0.194	-0.003	0.268	0.003
1.0	0.152	0.002	0.194	-0.002	0.263	-0.002
5.0	0.141	-0.009	0.188	-0.008	0.259	-0.007
10.0	0.134	-0.016	0.169	-0.027	0.238	-0.028
PHB						
	RH 43 %		RH 76 %		RH 92 %	
Concentration %	m/m norm	Δm/m norm	m/m norm	Δm/m norm	m/m norm	Δm/m norm
Blank	0.152	0.000	0.185	0.000	0.272	0.000
0.1	0.150	-0.001	0.178	-0.007	0.262	-0.011
0.5	0.149	-0.003	0.175	-0.010	0.263	-0.009
1.0	0.146	-0.006	0.178	-0.006	0.276	0.004
5.0	0.146	-0.006	0.180	-0.005	0.260	-0.012
10.0	0.141	-0.011	0.162	-0.023	0.250	-0.023

Table S3: Average desorption enthalpy of SOM water and the deviations from blank sample (Δ desorption enthalpy)

PET						
	RH 43 %		RH 76 %		RH 92 %	
Concentration %	Desorption enthalpy [J/mg]	Δ desorption enthalpy [J/mg]	Desorption enthalpy [J/mg]	Δ desorption enthalpy [J/mg]	Desorption enthalpy [J/mg]	Δ desorption enthalpy [J/mg]
Blank	2.261	0.000	2.027	0.000	2.120	0.000
0.1	2.123	-0.138	2.025	-0.002	2.081	-0.040
0.5	2.117	-0.143	2.004	-0.023	2.112	-0.009
1.0	2.060	-0.200	1.997	-0.030	2.048	-0.072
5.0	2.040	-0.221	1.982	-0.045	2.008	-0.112
10.0	1.982	-0.279	1.951	-0.076	1.913	-0.207
PHB						
	RH 43 %		RH 76 %		RH 92 %	
Concentration %	Desorption enthalpy [J/mg]	Δ desorption enthalpy [J/mg]	Desorption enthalpy [J/mg]	Δ desorption enthalpy [J/mg]	Desorption enthalpy [J/mg]	Δ desorption enthalpy [J/mg]
Blank	2.017	0.000	1.854	0.000	1.827	0.000
0.1	2.008	-0.009	1.800	-0.053	1.847	0.020
0.5	2.024	0.007	1.850	-0.003	1.789	-0.038
1.0	1.955	-0.062	1.821	-0.032	1.836	0.009
5.0	1.952	-0.065	1.766	-0.088	1.772	-0.055
10.0	1.877	-0.140	1.872	0.019	1.764	-0.063

Table S4: Average T_{onset} temperatures of WaMB and the deviations from blank sample (Δt)

PET						
	RH 43 %		RH 76 %		RH 92 %	
Concentration %	t [°C]	Δt [°C]	t [°C]	Δt [°C]	t [°C]	Δt [°C]
Blank	47.2	0.0	47.4	0.0	45.9	0.0
0.1	46.5	-0.7	47.1	-0.3	44.6	-1.3
0.5	46.3	-1.0	48.8	1.4	42.8	-3.1
1.0	46.1	-1.1	49.9	2.4	43.7	-2.1
5.0	45.9	-1.3	49.7	2.3	44.1	-1.8
10.0	45.8	-1.4	53.0	5.6	43.6	-2.3
PHB						
	RH 43 %		RH 76 %		RH 92 %	
Concentration %	t [°C]	Δt [°C]	t [°C]	Δt [°C]	t [°C]	Δt [°C]
Blank	49.8	0.0	48.7	0.0	46.3	0.0
0.1	48.5	-1.3	49.4	0.7	44.6	-1.6
0.5	49.6	-0.2	50.0	1.3	45.9	-0.4
1.0	49.5	-0.3	49.3	0.6	46.3	0.0
5.0	49.6	-0.2	48.2	-0.5	46.4	0.1
10.0	50.9	1.1	48.4	-0.3	46.8	0.5

Table S5: Average soil aliphatic crystallites melting enthalpy normalized with SOM and Microplastics and the deviations from blank sample (Δ Melting enthalpy)

PET						
	RH 43 %		RH 76 %		RH 92 %	
Concentration %	Melting enthalpy [J/mg]	Δ Melting enthalpy [J/mg]	Melting enthalpy [J/mg]	Δ Melting enthalpy [J/mg]	Melting enthalpy [J/mg]	Δ Melting enthalpy [J/mg]
Blank	2.301	0.000	2.066	0.000	2.440	0.000
0.1	2.226	-0.075	2.016	-0.050	2.230	-0.209
0.5	2.221	-0.080	1.906	-0.160	2.113	-0.327
1.0	2.017	-0.284	1.778	-0.288	2.125	-0.315
5.0	1.474	-0.827	1.653	-0.413	1.931	-0.509
10.0	1.395	-0.906	1.528	-0.538	1.710	-0.730
PHB						
	RH 43 %		RH 76 %		RH 92 %	
Concentration %	Melting enthalpy [J/mg]	Δ Melting enthalpy [J/mg]	Melting enthalpy [J/mg]	Δ Melting enthalpy [J/mg]	Melting enthalpy [J/mg]	Δ Melting enthalpy [J/mg]
Blank	2.598	0.000	2.657	0.000	2.688	0.000
0.1	2.348	-0.250	2.541	-0.116	2.229	-0.459
0.5	2.444	-0.154	2.417	-0.240	2.165	-0.523
1.0	2.418	-0.180	2.529	-0.128	2.095	-0.593
5.0	2.331	-0.267	2.159	-0.498	1.955	-0.733
10.0	2.029	-0.569	1.745	-0.911	1.916	-0.772

Table S6: Average soil aliphatic crystallites melting enthalpy normalized with SOM and the deviations from blank sample (Δ Melting enthalpy)

PET						
	RH 43 %		RH 76 %		RH 92 %	
Concentration %	Melting enthalpy [J/mg]	Δ Melting enthalpy [J/mg]	Melting enthalpy [J/mg]	Δ Melting enthalpy [J/mg]	Melting enthalpy [J/mg]	Δ Melting enthalpy [J/mg]
Blank	2.301	0.000	2.066	0.000	2.440	0.000
0.1	2.229	-0.073	2.018	-0.048	2.233	-0.207
0.5	2.232	-0.069	1.915	-0.151	2.124	-0.316
1.0	2.037	-0.264	1.796	-0.270	2.146	-0.294
5.0	1.552	-0.749	1.740	-0.326	2.033	-0.407
10.0	1.550	-0.751	1.698	-0.368	1.900	-0.540
PHB						
	RH 43 %		RH 76 %		RH 92 %	
Concentration %	Melting enthalpy [J/mg]	Δ Melting enthalpy [J/mg]	Melting enthalpy [J/mg]	Δ Melting enthalpy [J/mg]	Melting enthalpy [J/mg]	Δ Melting enthalpy [J/mg]
Blank	2.598	0.000	2.657	0.000	2.688	0.000
0.1	2.350	-0.248	2.543	-0.113	2.231	-0.457
0.5	2.457	-0.142	2.429	-0.228	2.176	-0.512
1.0	2.442	-0.156	2.554	-0.103	2.116	-0.572
5.0	2.454	-0.145	2.273	-0.384	2.058	-0.630
10.0	2.255	-0.344	1.939	-0.718	2.129	-0.559

Příloha D

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Chemical and Biological Technologies in Agriculture

RESEARCH

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Effect of biodegradable poly-3-hydroxybutyrate amendment on the soil biochemical properties and fertility under varying sand loads

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Abstract

Background: Poly-3-hydroxybutyrate (P3HB) is a bacterial intracellular carbon and energy storage polymer, used as a thermoplastic polyester in a wide array of industrial and agricultural applications. However, how the soil microbiome and fertility are altered by exogenously applied P3HB has been relatively unexplored. This study aimed to assess the effects of P3HB addition to nutrient restricted soil: its biological properties and lettuce (*Lactuca sativa* L. var. *capitata* L.) biomass production. The experiment was designed to evaluate impacts of spatial arrangement of the relatively organic-rich (soil organic matter, P3HB particles) versus poor fractions of the matrix with confounding factors such as variable microbial biomass, inherent nutrient/energy status, different water relations (due to variable hydrophysical properties of soil augmented by sand at different ratios).

Results: The results revealed that P3HB in soils induced inconsistent to contradictory changes in the microbial abundance as well as in most enzymatic activities. The differences were conditioned by the sand content both under P3HB presence or absence. On the other hand, dehydrogenase, urease activities, basal and substrate-induced soil respirations were mostly enhanced by P3HB addition, directly with increasing sand content (several respiration types). Nevertheless, P3HB significantly inhibited lettuce biomass production.

Conclusions: P3HB introduction to soil boosts the microbial activity owing to the preferential utilization of P3HB as C source, which depletes soil N and strongly inhibits the plant growth. Enhanced microbial activity in P3HB-amended soils with high sand content (60–80%) suggested that in nutrient-impoverished soil P3HB can temporarily replace SOM as a C source for microbial communities due to the shift of their structure to preferentially P3HB-degrading microbiome.

Keywords: Soil quality deterioration, Microbial community, Respiration, Biodegradation, Lettuce, P3HB

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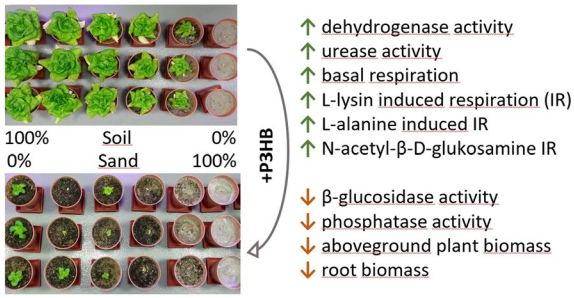
Graphical Abstract



Background

The non-degradable polymers derived from fossil fuels are in routine use worldwide. In face of the climate change and its environmental implications, the use of biodegradable polymers has been advocated recently [1, 2]. This has resulted in the increased production of various biodegradable polymers, such as polyhydroxyalkanoates (PHAs) and the most common type, poly-3-hydroxybutyrate (P3HB, for purpose of this publication), with presumably little or no negative effects on the environment. P3HB is a bacterial intracellular C and energy storage polymer, belonging to a family of biopolyesters polyhydroxyalkanoates (PHAs) [3]. PHAs are characterized by technologically promising properties such as biodegradability, biocompatibility, thermoprocessibility and flexible strengths [4]. PHB depolymerase enzymes are responsible for the biopolymer degradation [5] to monomer 3-hydroxybutyric acid which is completely microbially utilized [6, 7]. PHB has been further characterized as biodegradable under both aerobic and anaerobic conditions [8, 9], which adds a further plus to its utilization in a wide range of applications.

Given their advantages, biodegradable polymers are commonly used in agriculture [2, 10] as cover films (mulching), bands of sowing, in pots and containers and other horticulture materials and tools, for the controlled release of agricultural chemicals [11] and fertilizers [12, 13]. Owing to the wider use in daily products, other sources are similar in case of conventional plastics (i.e., compost, sewage sludge, irrigation, street runoff, littering and atmospheric deposition) [12–14]. By their degradation, the bioplastic particles are released into the soil, where their non-toxic character is assumed. However, up to now, the information on the influence of the PHB on soil quality and plant growth is scarce which demands further studies.

Zhou et al. [15] studied soil microbial community structure, growth, and exoenzyme kinetics in hotspots formed around microplastics of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). They observed that the addition of PHVB increased microbial activity and enriched specific bacterial taxa. Similar results have been observed by Deroiné et al. [16] in marine aquatic and sand environments. On the contrary, Sang et al. [17] observed reduced microbial activity in both environments.

So far, only few studies have focused on the effect of PHB amendment to soil on plant growth. The studies reported harmlessness of PHBV to maize (*Zea mays* L.) [18]. However, Mierziak et al. [19] found that an increase in the content of degradation product of PHB such 3-hydroxybutyrate (3-HB), in transgenic flax (*Linum usitatissimum* L. cv. NIKE) or after control plants treatment with 3-HB resulted in upregulation of genes involved in chromatin remodeling and activation of DNA de-/methylation. These changes were targeted to structural genes such as the phenylpropanoid pathway. Phenylpropanoid biosynthetic pathway is activated under abiotic stress conditions as the phenolic compounds attenuate the harmful impact of reactive oxygen species [20]. For example, drought stress in maize is accompanied by accumulation of phenylpropanoid [21]. Therefore, it may be considered an impact of excessive 3-HB in the soil environment on the plant ability to respond abiotic stress. In transgenic plant *C. sativa* designed to produce PHB in the plastids of seeds, high (up to 15% of mature seed weight) PHB production had varying effects on germination, emergence, and survival of seedlings; however, the survival was predominantly reduced [22]. Such finding also indicates the potential adverse impact of higher PHB levels in plants.

Under favorable conditions, the biodegradable plastic degrades fast within weeks to months [23]. These conditions occur mainly in favorable areas having soils with higher content of soil organic matter (SOM) and nutrients, higher microbial activity and C turnover, and water saturation. However, in regard to current spreadout

of soil degradation and SOM deficiency in soils, plastic particles may promote undesirable soil aggregation, which reduces the accessibility of organic matter to soil microbes [24], changes the proportion of soil nutrients [25], and subsequently could decrease plant primary production due to negative influencing of soil food web [26]. As a result, soil quality and productivity decreases, which includes a decrease in microbiological activity, deceleration of nutrient turnover and a decrease in SOM content [27]. To continue the biodegradation of bioplastics, which are composed mostly of C, H and O, the degrading organisms presumably exploit other sources of nutrients in soils including those normally used by other biota including plants. The scenario of bioplastics degradation under changing conditions of soil is hard to predict or model and only partial information on these processes are currently available, especially the soil textural differences for plastics biodegradation should be further considered.

Therefore, the aim of this study was to test the influence of introduction of P3HB into the arable soil and its effects on the soil quality and fertility at different levels of sand load. Increasing sand content represents a trajectory of soil degradation—a decrease in SOM, nutrient content, and different hydrophysical conditions represent a model of declining soil quality. Therefore, using sand, the number of biodegrading microorganisms and fungi in the arable soil was diluted and the conditions for biodegradation were changed. The study aims to answer the following questions: (a) How and to what extent soil fertility and microbial communities are affected by the P3HB amendment? (b) Does the dilution influence soil fertility and microorganisms' activity proportionally? (c) How will the increasing sand proportion influence the activity of the microorganisms after P3HB amendment?

Materials and methods

Experiment design

The growth substrates used for the pot experiment were prepared by mixing a fine quartz sand (0.1–1.0 mm; $\geq$95% SiO_2) with an arable soil. The soil was a silty clay loam (USDA Textural Triangle) Haplic Luvisol (WRB soil classification) sampled (0–15 cm) near the town Troubsko, Czech Republic (49°10′28″N 16°29′32″E). The soil properties were as follows: soil macronutrients ($g \cdot kg^{-1}$)—total C 14.0, total N 1.60, P 0.097, S 0.145, Ca 3.26, Mg 0.236, K 0.231; N forms ($mg \cdot kg^{-1}$)—$N_{mineral}$ 62.8, N-NO_3 56.8, N-NH_4 6.04; pH ($CaCl_2$) 7.3. Poly-3-hydroxybutyrate (P3HB) material ENMAT Y3000 (particles < 63 µm) in the form of microparticles was obtained from TianAn Biologic Materials Co., Ltd. (Ningbo City, China). The particles had spherical or spherical-like shapes. The contact angle of P3HB was reported between 70° and ~81°, which makes it slightly hydrophobic. Further specification was reported in Fojt et al. [28].

To remove the coarse particles, the soil was sieved through a sieve with mesh size 2 mm. The sieved soil was mixed with the sand in the following weight ratios: (I) 100% soil; (II) 80% soil + 20% sand; (III) 60% soil + 40% sand; (IV) 40% soil + 60% sand; (V) 20% soil + 80% sand; (VI) 100% sand. Each treatment was prepared in two scenarios: (A) with 1 wt% P3HB; (B) without any amendment (control). The content of 1 wt% P3HB was chosen in according to results of previous experiment with P3HB [28]. In total, 6 treatments were prepared. One kg of each thoroughly mixed growth substrate type was used to fill experimental plastic pots (volume 1 L, top diameter 11 cm, bottom diameter 9 cm, height 13 cm). Each treatment was carried out in 3 replicates (pots).

The pot experiment with crop lettuce (*Lactuca sativa* L. var. *capitata* L.) cv. Brilliant took place according to the following controlled conditions: cultivation in growth chamber Climacell EVO (BMT, Czech Republic)—full-spectrum LED lighting, intensity 20 000 lx; photoperiod 12 h; temperature 18/22 °C (night/day); relative humidity 70%. A 2-day sprouting of the lettuce seeds on wet filter paper preceded sowing to the depth of approximately 2 mm in each pot. After sowing, each pot was watered with 100 mL of distilled water. The 10-day-old seedlings were reduced to only one plant (the most robust) per pot. Pot placement in the growth chamber was randomized. Manual watering of each pot with 50 mL of distilled water was done every other day. Soil humidity was controlled, and water content was maintained during the experiment. The pots were variably rotated once per week. The plants were harvested 8 weeks after sowing.

The lettuce shoots were cut at ground level, and the roots were gently cleaned of soil and washed with water. The lettuce shoots and roots were dried at 60 °C to a constant weight, and dry aboveground and root biomass were estimated gravimetrically by weighing on the analytical scales.

Soil analysis

A mixed soil sample was taken from each pot after harvesting the lettuce. Soil samples were homogenized by sieving through a sieve with mesh size 2 mm. Air dried samples were analyzed for pH [29]. Freeze-dried samples were used for the analyses of enzymatic activities: β-glucosidase (GLU), arylsulfatase (ARS), phosphatase (Phos), urease (Ure) and N-acetyl-β-D-glucosaminidase (NAG) [30]. The *p*-nitrophenol (PNP)-derivatives of the specific soil substrates were used for Vis spectrophotometric measurement (Infinite M Nano, Tecan Trading AG, Switzerland) at $\lambda = 405$ nm (β-glucosidase,

arylsulfatase, phosphatase, and N-acetyl-β-D-glucosaminidase). Urease activity was determined as an amount of ammonium produced from the substrate urea, detected Vis spectrophotometrically by the reagent cyanurate ($\lambda = 650$ nm). Each soil sample was measured in nine replicates. The samples stored at 4 °C were used for determination of dehydrogenase activity (DHA) [31], soil basal respiration (BR) and substrate-induced respirations (IR). DHA was measured by 2,3,5-triphenyltetrazolium chloride (TTC)-based method. Respiration types—BR and induction with D-glucose (Glc-IR), D-trehalose (Tre-IR), N-acetyl-β-D-glucosamine (NAG-IR), L-alanine (Ala-IR), L-lysine (Lys-IR) and L-arginine (Arg-IR)—were measured using MicroResp® device (The James Hutton Institute, Scotland) and spectrophotometer (Infinite M Nano, Tecan Trading AG, Switzerland) [32].

DNA Extraction and Real-Time qPCR: DNA was extracted from 0.5 g of freeze-dried soil sample using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, USA). Isolated DNA was quantified using Nanodrop One (Thermo Scientific, USA). The SYBR-Green platform was used on a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories, USA). Real-time PCR was performed to quantify partial bacterial (16S rDNA) and fungal (18S rDNA) genes coding for ribosomal RNA, and gene *phaZ* (coding for polyhydroxybutyrate depolymerase) in soil DNA extracts. The primers used were 1108F (5′ ATGGYTGTCGTCAGCTCGTG 3′) and 1132R (5′ GGGTTGCGCTCGTTGC 3′) for bacteria [33], FF390 (5′ AICCATTCAATCGGTAIT 3′) and FR1 (5′ CGATAACGAACGAGACCT 3′) for fungi [34], PHBf (5′ CGTCTACCGCAACGGCACCAAGG 3′) and PHBr (5′ TGGGCGTAGTTGCTGGCCGT 3′) for *phaZ* [35].

Statistical analysis

Data processing and statistical analyses were performed using freely available software R, version 3.6.1. [36]. For characterization the relationship between the treatments and selected soil properties was used principal component analysis (PCA), multivariate analysis of variance (MANOVA at 0.1% significance level) and one-way analysis of variance (ANOVA) type I (sequential) sum of squares at 5% significance level [37]. For detection the statistically significant difference among factor level means, it was used Tukey's HSD (honestly significant difference) test and "treatment contrast" to calculate factor level means for each treatment. The results were also graphically presented with Rohlf biplot for standardized PCA. Pearson correlation analysis was performed for measuring the linear dependence between soil properties. Pearson correlation coefficient was interpreted as follows: $0.0 < r < 0.3$ (negligible correlation), $0.3 < r < 0.5$ (low correlation), $0.5 < r < 0.7$ (moderate correlation), $0.7 < r < 0.9$ (high correlation), and $0.9 < r < 1.0$ (very high correlation) [38].

Results

Soil microbial communities

As expected, the effect of P3HB on 16S rDNA was variable depending on the sand content (Fig. 1A). At 0–40% of sand, the P3HB effect was neutral to negative, while at 60–100% sand content, the effect was neutral to positive. In control, the effect of soil dilution by sand on 16S rDNA was significant from 60% of sand. The similar patterns

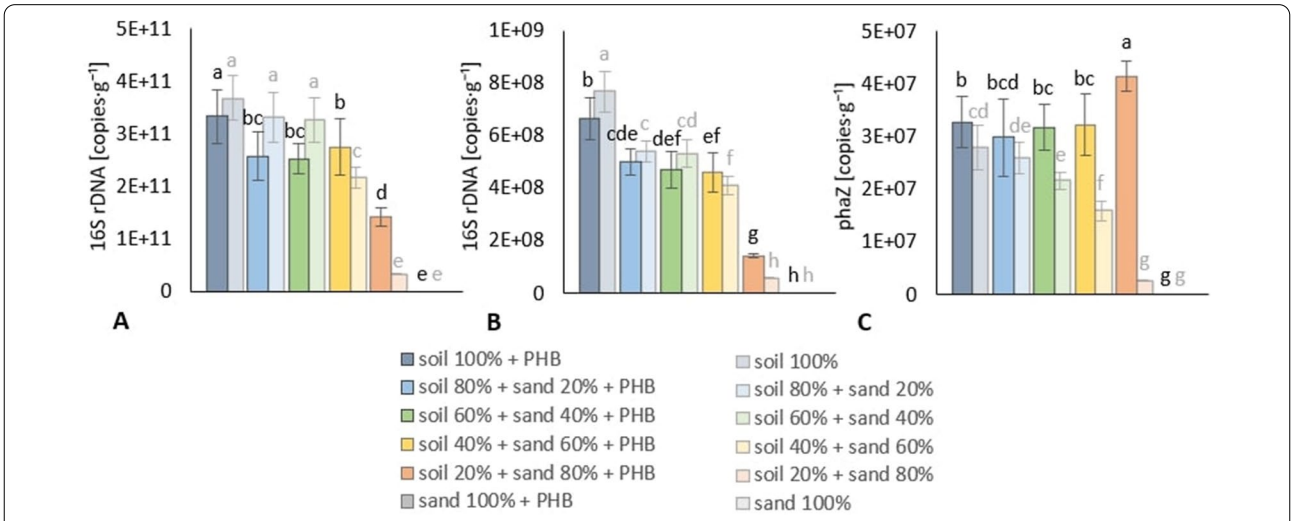


Fig. 1 **A** Bacterial (16S rDNA), **B** fungal (18S rDNA), **C** PHB-degrading microorganisms (*phaZ*) gene markers in the growth substrates; the lowercase letters indicate significant differences at $p \leq 0.05$

were found for 18S rDNA (Fig. 1B), however the neutral-to-negative effect of P3HB prevailed until 60% of sand. In control, the effect of sand was more significant as already 20% of sand significantly reduced 18S rDNA. Presence of P3HB had neutral-to-positive effect on PHB-degrading microorganisms in all growth substrates (Fig. 1C). Furthermore, presence of P3HB alleviated the negative effect of increasing sand content (up to 80%).

Soil enzyme activities

DHA was significantly higher in P3HB-amended variants (Fig. 2A). The only exception was 100% sand variant with barely detectable DHA regardless of P3HB presence. The increasing proportion of sand predominantly led to a decrease in DHA despite its moderation to stimulation by P3HB between 60 and 80% of sand. The effect of P3HB on ARS was ambiguous depending on the sand content (Fig. 2B). At 0–40% of sand, the P3HB effect was neutral to negative, while at 60–100% of sand, the effect was neutral to positive. In the case of NAG, the P3HB effect was not clear as well, however, it was rather positive as four P3HB-amended variants had significantly increased NAG values (Fig. 2C).

P3HB increased Ure in all the variants (Fig. 2D). Decrease in Ure values related to increasing sand–soil ratio was noticeable in controls; less steep but still decreasing trend in Ure values indirectly related to raising sand content was found in the P3HB-amended variants. P3HB-amendment decreased GLU at 0–60% of sand (Fig. 2E). The effect of P3HB on Phos was differentiated depending on the sand content (Fig. 2F). At 0–40% of sand, the P3HB effect was neutral to negative, at 60–100% of sand, the effect was positive. In general, the increasing proportion of sand caused a significant gradual decrease in ARS, NAG, GLU and Phos (Fig. 2).

Soil respiration

P3HB amendment significantly increased BR at 0–80% of sand (Fig. 3A). In addition, BR increased with increasing sand content (up to 80%) in the presence of P3HB. Except

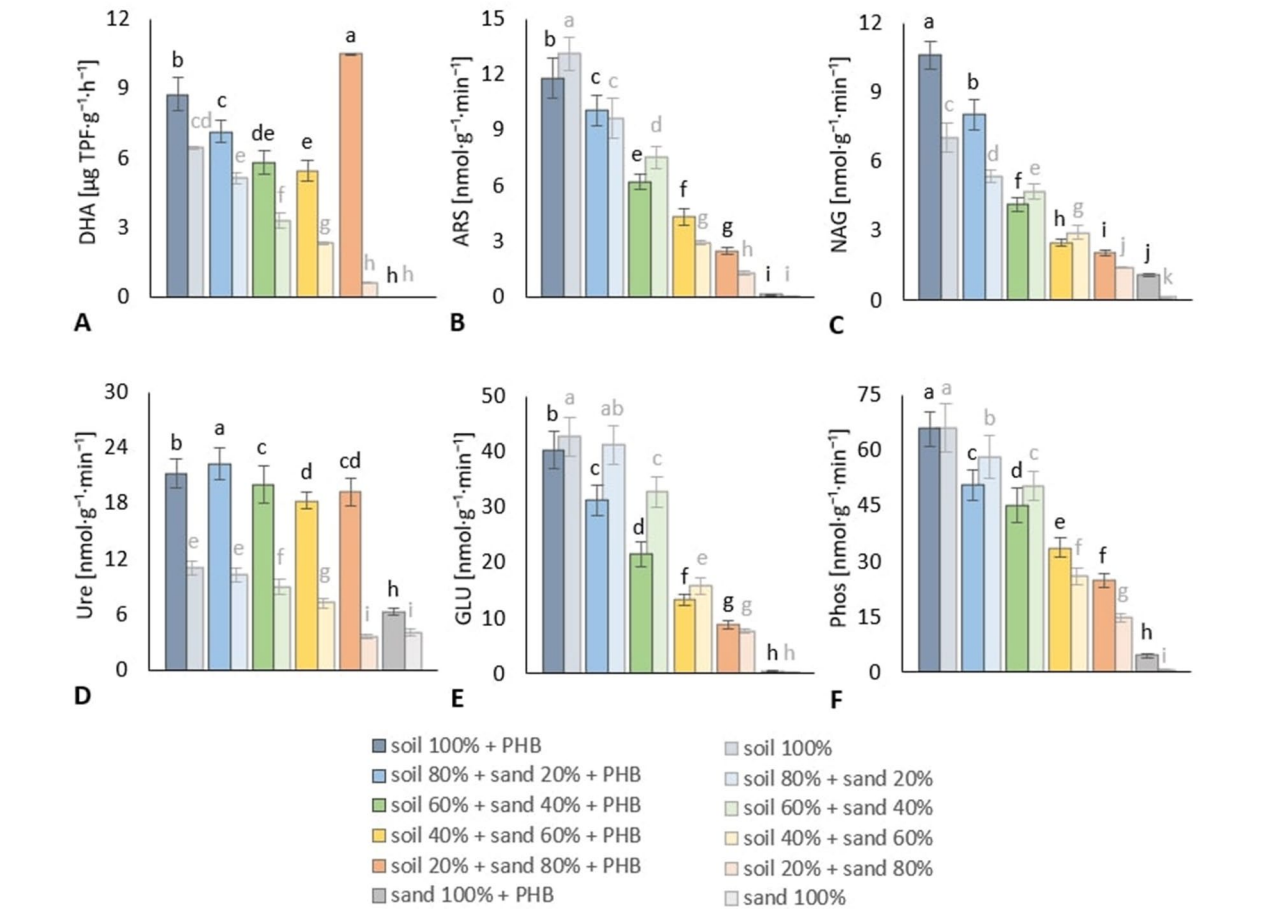


Fig. 2 Soil activities of **A** dehydrogenase activity, **B** arylsulfatase, **C** N-acetyl-β-D-glucosaminidase, **D** urease, **E** β-glucosidase and **F** phosphatase in the growth substrates; the lowercase letters indicate significant differences at $p \leq 0.05$

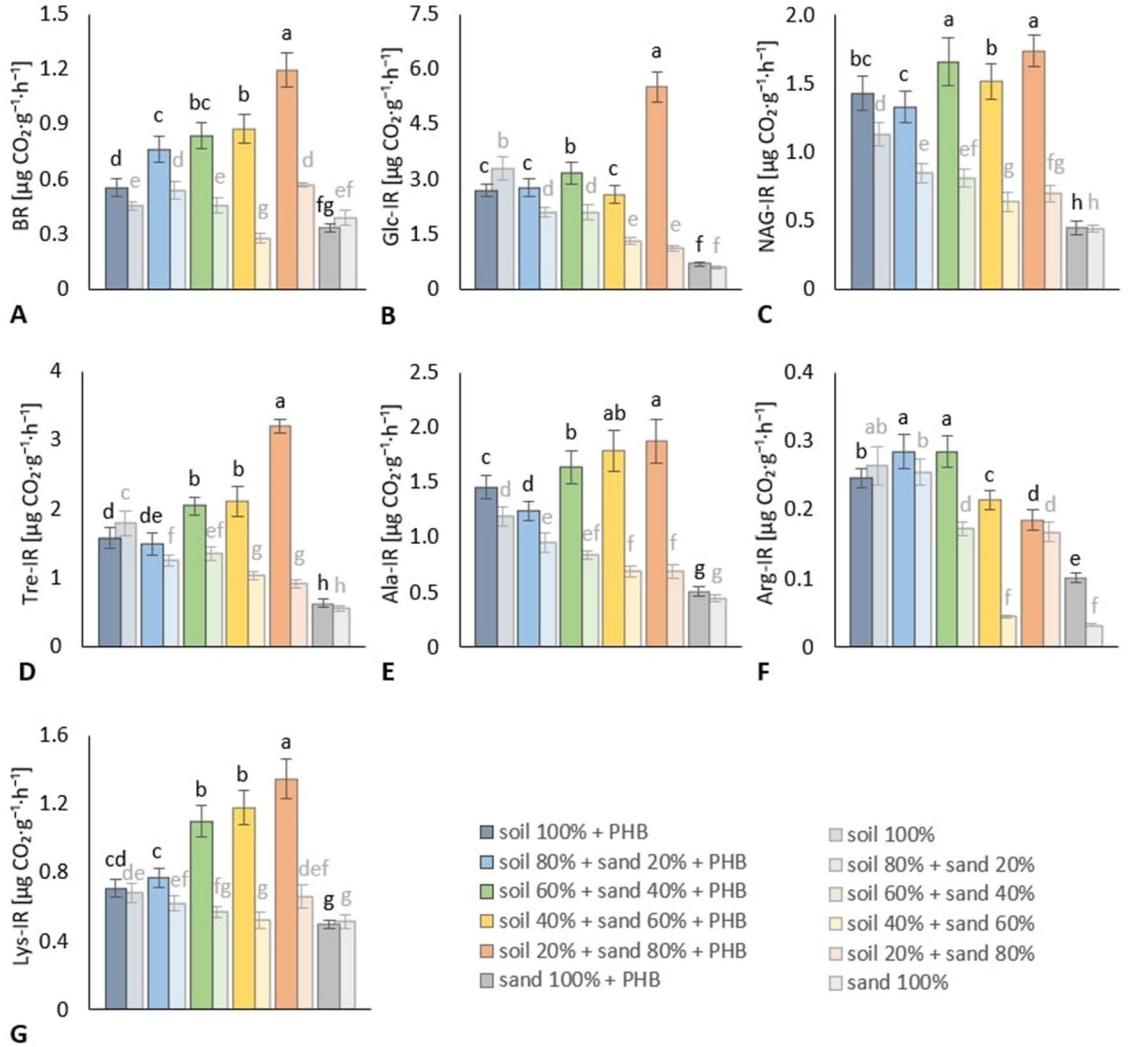


Fig. 3 **A** Basal, **B** D-glucose, **C** N-acetyl-β-D-glucosamine, **D** D-trehalose, **E** L-alanine, **F** L-arginine and **G** L-lysine induced respiration in the growth substrates; the lowercase letters indicate significant differences at $p \leq 0.05$

for pure soil and sand variants, P3HB had a positive effect on Glc-IR (Fig. 3B). In contrast to the control, the presence of P3HB stabilized or even increased Glc-IR with increasing sand content up to 80%.

P3HB significantly increased NAG-IR in all variants containing soil (Fig. 3C). In addition, the negative effect of increasing sand content on NAG-IR in the control contrasted with P3HB-amended variants up to a sand level of 80%. The same trend was visible in Tre-IR (Fig. 3D) as both respirations highly correlated (Additional file 1: Fig. S1). P3HB effect was positive as well, except pure soil and sand variants. The results' trends of Ala-IR and Lys-IR (Fig. 3E, G) followed the results of Glc-IR and Tre-IR as is also reflected by their positive significant correlations (Additional file 1: Fig. S1).

Arg-IR slightly differed compared to other respirations (Fig. 3F). The effect of P3HB was neutral to positive as well, however, the respiration increase with increasing sand content in the presence of P3HB was not recorded. Although there was a significant increase in Arg-IR at 20% of sand, further sand content increase was followed by unchanged or decreasing Arg-IR.

Plant biomass

Fresh and dry aboveground plant biomass (Fig. 4A, B) as well as dry plant root biomass (Fig. 4C) were seriously negatively affected by P3HB as all soil containing variants showed significantly lower values compared to the control. The only exception was 100% sand variant, which had similar negative effect on plant biomass production

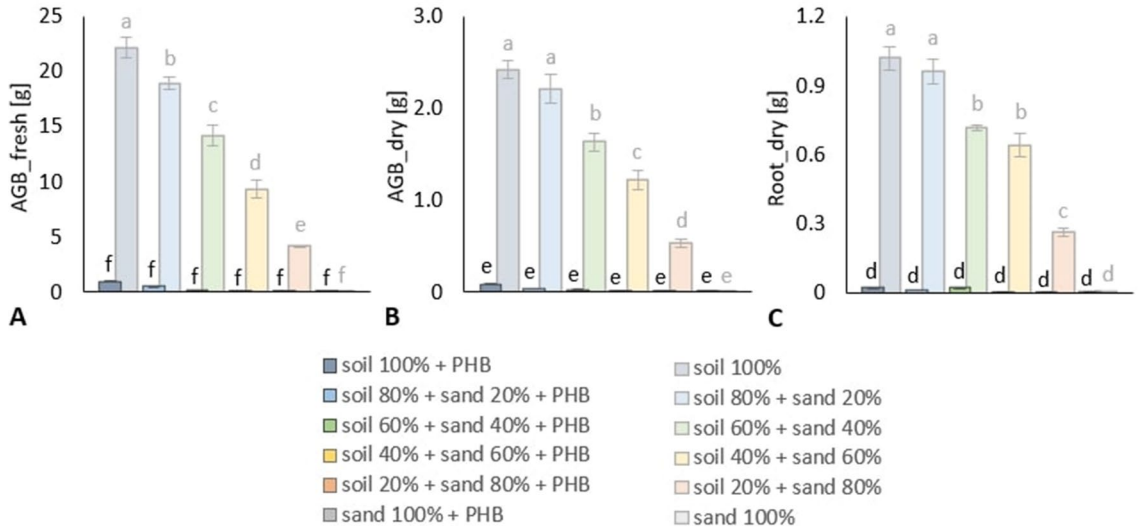


Fig. 4 **A** Fresh and **B** dry aboveground plant biomass (AGB) and **C** dry root plant biomass in the growth substrates; the lowercase letters indicate significant differences at $p \leq 0.05$

regardless of P3HB presence. In control, decreasing plant biomass production followed increasing sand content; in P3HB-amended growth substrates, this trend was suppressed.

Discussion

Effect of P3HB on soil microbial activity

The microbial behavior after addition of P3HB was similar as after PHBV addition [15] with specifics caused by dilution of soil or SOM by increasing sand content. The effect of P3HB on 16S rDNA and 18S rDNA was ambiguous, as P3HB addition reduced, increased, or did not affect bacterial and fungal content (Fig. 1A, B). The results suggest that the mechanism of their interaction and resulting effect may be related to changing physical and chemical properties of soil (see further discussion). PHB-degrading microorganisms (Fig. 1C), on the other hand, were predominantly positively affected by P3HB addition to soil. This increase is probably directly related to the supply of the preferred energy source in the form of a biodegradable polymer [5, 39]. Due to the suppression of the influence of P3HB in the sandy (100%) substrate due to the assumed negligible content of SOM and microbiota (Fig. 1), we do not discuss this variant in this chapter.

The individual enzymatic activities shed light on the processes occurring in soil after addition of P3HB. Ure is an important extracellular enzyme that hydrolyzes urea and regulates the early nitrification process in soil and is closely related to the SOM content [40]. Therefore, it is related to the N availability. Ure is the only enzyme that showed disproportional decrease with increasing sand content (Fig. 2) as in soils without P3HB. This indicates that the P3HB increases activity and abundance of nitrifying microbes which cause enhanced Ure activity independently of SOM content. Thus, P3HB can replace (temporarily) the SOM in sandy soil.

The P3HB is composed of C, O and H, therefore the immobilization of essential nutrients (mainly N and P) is necessary for microbial degradation [15, 41]. This comes from the notion that an optimal C:N ratio in soil is around 25.0 [42]; above this value N is immobilized and below this value is N mineralized [43]. In this work, P3HB application increased soil C:N ratio. During biodegradation, this increase caused N shortage around PHB particles, and the microbes immobilize N from their environment [44]. This generally results in a decrease in plant-available N in soil. If there is no N to immobilize, microbial growth is slowed down. By addition of P3HB is the C:N disturbed, while the disruption seems to be the most pronounced in samples with low content of SOM. Noteworthy, the imbalance in C:N ratio influences the soil organisms in a different way; fungi have wider C:N ratios in their tissues than bacteria and archaea and therefore, they can grow more efficiently on low N substrates and will thus mineralize N more readily. This is also the case of PHB, which is biodegraded preferably by fungal communities at the phylum level dominated by *Ascomycota* [45]. The product of urea hydrolysis is CO_2 and NH_4^+; the latter is either used by plants or microbes, which can eventually convert it into NO_3- via nitrification processes

[46] However, nitrification rates are typically low as the nitrifiers are relatively poor competitors for NH_4^+ in the soil solution and occurs when the NH_4^+ supply exceeds plant and other heterotrophs demand [47]. As the plant growth was largely suppressed under P3HB addition (Fig. 4), it can be anticipated that majority of NH_4^+ was used for growth of soil microbes, especially of PHB-degrading microorganisms (Fig. 1C). This enhanced consumption of ammonium nitrogen putatively more decreased the content of plant available, inorganic nitrogen, similarly as referred to polylactic acid (PLA) contaminated soil during vegetative state of bean [48]. This hypothesis is supported by the enhanced Ure activity (Fig. 2D) and high correlation of Ure activity with the total degradation level indicated by DHA and PHB-degrading microorganisms (Additional file 1: Fig. S1). In addition, Zhou et al. [15] reported an increased activity of *Acidobacteria* and *Verrucomicrobia* phyla in soils degrading PHBV. In fact, neither *Acidobacteriota* [49] nor *Verrucomicrobia* [50] are involved in soil N-cycle processes such as nitrification, denitrification, or nitrogen fixation. Thus, it can be concluded that N (or its vast majority) is used for growing of microorganisms' population.

This finding confirms the earlier assumption of Hoshino et al. [51] who explained better correlation of the biodegradable polymers degradation with the total N content than with the total C content by the necessity of soil N for the degradation by microorganisms as it is absent in bioplastics.

Importantly, unlike the Ure the phosphatase activity showed proportional results in terms of sand content (Fig. 2F). Phosphatase is an extracellular enzyme that mineralizes organic P into phosphate by hydrolyzing phosphoric (mono) ester bonds [52]. Similarly to all extracellular phosphatases, enzyme expression is induced by P deficiency [53]. Notably, Fig. 2F shows higher demand for P in soils containing P3HB and high sand content comparing to soils with lower sand content.

DHA is a basic indicator of microbial activities coupled with SOM degradation in soil [54]. The most important function of DHA is the biological oxidation of SOM, achieved by transferring protons and electrons from organic substrates to inorganic acceptors [55]. For this reason, DHA positively correlates with SOC and C_{org} as it reflects the activity of living cells and not of DHA stabilized in soil complexes [53]. This explains its positive correlation with other microbe-related soil properties (Additional file 1: Fig. S1). In addition, significantly increased DHA values in P3HB-amended variants (Fig. 2A) assumed that PHB addition enhanced soil degradation rate due to its utilization as an energy/C source [15, 56, 57]. This assumption was confirmed especially by the prevailing increase in PHB-degrading microorganisms (Fig. 1C) and their high positive correlation with DHA (Additional file 1: Fig. S1).

ARS is involved in the S mineralization process which cleaves organosulphates [58] and is used as a measure of soil health and soil microbial activity [59]. ARS activity is correlated with soil microbial biomass and the rate of S immobilization [60], pH and SOC [61]. Here, similarly as phosphatase, its activity is elevated in sandy soils (>60% sand) with P3HB (Fig. 2B), which reflects higher demand for immobilization of S in less buffered system.

NAG is an enzyme catalyzing the hydrolysis of terminal 1,4 linked N-acetyl-beta-D-glucosaminide residues in chitooligosaccharides, i.e., it is involved in degradation of chitin, the key polysaccharide of fungal cell wall [62]. Its activity was enhanced in most P3HB-amended variants (Fig. 2C), which underlines higher demand of degrading organisms for N acquisition from available sources, but different than Ure, as suggested by their low correlation (Additional file 1: Fig. S1). Enhanced NAG activity supports the view that plants or microbes may use N-containing monomers and not only inorganic N [63].

GLU is an enzyme that catalyzes the hydrolysis of terminal 1,4 linked β-D-glucose residues from β-D-glucosides, including cellulose oligomers and thus it is an indicator of SOM degradation and soil C utilization [64]. Its activity was either the same or even lower in soils with P3HB (Fig. 2E). This may be attributed to either preferable cleavage of PHB rather than cellulose or a high C:N ratio [64].

BR is a key indicator of aerobic catabolic activity in soil, and accessibility and degradability of organic C in SOM [65]. The P3HB application positively influenced BR (Fig. 3A) according to the mechanism described above in the DHA-related part. Substrate-induced respiration is used to measure the activity of specific microorganisms responding to addition of substrates with specific composition. Response induced by Glc, NAG, Tre, Ala and Lys corroborated with results of BR (Fig. 3). Similar mechanisms and involvement of the same organisms is a probable reason of the grouping of all these respirations (Additional file 1: Fig. S2) and their positive significant correlation (Additional file 1: Fig. S1). On the contrary, arginine substrate showed slightly different results comparing to them.

The results of Arg.IR, an indicator of fungal respiratory activity in soil, suggest neutral-to-positive effect of P3HB as well (Fig. 3F). However, a slight deviation in the trend of values from the results of other respirations is visible (Fig. 3) and evident also from the PCA (Additional file 1: Fig. S2). The respiration decreases with increasing sand content. This indicates the relation of Arg-IR to soil texture. In sandy soils, the pores are better aerated, water

content and water holding capacity are significantly reduced. These conditions probably limited the use of arginine substrate by microorganisms.

Effect of P3HB on soil fertility

The results clearly indicate an adverse effect of P3HB on both above- and below-ground biomass production of *L. sativa* (Fig. 4). These results are in accordance with previous works that degradation of biodegradable plastics might negatively affect plant growth [66, 67]. In general, for biodegradation of P3HB, two major explanations may be attributed to the observed effects: (i) phytotoxicity of P3HB microplastics or their degradation products and (ii) the effect on soil properties and/or inhibition of nutrients.

Alternative (i) was thoroughly discussed by Zhou et al. [15] who observed similar results as in this work by testing PHBV, which is a common derivative of PHB. The authors speculated about possible phytotoxic effect of PHB biodegradation products due to acidification of soil caused by released of 3-hydroxybutyric acid during PHBV degradation, but this speculation was in the cited work rejected. The rejection of the hypothesis is partially in line with our results showing the resulting pH above 7 in soil without P3HB (in other soils was probably increased due to sand content). Moreover, the pKa of 3-hydroxybutyric acid is 4.41 [68] indicating that the acidification effect of this acid is very low. Influence of 3-HB effect on the phenylpropanoid pathway regulation related to reaction on abiotic stress [19, 20] was also mentioned. Liwarska-Bizukojc [69] used *S. saccharatum, S. alba* and *L. sativum* as phytotoxicity bioindicators of PHB and found no effect on seed germination even at concentration as high as 11.9% w/w. Nevertheless, the presence of PHB in soil caused root growth inhibition mainly on *Sinapsis alba* and *Lepidium sativum*. On the contrary, Dahal et al. [18] did not find any significant effect of PHBV on plant growth. Possible adverse effect of PHB due to reduced seedlings survival was indirectly suggested by [21].

Alternative ii) was discussed by Silveira Alves et al. [70] who stated that the role of PHB in plant-bacterium interactions is still poorly understood, however, their study suggested that PHB metabolism may contribute to bacterial plant growth promotion and that deletion of genes involved in the synthesis and degradation of PHB reduce the bacterial ability to enhance plant growth [70]. Here, despite the prevailed P3HB-related promotion of microorganisms' abundance and community structure, the suggestion of plant growth promotion must be rejected. Furthermore, due to the hydrophobic nature of PHB [71], a higher water repellency and increased drain-off may be expected in PHB-amended substrates. Zhou et al. [15] concluded that PHBV addition increased microbial activity, growth, and exoenzyme activity, changed the soil bacterial community at different taxonomical levels and increased the alpha diversity, which most likely led to the enhanced mineralization of native SOM and negatively influenced the growth of *Triticum aestivum* L.

Our results, i.e., serious P3HB-related growth inhibition of *L. sativa* (Fig. 4) and enhanced microbial activity, are supported by the results reported in [15]. As indicated by the low negative correlations between plant biomass and BR, Lys-IR and Ure, respectively (Additional file 1: Fig. S1), growth inhibition could result from an adverse consequence of plant-microbiota interaction, such as competition for nutrients. The most likely scenario appears to be competition for N, which was probably utilized by PHB-degrading microorganisms (as discussed above). Similarly, suppressed growth of common bean shoots and roots in PLA-treated sandy soil, reported by [48], was likely caused by significant deficit of plant available (mainly nitrate nitrogen) and disproportion in dissolved organic carbon (DOC) and nitrogen (DON), leading to increased C:N ratio. Noteworthy, in our case, we can exclude the negative effect of drought stress caused by hydrophobicity of PHB as the design of pot experiments (i.e., regular irrigation) exclude the possibility of shortage of moisture in soils due to regular irrigation.

Effect of P3HB under changing SOM content as an implication of soil degradation

The results of multivariate analysis of variance (MANOVA) showed significant ($p < 0.001$) differences among experimental variants in all determined properties confirming the importance of increasing sand content in arable soils for the use of P3HB in agricultural soils. However, the consequences were variable, as they were positive, neutral, and negative.

16S rDNA and 18S rDNA (Fig. 1A, B) were very highly positively correlating (Additional file 1: Fig. S1) and followed similar pattern of overall decrease in both P3HB-amended and control substrates. Despite clear disproportionality, the decrease was probably caused by the decrease of the SOM content and number of soil microorganisms and fungi following soil dilution by sand. This also negatively affected all enzymatic activities, which showed a similar overall trend (Fig. 2). Therefore, the progressive deterioration of soil quality by the increase in the sand fraction will have a negative impact on microbial and fungal biomass and enzymatic activities, whether the soil is contaminated with P3HB or not.

However, the degree of sand influence is significantly affected by the presence of P3HB. Compared to control, bacterial and fungal biomass content (Fig. 1A, B)

followed similar pattern of initial stagnation or decrease after P3HB addition replaced by stagnation or growth at higher (≥ 60%) sand load. In PHB-degrading microbiome, the negative effect of increasing sand content was alleviated by presence of P3HB (Fig. 1C). Moreover, the results suggest that presence of P3HB together with increasing sand content (up to 80% of sand) can even stimulate PHB-degrading microorganisms. Thus, the partial increase of 16S rDNA and 18S rDNA was probably related to this part of the microbiome. The reason may be better aeration of the substrate accompanied by the necessary presence of the P3HB energy source substituting declining SOM content, resulting in the booming of PHB-degrading microorganisms and overall shift in microbiome structure. This hypothesis is supported especially by the similar phenomenon in DHA (Fig. 2A), increasing respirations with increasing sand content (Fig. 3) and the grouping of these factors in the PCA (Additional file 1: Fig. S2).

Some other enzymes (ARS, NAG, Ure, GLU, Phos) also showed partial deviations indicating a different effect of P3HB depending on the sand content (Fig. 2). For example, at lower sand content (0–40%), the effect of P3HB on ARS (Fig. 2B) and Phos (Fig. 2F) was neutral to negative, while at higher sand content (60–100%), the P3HB effect was neutral to positive. This suggests that the expected PHB-degrading microorganisms boom related to the increased aeration had a positive effect on these enzymatic activities as well. Therefore, although P3HB acts as a potential selective microbial inhibitor in a favorable state of soil due to the dominance of different (natural) functional groups of microorganisms, in unfavorable conditions of increasing sand content, P3HB can maintain or even stimulate the activity of some enzymes associated especially with the PHB-specific microbes as an alternative energy source.

As already mentioned, predominant stimulation of BR, Glc-IR, NAG-IR, Tre-IR, Ala-IR and Lys-IR with increasing (up to 80%) sand content in P3HB-amended substrates (Fig. 3) was probably caused by better aeration coupled with P3HB utilization. This phenomenon could also be explained by the increasing rate of preferable utilization of P3HB with the increasing portion of PHB-derived C regarding the total soil organic C. This feature might be comparable to the observation of Kuzyakov and Bol [72], who described a metabolism switch from the hardly utilizable recalcitrant C in SOM to the easily available carbonaceous compound leading a positive priming effect. The study carried out with bean-planted PLA-contaminated sandy soil also showed increasing values of readily oxidizable carbon (POXC) with ascending content of plastics (up to 2%) [48]. However, this can be considered as a necessary side effect rather than a cause, as it would have a similar graded positive effect on the other characteristics studied.

The increase in sand content significantly reduced the production of plant biomass in the control (Fig. 4), however, the presence of P3HB suppressed this phenomenon and limited biomass production to a minimum. Therefore, the gradual degradation of P3HB-contaminated arable soils by increasing sand content is not determining for *L. sativa* yields; P3HB presence limits the growth of *L. sativa* to the same extent as growing in pure sand. It is important to keep in mind that the possible contamination of soil by 1% of P3HB is realistic in the case of application PHB-based fertilizer coatings, delivery systems and mulching films. The SOC content in the soil was 14 g kg^{-1}, therefore, applied dose of P3HB is very high in terms of SOM-to-P3HB ratio. As the P3HB is easily available substrate, we speculate that even significantly lower dose may have an adverse effect on plant growth. A decrease in SOM (represented here by sand dilution) can worsen this adverse effect .

In summary, the effect of changing sand content is also well reflected in the PCA (Additional file 1: Fig. S2). Sand's dominant property, high pH (Additional file 1: Fig. S3), is clearly related to the sandy variants, which form one separate group of substrates unsuitable for soil organisms and plant biomass production. The absence of P3HB and high soil content are clearly the most important factors for high *L. sativa* biomass production. High soil content is key for high level of bacterial and fungal biomass, as well as most enzymatic activities. The presence of P3HB and a more balanced soil:sand ratio is both crucial for high soil respiration and content of PHB-degrading microorganisms.

Conclusions

The changes in the microbial abundance and community structure after P3HB addition were variable; however, there was a growth and amplification of specific PHB-degrading microbial population. The results of enzymatic activities were also ambiguous depending on the sand content. The basal and substrate-induced respirations as well as DHA were mostly enhanced by the P3HB addition, which seemed to be preferable source of C and energy for soil aerobic microbes. In conclusion, although P3HB acts as a potential selective microbial inhibitor in a favorable state of soil, in unfavorable conditions of increasing sand content (approximately 60–80%), P3HB can maintain or even stimulate the microbial abundance and community structure as well as enzyme activities and soil respiration. However, these potentially positive effects on the soil microbiome were contrasted by the significant adverse effects on the plant growth. The results show that soils may have the capacity to degrade

the bioplastics, but at the cost of nutrient availability to plants and negative impact on their growth. This capacity may be affected by a gradual nutrient-related deterioration of soil quality. Moreover, at low SOM content (increased sand-to-soil ratio), the P3HB can replace the SOM as a main substrate, which shifts the composition of microbial community towards this only available substrate. We conclude that, although the biodegradable bioplastics are C neutral, further research should answer if they do not induce positive priming effect on SOM, as the overall activity of soil organisms were disproportionately boosted in P3HB-amended sandy soils. Most importantly, future studies should find the conditions under which can biodegradable plastics enter the soils without any adverse effect on soil fertility and other properties.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-022-00345-9.

Additional file 1: Fig. S1. Correlation matrix of soil properties; numbers indicate the Pearson's correlation coefficient r. **Fig. S2.** Rohlf PCA biplot of individuals and variables. **Fig. S3.** pH in the substrates; the lowercase letters indicate significant differences at $p \leq 0.05$

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Author contributions
Conceptualization, TH, JH and MB; methodology, TH, JH and MB; software, TH, TB, JF; validation, VP, MB, AM, OM, AK and JK; formal analysis, JF, TB; investigation, JH, AM and MB; resources, JH, TH, and AK; data curation, TH, MR and JF; writing—original draft preparation, JH, MB; writing—review and editing, VP, TH, JH, AM, JK and MB; visualization, TH and AK; supervision, JK, VP; project administration, JF, JH, MB, AK; funding acquisition, MB, AK, JH, JK. All authors have read and agreed to the published version of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

Effect of biodegradable poly-3-hydroxybutyrate amendment on the soil biochemical properties and fertility under varying sand loads

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Supplementary material

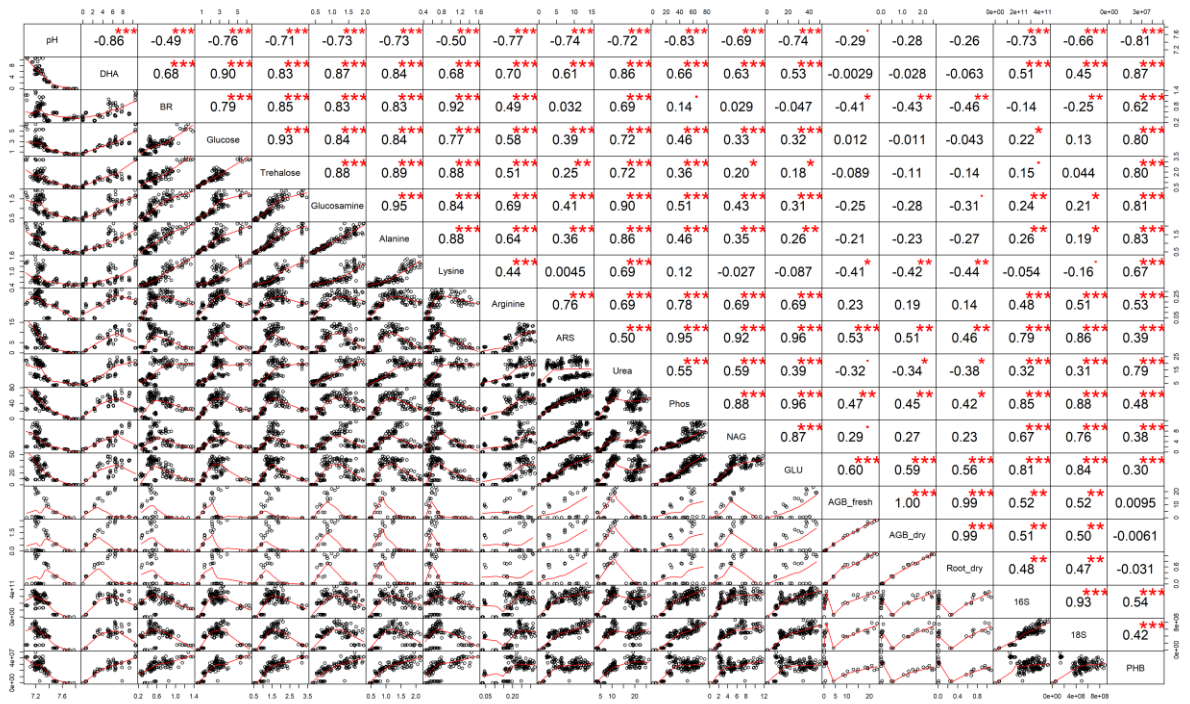

Fig. S1 Correlation matrix of soil properties; numbers indicate the Pearson's correlation coefficient r.

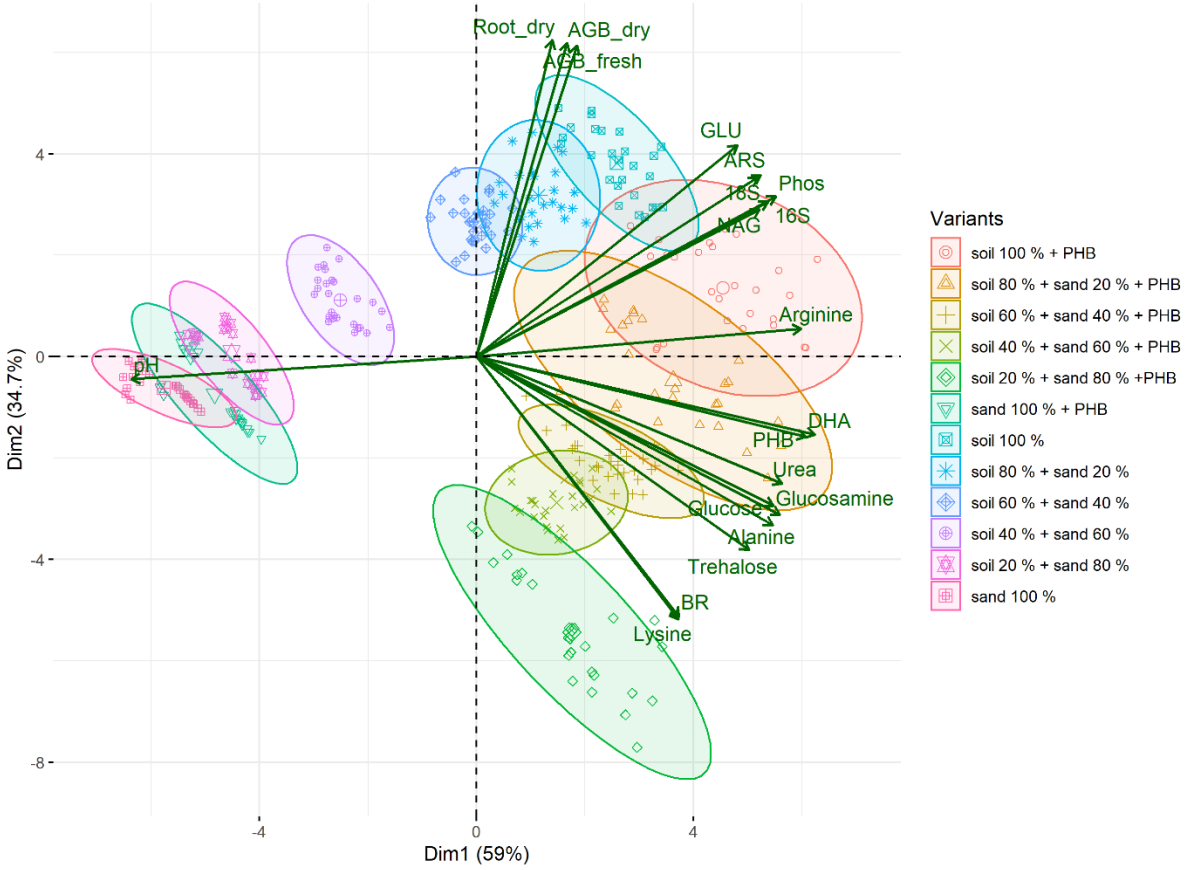

Fig. S2 Rohlf PCA biplot of individuals and variables.

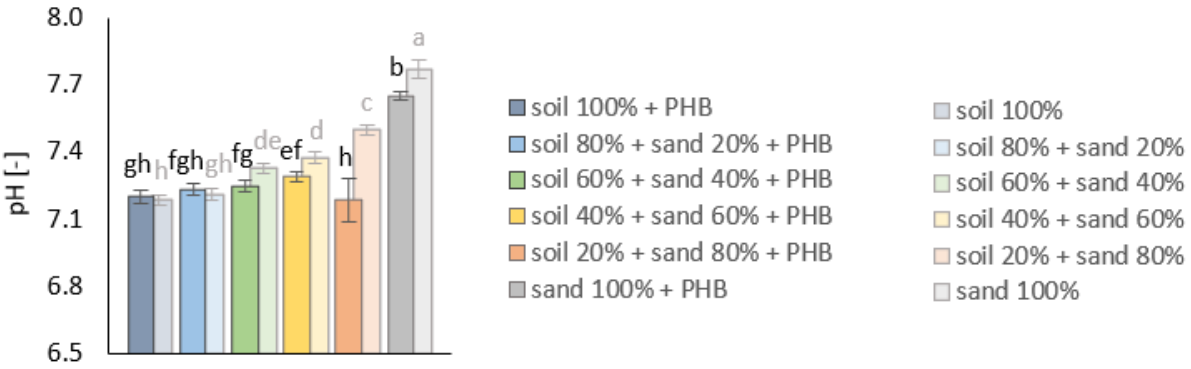

Fig. S3 pH in the substrates; the lowercase letters indicate significant differences at $p \leq 0.05$.

Příloha E

Does the biodegradation of poly-3-hydroxybutyrate cause the degradation of soil organic matter?

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Abstract

Biodegradable bioplastics are developed because of their ability to biodegrade, i.e. to be an environmentally friendly alternative of conventional plastics. However, the recent studies suggested that the biodegradation and residual bioplastics in soil may pose thread to some physical, chemical, and microbial soil properties. Currently, it is unclear what are adverse effects of different stages of biodegradation and which soils are more endangered by bioplastic biodegradation. In order to clarify these research questions, we conducted ten-months experiment with chernozem, cambizem and phaeozem spiked with model bioplastic poly-3-hydroxybutyrate (P3HB) at concentration of 0.1, 1, 5 and 10%. All soils were incubated in respirometer where evolved carbon dioxide was determined, then thermogravimetry was used for estimation of residual PHB and evaluation of changes in soil organic matter. For carbon balance was used also elemental analysis, enzyme analysis served for analysis of activity of soil microorganisms. It was observed that the biodegradation of P3HB lead to higher CO_2 evolution than theoretical values which indicated possible degradation of soil organic matter; thermogravimetry analysis showed residues of nondegraded PHB which required at highest concentration more time to fully decompose. The biodegradation was accompanied by a loss of soil organic matter which was a second indicator of primming effect; this was also supported by a change in enzymatic activity of five key soil enzymes. We conclude that biodegradation of bioplastics in soil might have serious adverse effect on organic matter in various soil types.

Introduction

Today's annual plastic production exceeds 360 million tons per year (Yang et al., 2022). Improper waste management leads to environmental contamination by microplastics (particles with a diameter of less than 5 mm)(Thompson, 2004). Microplastics can pose a threat to the environment including adsorption organic pollutants such as pesticides and pharmaceuticals to their surface, and acting as vectors og those chemicals (Miranda et al., 2022), bringing oxidative stress and changes in the behavior of earthworms and springtails (Cao et al., 2017; Cui et al., 2022; Kim and An, 2019; Yu et al., 2022), alteration of soil structure and properties such as soil pore size, soil particle aggregation and soil water desorption (De Souza MacHado et al., 2018; Zhang et al., 2019), affecting soil microbial composition and nutrient cycles (Zhao et al., 2022). In addition, it is also speculated that the microplastic contamination can increase soil respiration and thus accelerate mineralization of soil carbon due to higher porosity (Yang et al., 2018) or an increased stress causing enhanced activity of microbial biomass (Gao et al., 2021). Nevertheless, a higher concentration of microplastics in soil enhanced nutrients concentration in dissolved organic matter, which makes them better available to plants (Liu et al., 2017).

Biodegradable bioplastics (hereafter referred to as bioplastics) are being developed as a greener alternative to conventional plastics to reduce the formation of microplastics (Zimmermann et al., 2020). This type of material can be made from petroleum (polybutylene adipate terephthalate - PBAT) or from natural sources preferably from waste biomass (polylactic acid, polyhydroxybutyrate (PHB))(Obruca et al., 2010). Biodegradable bioplastics are characterized by their ability to biodegrade, i.e. under ideal conditions mineralize into CO_2 (aerobic environment), water or methane (anaerobic conditions)(Karan et al., 2019a). In real environments, ideal conditions are not always achieved (changes in seasons, seasonal drought, environments with low microbial activity, etc.) and therefore biodegradation may be slow or stopped. In this case, the bioplastics only break down more rapidly into smaller particles of micro-bioplastics (MBP) or nano-bioplastics (Fojt et al., 2020; Qin et al., 2021; Shruti and Kutralam-Muniasamy, 2019). These particles then enter the soil most often through agriculture, for example through the application of contaminated compost, bio mulch films or fertilizers with a biodegradable coating (Harmaen et al., 2016; Jeszeová et al., 2018; Kale et al., 2007; Steiner et al., 2022).

Although most research has focused on the environmental impact of conventional microplastics, early studies suggest that the environmental behavior of microplastics is similar to that of conventional ones, and so attention should also be paid to preventing them from entering the environment (Fojt et al., 2022a). A study conducted on particles formed from shopping bags containing PBAT and polyethylene (PE) examining the adsorption of phenanthrene found that PBAT-containing particles had up to three times the sorption capacity of PE (Zuo et al., 2019). Fan et al. conducted an adsorption experiment with antibiotics, where they compared conventional polyvinyl chloride PVC microplastics with polylactic acid (PLA) MBPs, showing that PLA has a higher sorption capacity, which increases with aging of PLA

(Fan et al., 2021). This suggests that MPBs may be an even stronger vector for micropollutants than conventional microplastics (Torres et al., 2021). A study conducted by Ding et al. on earthworms in soil contaminated with two conventional microplastics PE and PPC and MPB PLA found that these particles have more or less similar effects on earthworm reproduction, behavior and mortality and depend more on their concentrations (Ding et al., 2021). The interaction of both microplastic polyethylene terephthalate and MBP PHB with the soil organic matter of peat was similar and led to a change in the soil pores, causing soil drying (Fojt et al., 2022a).

Bioplastics consist mainly of C, O and H atoms, connected via bonds which can be easily cleaved by microorganisms (Meng et al., 2022). Their entry into the soil causes an increase in the C:N ratio, leading to a boost in bacterial activity and subsequent immobilization of soil nitrogen (Meng et al., 2022; Zhou et al., 2021). MBP or another biodegradable organic carbon-rich substance (e.g. cellulose) induces bacteria to produce higher levels of extracellular enzymes capable of degrading not only contaminating compounds but also soil organic matter (SOM)-like compounds (Bher et al., 2019; Blagodatskaya et al., 2014). When the substrate supplied to the soil is depleted, some of the micro-organisms then die and become a source of readily available nutrients. This releases the carbon stored in the SOM and potentially results in a deficit of N available to plants and shifts in the biodiversity of soil micro-organisms (Bher et al., 2019; Blagodatskaya et al., 2014).

The aim of this work was to clarify the effect of biodegradation process and residues of undegraded micro-bioplastic on some soil parameters and soil organic matter in different unplanted soil types such as Cambisol, Phaeozem and Chernozem. Respirometry was used to determine the degree of biodegradation followed by evaluation according to EN ISO 17556:2012. The second method was thermogravimetry, which can directly determine the amount of residual PHB in the case of PHB. Thermogravimetry was also used to assess changes in the proportion of stabilized and stable soil organic matter and soil water content. Elemental analysis is used to monitor the total carbon and nitrogen content before and after biodegradation. The activity of soil organisms in each soil type was monitored by enzyme activity.

Materials and methods

Preparation and sample analysis

Three types of soils were used for all experiments (topsoils, sampling from 10 to 15 cm); Phaeozem was sampled near the village Šaratice, Cambisol was sampled near Postoupky near Kroměříž and Chernozem was sampled near village Sloveč. Their properties are listed in Table 1. pH was determined according to standard (ISO 10390, 2005), clay content was determined the same way as in (Kučerík et al., 2020), porosity of soil was determined using method from (Hladký et al., 2016), available phosphorus was determined using the same method as (Kintl et al., 2016), humification index was calculated as ratio of humic acid (HA) and fulvic acid (FA) content (Huang et al., 2008) and soil water

retention pF1.8 was determined by the method described in (Kučerík et al., 2013). These soils were air dried and sieved at 2 mm sieve. 5 g of each soil was transferred to a glass beaker, where P3HB micro-bioplastics with particle sizes between 0.6 and 1.6 μm were added (Y3000, TianAn Biologic Materials, Ningbo City, China) to achieve concentrations 0, 0.5, 1 and 3 w/w%, subsequently, the mixtures were thoroughly mixed and distilled water was added in an amount corresponding to 76% of pF 1.8.

Table 1: *An overview of selected properties of the soil used for experiments.*

	Phaeozem	***Cambisol***	***Chernozem***
Sampling locality	Sloveč	Postoupky	Šaratice
Coordinates	N 50°14.256' E 15°20.705'	N 49°18.685' E 17°21.967'	N 49°06.641' E 16°49.347'
pH	7,18	4,86	7,09
Clay content [%]	48	22	46
Porosity [%]	44	38	46
Available phosphorus (Mehlich) [mg/kg]	67	97	37
Humification index [HA/FA]	0.59	0.89	0.56
Soil water retention pF1.8 [%]	28.6	25.8	32.1

Respirometry

Respirometry is a standardized method used to determine the biodegradation of substances in certain environmental compartments. The first biodegradation standard was developed for aquatic ecosystems by the OECD in 1981, and since then biodegradation standards have been introduced by other standards organizations and for other ecosystems (Reuschenbach et al., 2003). Biodegradation tests are carried out in a closed respirometer under defined conditions. Respirometric determination of biodegradation is performed by measuring the oxygen consumed or carbon dioxide evolved.

In this work, the EN ISO 17556:2012 method was used which determines the amount of evolved CO_2, this reflects the metabolism of the microorganisms in the mineralization of the substrate, the end of the experiment is interpreted as 90% of the plateau phase value at respiration. The plateau phase indicates the equilibration of the sample and control respiration rates. The amount of CO_2 evolved from blank samples was subtracted from the amount of CO_2 evolved from the sample to determine the end of biodegradation, and the beginning of the plateau was determined as the end of biodegradation, i.e., 100% degradation and not 90% as recommended by EN ISO 17556:2012 (Figure 1). Specifically, the end point of biodegradation was determined when the amount of released CO_2 per hour from the blank and samples ceased, i.e., the rate of CO_2 release from the blank and the sample was equilibrated.

The biodegradation experiment was carried out in a Respicond VI respirometer (Nordgren Innovations, Sweden). The carbon dioxide released during biodegradation was captured in a 0.6M KOH solution in a small container under the lid of the reaction vessel. The conductance was automatically recorded every

hour using platinum black electrodes immersed in the KOH solution. The instrument software automatically calculated the amount of CO_2 captured from the conductance. The KOH was replaced each time more than 70 mg of CO2 was captured. The experiment was conducted in a thermostatic water bath without access of light in hermetically sealed measuring vessels at a temperature of 20.00±0.01 °C for 300 days. All samples were duplicated and the results averaged and microcrystalline cellulose (Dr.Hoffmann, Cítov, Czech republic) was used as a positive control. Biodegradation was calculated according to Equation 1 and 2. Subsequently, soils were removed and analyzed by thermogravimetry, elemental and enzymatic analysis.

$$\mathrm{ThCO_2} = \frac{\mathrm{M_r(CO_2)}}{\mathrm{A_r(C)}} \cdot \mathrm{m} \cdot \mathrm{w_c} \quad (1)$$

$ThCO_2$ is theoretical amount of carbon dioxide evolved by test material during biodegradation, $M_r(CO_2)$ is relative molecular mass of carbon dioxide**,** $A_r(C)$ is relative atomic mass of carbon**,** m is mass of the test material, in milligrams, introduced in the system**,** w_c is carbon content of the test material, determined from the chemical formula, expressed as a mass fraction (ISO, 2019a).

$$\mathrm{Biodegradation(\%)} = \frac{\sum m_t - \sum m_B}{ThCO_2} \cdot 100 \quad (2)$$

$\sum m_t$ is amount of carbon dioxide, in milligrams, evolved in the test vessel, $\sum m_B$ is the amount of carbon dioxide, in milligrams, evolved in the blank test vessel(ISO, 2019b).

Thermogravimetry

Thermogravimetry (TG) is a method based on monitoring the change in mass of a sample as a function of temperature during a selected temperature program. In a TG soil record, weakly bound water evaporates between 30 and 100 °C; at 100 and 200 °C, strongly bound water is released. Total moisture content can therefore be determined directly by thermogravimetry using thermal mass loss (TML) 30-200 °C (Tokarski et al., 2020). It is also possible to determine the total SOM content using thermogravimetry (TML 110-550 °C), stabilized fraction content 300-450°C and Organic clay minerals content and black carbon (persistent) int temperature over 450°C (Kučerík et al., 2018a).

Thermogravimetric analysis was performed to detect undegraded PHB and to monitor changes in soil properties caused by PHB biodegradation. A thermogravimeter was used for the analysis TGA 550 (TA Instruments, New Castle, Delaware, USA). All soils were measured before and after the biodegradation experiment. Prior to measurement, each sample was equilibrated in a desiccator at relative humidity 43±2 % controlled by saturated solution of K_2CO_3 and under temperature 20±2 °C. Approximately 200 mg of each sample was then dispensed into Al2O3 pans and placed in an autosampler that was at the same relative humidity as the desiccator. Also, air supplied to the oven was bubbled through the wash flasks to ensure that the samples were exposed to the same relative humidity throughout the experiment.

The oven was heated at rate 5 °C per minute until temperature 740 °C in a dynamic air atmosphere (90 mL/min).

Elemental and enzymatic analysis

The elemental analysis was measured with automatic analyser LECO CNS 2000. The soil enzyme activities were determined according to ISO 20130:2018. Fresh soil samples after probing were sieved to size 2 mm and freeze-dried. Each soil sample was measured in nine replicates, using *p*-nitrophenyl (PNP) derivatives ($\lambda = 405$) of the specific enzyme substrates for spectrophotometric (Vis) measurement of β-glucosidase (GLU), arylsulfatase (ARS), phosphatase (Phos), and N-acetyl-β-D-glucosaminidase (NAG), while urease (Ure) was measured using its natural substrate urea a detecting cyanurate ($\lambda = 650$). The activity values were expressed in μg (*p*-nitrophenol) $PNP \cdot g^{-1} \cdot h^{-1}$ and in μg $NH_3 \cdot g^{-1} \cdot h^{-1}$ (urease).

Results and discussion

Elemental analysis results

Table 2 summarizes the results of elemental analysis. Nitrogen content was the same in each soil (variations are within experimental error) in the blank before biodegradation and in all samples after biodegradation. In contrast to the nitrogen content, the carbon content changed in each soil. There was always a loss of carbon in all blank samples compared to soils with an original PHB content of 0.5% and in soils rich in soil carbon originally with a PHB concentration of 1%, where it was respired as CO_2. In the samples with higher initial PHB content, the carbon content increased due to undegraded residual PHB.

***Table 2**: Results of elemental analysis (deviation was calculated separately for each value, average standard deviations: 0.006% for N, 0.021% for C).*

Soil	Concentration of added P3HB [%]	Time of the analysis	Total N [%]	Total C [%]
Phaeozem	0	Before degradation	0.321	3.179
	0	After degradation	0.319	2.971
	0.5	After degradation	0.316	3.085
	1	After degradation	0.309	3.062
	3	After degradation	0.312	3.556
Cambisol	0	Before degradation	0.125	0.987
	0	After degradation	0.110	0.957
	0.5	After degradation	0.111	0.935
	1	After degradation	0.125	1.013
	3	After degradation	0.126	1.623
Chernozem	0	Before degradation	0.323	3.048
	0	After degradation	0.283	2.555
	0.5	After degradation	0.282	2.694
	1	After degradation	0.274	2.765
	3	After degradation	0.288	3.334

Biodegradation determined using respirometry

The biodegradation consists of several phases, such as biodeterioration and biofragmentation (Bhagwat et al., 2020; Karan et al., 2019b; Zhao et al., 2020), during which the plastic undergoes fragmentation into debris. Subsequent stage is bioassimilation (Meereboer et al., 2020), in which the lower-molecular-weight products formed during the previous stage are transported into cells. The last phase is mineralization (Meereboer et al., 2020), which leads to low-molecular-weight products. The biodegradation is also accompanied by an increase in the number of biodegrading microorganisms and therefore also in the production of microbial biomass.

As already mentioned, the end point of biodegradation was determined as the point at which the CO_2 evolution align to evolution from the values provided by the blank, i.e., the CO_2 release rate from the blank and the sample was equilibrated. Figure 1 illustrates this situation for a Chernozem soil sample contaminated with 0.5% PHB. A similar procedure was followed for the biodegradation determination for the other samples. Table 3 summarizes the times at which biodegradation ended according to EN ISO 17556:2012 and the amount of CO_2 absorbed in the experiments. Table 3 also shows that in the case of the 0.5% variant, respiration indicated that the end of biodegradation occurred in all cases at 96 days (soil Cambisol), 131 days (Chernozem) and 146 days (Phaeozem). This is similar for the 1% variant where the end of biodegradation occurred after 221 days for Cambisol, 224 days for soil Chernozem and 260 days for soil Phaeozem. For the 3% variant, complete biodegradation did wasn't achieved during the 300 days of the experiment.

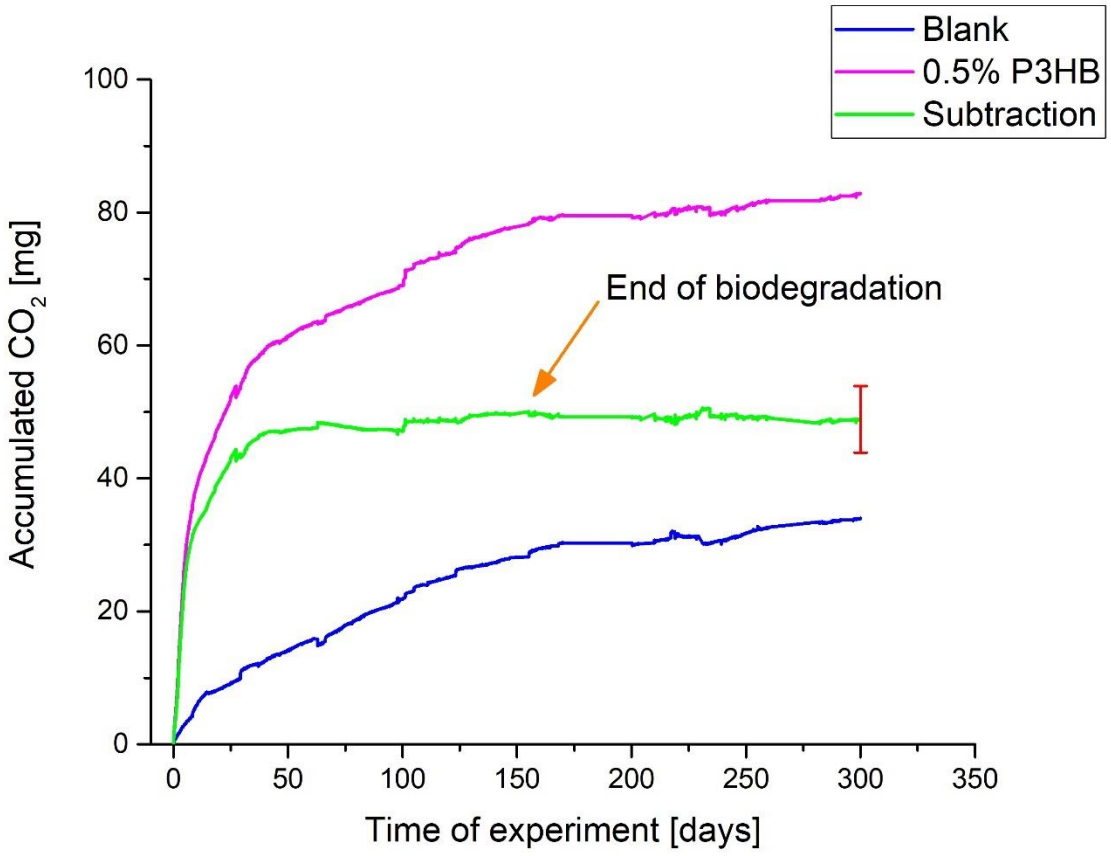


Figure 1: Exemplary evolution of CO_2 during the biodegradation experiment of Chernozem spiked with 0.5% PHB and blank soil, the orange arrow indicates the end of biodegradation and the beginning of the plateau phase; the interval indicates the experimental error.

The theoretical amount of CO_2 released during MBP biodegradation used to assess the progress of biodegradation for all samples was calculated after 300 days of experiment using the molecular formula of PHB and the amount of PHB added to the soil (Equation 1). The percentage of biodegraded PHB

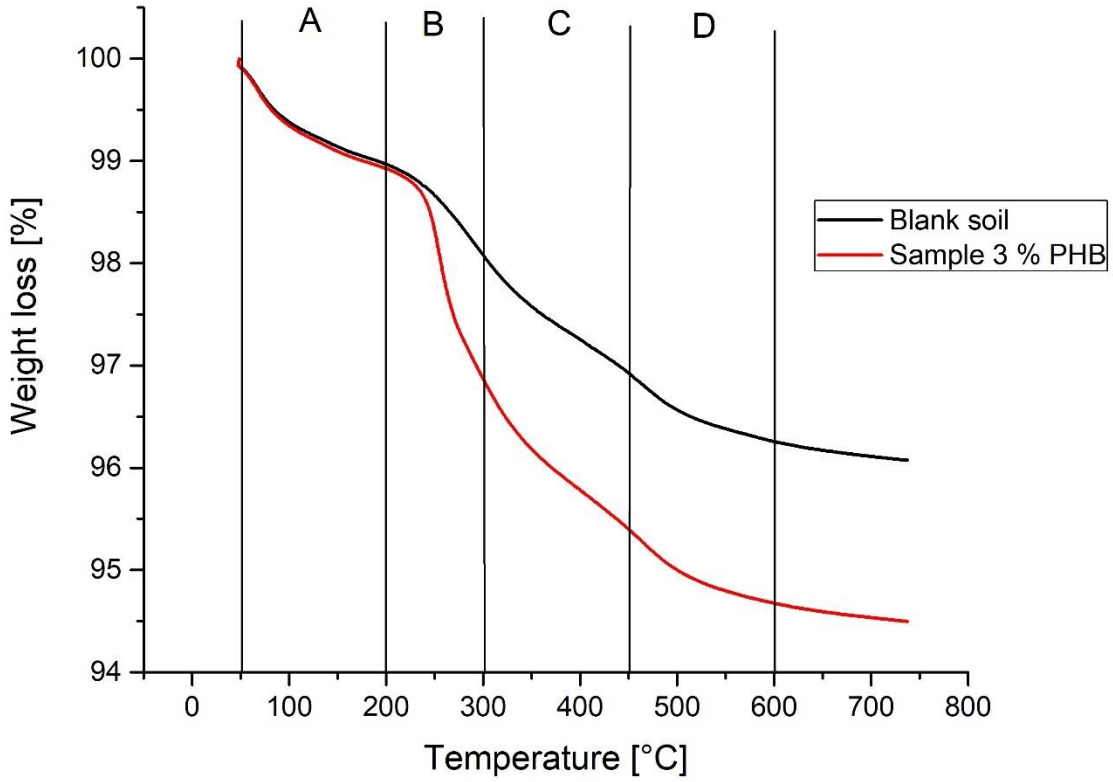
(Table 3) was calculated from ratio of theoretical CO_2 evolution and measured CO_2 respiration after 300 days (Table 3). According to the respirometry data, 100% biodegradation was not achieved in any case. Calculations indicated that the PHB most degraded in 300 days in soils Cambisol and Chernozem at 0.5% concentration when 94±4.7% and 93±4.6% were degraded respectively. The least degradation of PHB was observed in Chernozem and Cambisol containing 3% PHB where only 56±2.8% and 49±2.4% degraded respectively. Respirometric measurements indicate that although biodegradation in the 0.5% and 1% variant ended in all soils, none of the soils had 100% PHB carbon mineralization. This suggests that the Carbon remains in the soil in the form of biomass, or hypothetically also in the form of PHB fragments, which are not addressed by the EN ISO 17556:2012 method.

***Table 3**: CO_2 respired in the biodegradation experiment from each soil, the biodegradation of the PHB in these soils and the number of days required to complete biodegradation.*

		Original PHB content			
	soil	**0 %**	**0.5 %**	**1 %**	**3 %**
CO_2 respired [mg]	Phaeozem	37±1.8	77±3.9	112±5.6	215±11
	Cambisol	26±1.3	76±3.8	117±5.9	179±9
	Chernozem	36±1.8	84±4.2	110±5.5	306±15
Biodegradation of PHB from respirometry [%]	Phaeozem		74±3.7	71±3.6	56±2.8
	Cambisol		94±4.7	88±4.4	49±2,5
	Chernozem		93±4.6	76±3.8	86±4.3
Time of biodegradation end (days)	Phaeozem		146	260	not complete
	Cambisol		96	221	not complete
	Chernozem		131	249	not complete

Biodegradation determined by thermogravimetry and its influence on soil organic matter

Figure 2 shows exemplary comparison of the thermogravimetric blank sample record with Cambisol soil contaminated with 3% PHB after biodegradation. From Figure 2, it is clear that the 200-300°C region can be used for the quantitative determination of residual PHB, this interval would shift to higher temperatures in inert atmosphere (Fojt et al., 2022b), which indicates that the complete degradation of PHB takes place in lower temperatures than 300 °C.



224

225 *Figure 2: Comparison of blank and sample thermogravimetric record of 3% PHB Cambisol after*
226 *biodegradation. The weakly and strongly bounded water is evaporated between temperatures 30 and*
227 *200 °C (A) PHB thermal degradation occurs in the interval 200-300 °C (B). Temperature interval*
228 *between 300 and 450 °C is specific for stabilized SOM thermal degradation (C) and temperature*
229 *interval between 450 and 600 °C is specific for thermal degradation of stabile SOM (D). Temperature*
230 *intervals are taken from the work* (Kučerík et al., 2018b).

231 Table 4 summarizes the amount of undegraded PHB in the soils, which was determined by subtracting
232 the sample TML 200-300 °C from the blank TML 200-300 °C and then it was converted to the percent
233 biodegradation of PHB. Table 4 shows that TG indicated complete PHB degradation in all soils spiked
234 with 0.5% PHB. Biodegradation greater than 100% in Table 4 means that in the 200-300°C interval, the
235 mass loss is higher than would correspond to thermal degradation of PHB alone. This suggests that there
236 was also a loss of SOM indicating a possible effect on the quality and amount of SOM, which will be
237 discussed later in this paper. The phaeozem and cambisol spiked with 1% PHB probably achieved almost
238 complete biodegradation (and SOM alternation cannot be excluded) and the chernozem soil also shows
239 a possible impact on SOM quality. According to TG records, the soils spiked 3% did not reach complete
240 degradation after 300 days, with the least degradation of PHB in cambisol soil (62%) and the most in
241 phaeozem soil (91%). The PHB biodegradation results determined by thermogravimetry are higher than

the biodegradation determined by respirometry in all cases, confirming the above-mentioned assumption that some of the carbon from PHB was not respired but was immobilized in the SOM (e.g., used for microbial biomass formation). TG also confirms the respirometric results that soils spiked with different concentrations of PHB are at different levels of biodegradation. Samples spiked with 0.5% PHB represent soils longer after complete biodegradation; samples with an original PHB content of 1% are soils in which biodegradation has just ended; samples spiked with 3% PHB are in the process of biodegradation, i.e., contain increased biomass and residual PHB.

***Table 4**: Biodegradation of PHB in soils determined by thermogravimetry. Standard deviation never exceeded 1-2%.*

	Biodegradation using TG [%]		
PHB [%]	**Phaeozem**	**Cambisol**	**Chernozem**
0.5	141	111	111
1	100	99	104
3	91	62	86

As already mentioned, the total SOM content can be determined as the mass loss of the sample over the temperature interval from 110 to 550 °C. Figure 3 summarizes the data obtained for all variants after respiration. Figure 3 A, B and C show that in all three soils a decrease in SOM was observed for the 0.5 and 1% variants. For the 3% variant, the total SOM content is affected by the presence of residual PHB, hence a significant increase in SOM content can be seen in all soils.

The decreases of TML in the 0.5 and 1% variants suggest that these contaminated soils (except the 1% variant of cambisol) showed a decrease in SOM content in comparison with the uncontaminated soil due to PHB biodegradation. This confirms the effect observed when PHB biodegradation was determined by thermogravimetry in the 200-300°C interval. In general, there is a traceable trend of decreasing in SOM content with increasing progression of biodegradation, i.e. the samples with the least SOM are those with the earliest complete degradation. This implies that SOM-rich soils might be more prone to this degradation than carbon-deficient soils, and furthermore that soil degradation might occur only after a longer period after substrate (PHB) depletion.

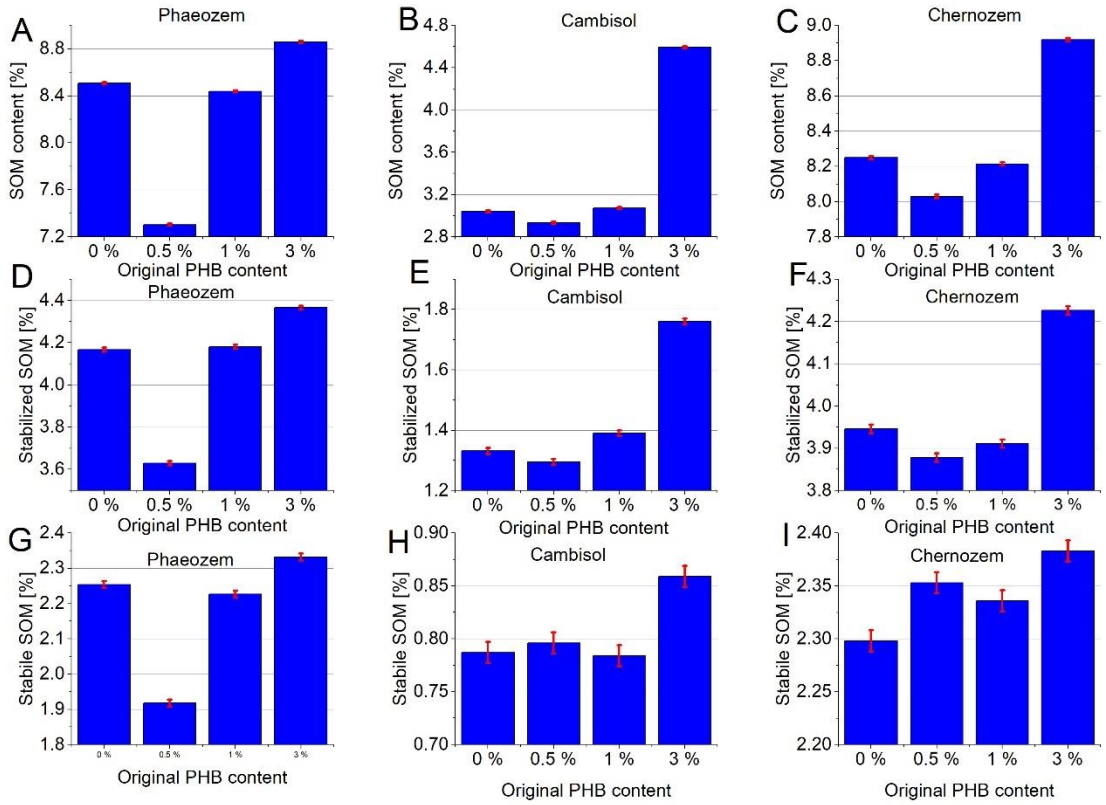


Figure 3: SOM thermogravimetry analysis in all soils. Total SOM content is shown in figures A) B) and C), stabilized SOM content is shown in figures D), E) and F) and stable SOM content is shown in figures G), H) and I). The figures also contain standard deviations calculated from repeated measurements.

The temperature interval 300-450 °C (Figure 3 D, E and F) is characteristic of the thermal degradation of stabilized SOM and changes in this interval indicate shifts in SOM quality (Kučerík et al., 2018b; Tokarski et al., 2020). Even there, the content of this fraction has been reduced for variant 0.5%. For the 1% variant, this decrease was observed only in the Chernozem, the Cambisol soil showed an increase and the Phaeozem showed no change. The 3% variant showed an increase for all soils. The mass loss in this interval is not so much influenced by residual PHB, yet the soils with the highest initial PHB concentration and highest residual PHB content also show the highest SOM content. This implies that biodegradation of biodegradable plastics initially results in an increase in SOM, which gradually decreases with time, and that some of the original SOM is consumed that would not have been consumed without the presence of PHB.

Stable SOM was determined from mass loss over the temperature interval 450-600 °C (Kučerík et al., 2018b) (Figure 3 G, H and I). Significant changes occurred only in phaeozem (decrease in 0.5% variant) and cambisol (increase in 3% variant). Thus, during biodegradation of PHB in phaeozem, a part of SOM is immobilized first, but after biodegradation is mineralized. The slight increase in cambisol may be related to the increase in the number of organisms and the formation of carbonates, which are degraded

in this temperature range (Pribyl, 2010). However, compared to previous temperature intervals, the changes measured in this temperature interval are minimal, therefore it can be argued that PHB degradation does not significantly affect the stable SOM content and mainly affects the labile part of the SOM.

Biodegradation – carbon balance

One of the ways to evaluate the effect of PHB biodegradation on soil is to analyze the carbon content before and after biodegradation. In variants where there is still residual PHB, the analysis is more complicated, but respirometry and TG data can be used to subtract carbon from PHB. Figure 4 summarizes the data obtained. The blue columns indicate the total SOC, for Figure 4 A, B and C the readings provided by respirometry (from respired CO_2) and for Figure 4 D, E and F the thermogravimetry data (from residual PHB) are shown in orange. Very important is the fact that the N content did not change during the experiment in any of the variants, which is in line with the observation that N mineralization does not occur during N deficiency in the soil but only immobilization (Brtnicky et al., 2022). A detailed description of the processes is described in the soil enzymes section.

The blue columns indicates that in all soils of the 0.5 and 1% variants, the total carbon content always decreased during PHB biodegradation. This corresponds with the TG data, which indicated a reduction in total SOM after PHB biodegradation. 3% variant samples show an increase which, is related to residual PHB. There is an increase in SOC in all cases after deducting, which is related to the still high biomass content in this variant. It will start to decrease relatively quickly after the end of biodegradation. The respirometry data are not completely consistent with TG, as they showed a decrease in SOC in all variants except the 3% Chernozem.

We assume that the overgrowth of PHB-degrading microorganisms leads to SOM degradation at a later stage. In other words, for all soils, there was a decrease in labile SOM in the sample containing 0.5% PHB compared to the blank, where data from thermogravimetry indicated complete biodegradation of PHB. For the sample originally containing 1% PHB, the SOM content is comparable to the blank. In this case, the contribution of residual PHB is minimal, since the samples are in the stage of almost complete biodegradation. A large part of the labile SOM at this point consists of the bodies of microorganisms that have multiplied rapidly due to the abundance of easily degradable substrate for them. After the complete consumption of the substrate, a part of the microorganisms that without a suitable food source will subsequently die and then will degrade by the surviving microorganisms, so that the content of labile SOM will most likely decrease as in the samples with an original PHB content of 0.5%. For samples spiked with 3% PHB, a substantial fraction of PHB is still present in the soil, which has a similar thermal degradation interval to labile SOM, thus significantly biasing the analysis. Thus, MBPs can cause not only the bias in the determination of total carbon, as described by (Rillig, 2018), but also complicates the determination of labile SOM using thermogravimetry. Thus, a similar

trend is observed for all soils, where the strongest effects are again observed for soils with higher total organic carbon content, with phaeozem is the most affected.

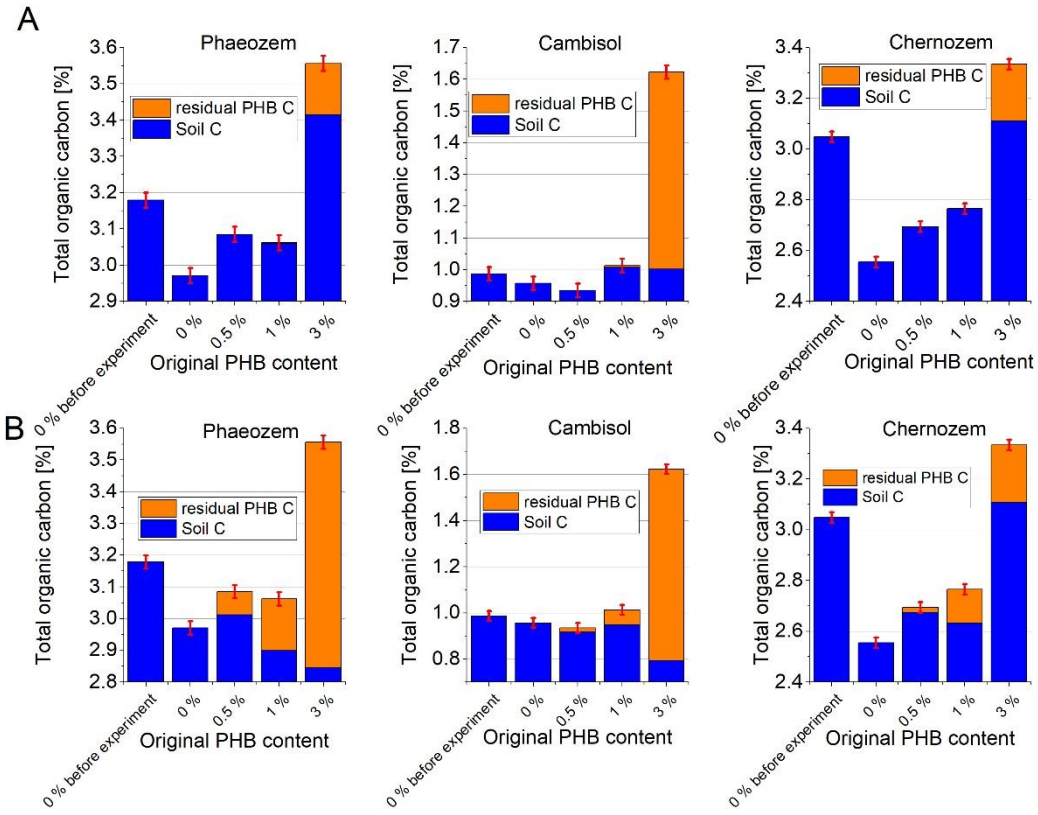


Figure 4: Graphical representation of total organic carbon content in soils before and after the biodegradation experiment based on the results of total C determination and A) respirometry and B) thermogravimetry. Residual PHB carbon determined by respirometry and respirometry are in orange; other soil carbon is shown in blue. The figures also include standard deviations calculated from repeated measurements.

Effect of biodegradation and residual PHB on soil moisture content

The free and bound water content of the soil corresponds to a mass loss in the interval 30-200 °C (Figure 2). The highest water content was determined in the phaeozem and chernozem, because they also contain the highest SOM (Figure 5 and Table 1). For these soils, the samples with an initial 0.5 % PHB content showed a decrease in loss from 4.02 % to 3.46 % and from 3.96 % to 3.65 %, respectively. This suggests that the soils with higher SOM content have a decrease in soil water content in the post-biodegradation period, which may be due to the loss of SOM in the soils compared to the blank. For the phaeozem and chernozem soil samples with an initial PHB content of 1%, the mass loss increased to 3.91% and 4.00%, respectively, which is almost a return to the level of the blank, because of higher SOM content. Although the SOM content of the phaeozem and chernozem soil samples is the highest in 3% PHB variant, there is a decrease in water content to 3.85% and 3.46% respectively. This can be explained by the fact that

part of the SOM is composed of residual PHB, which due to its relative hydrophobicity does not contribute to the increase in water content. In contrast, the cambisol with an initial PHB concentration of 0.5% shown an increase in mass loss from 1.00% to 1.65%. For this soil spiked with 1% PHB, there was then a decrease in mass content to 0.95% and for the soil spiked with 3% PHB, the mass loss increased slightly to 1.04%. Due to the smaller change in SOM content, a direct effect of biodegradation on soil water capacity can be observed here. The effect of PHB on soil water content immediately after the MBPs enter the soil has already been discussed by Fojt et al. where a possible decrease in soil water capacity with increasing MBPs content was observed (Fojt et al., 2022a). In this case, a long-term effect of the presence of PHB in the soil on soil water capacity is observed, which in soils with higher SOM content strongly depends on SOM loss. In lower SOM soils, the loss of SOM itself is not so significant and there is a slight increase in equilibrium moisture content after biodegradation.

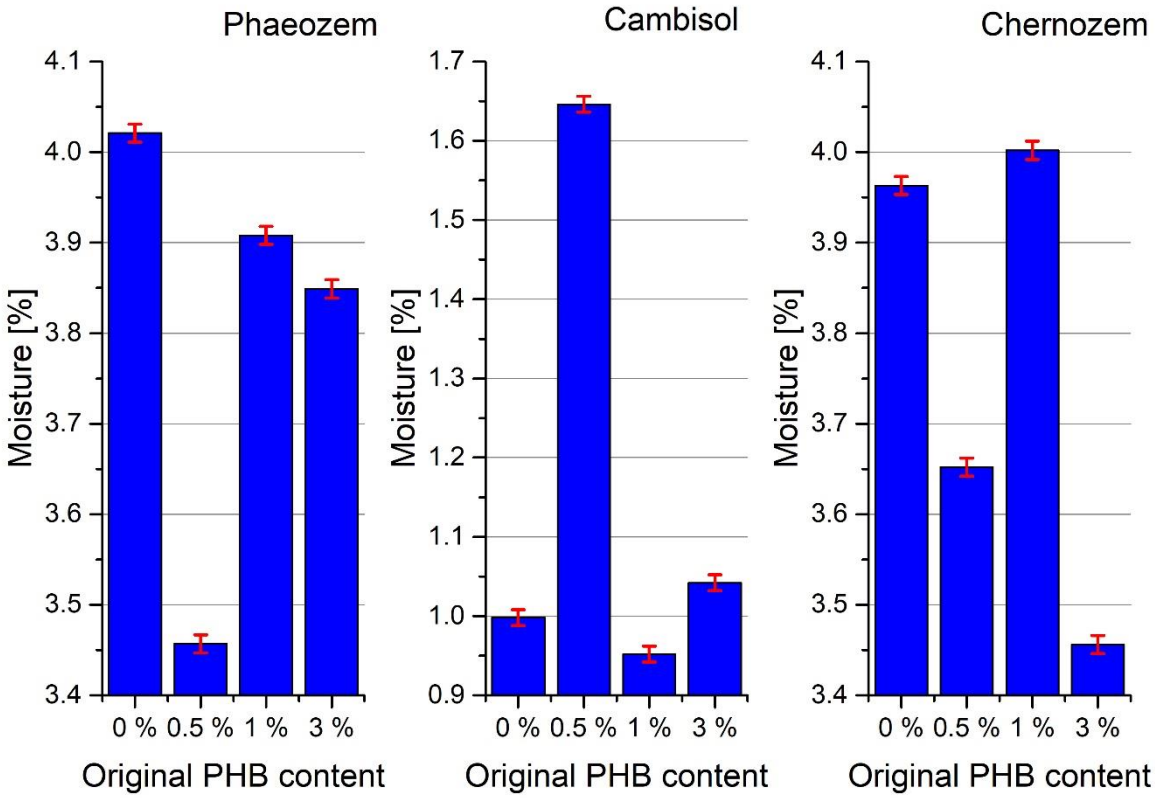


Figure 5: Soil moisture content determined by thermogravimetry from the interval 30-200 °C. The figures also include standard deviations calculated from repeated measurements.

Enzymatic analysis

Content of soil enzymes and their activity are affected by soil properties, energy and nutrient availability (Sander, 2019; Zhou et al., 2021). We observed this phenomenon in our experiments in analysis of five key enzymes activity changes: arylsulfatase, urease, phosphatase, β-N-Acetylglucosaminidase (NAG) and glucosidase. In all soil samples, were observed a significant response to bioavailable carbon from

PHB MBPs in comparison with blank sample. This addition led to boost carbon mineralizing microbial activity. Each enzyme was plotted in bar graph for all soil types including error bars (see Figure 7). The results of 3% PHB samples were expected to be affected by non-complete biodegradation and residual PHB MBPs.

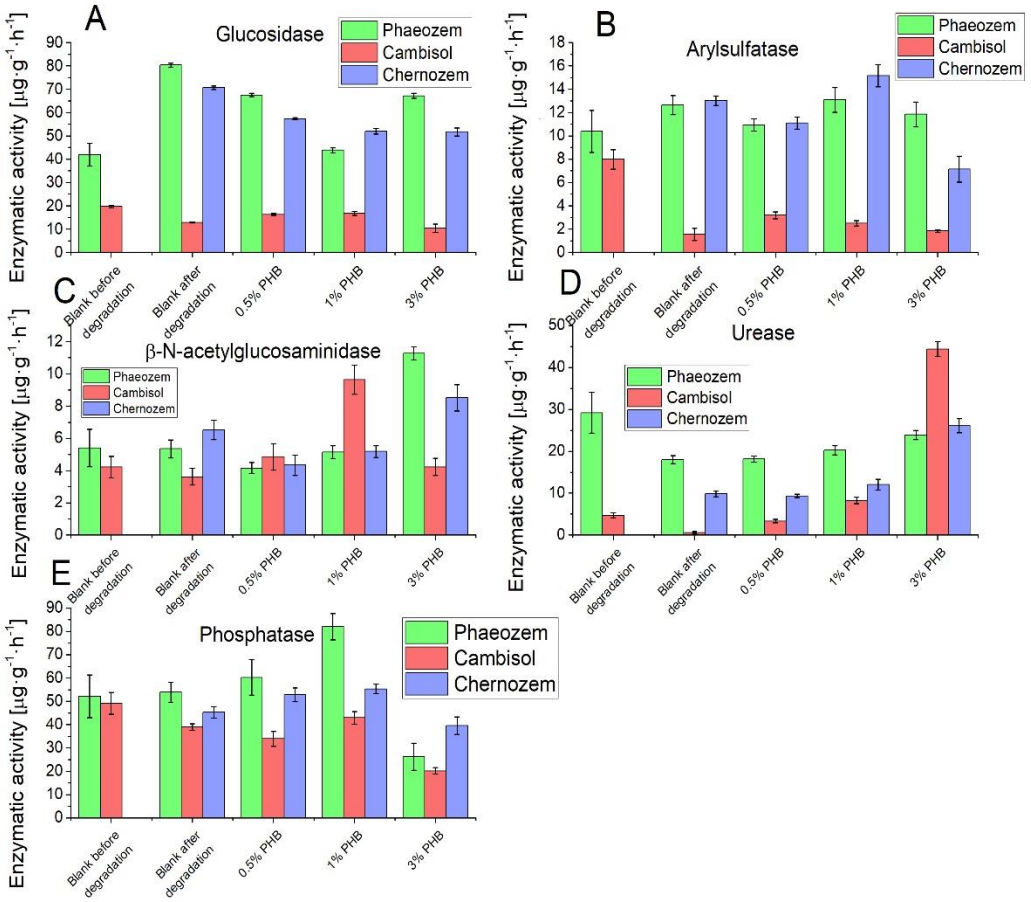


Figure 7: Enzymatic activity of five key enzymes Glucosidase (A), Arylsulfatase (B), NAG (C), Urease (D) and Phosphatase (E) in all three soils in blank sample before and in all spiked samples after biodegradation. The data chernozem blank before degradation were not available. The figures also include standard deviations calculated from repeated measurements.

Glucosidase activity (see Figure 7A) is linked with soil carbon source acquisition during decomposition (Turner et al., 2002). Our results show that, the activity of glucosidase enzyme decreased in both Chermozems and Phaeozem after PHB MBP contamination. Relatively high original β-glucosidase activity was reduced because added PHB is a preferential source of carbon comparing to SOC. Only in cambisol, which contained less SOM, PHB addition boosted β-glucosidase activity comparing to the less active blank. This in agreement with other studies where biodegradation of other naturally occurring polymers (e.g. cellulose) may cause a positive priming effect (Blagodatskaya et al., 2014). On the other hand, PHB in cambisol caused an increase in β-glucosidase activity, however, also in this case was observed decomposition of SOM (see Figure 3).

Soil arylsulfatase (ARS, Figure 7B) has impact on the organic sulphus acquisition and thus the cycle of soil sulphur. The resulst displayed in the Figure 7 reveal that the enzyme activity at 1% PHB of all soil types increased in comparisson with the blank sample after experiment. In case of phaeozem, the ARS activity was even higher than in blank before experiment. On the other hand, in cambisol soil, ARS activity decreased after experiment. However, the enzymatic activity after the addition of PHB caused significantly higher values than in case of blank sample after experiment and still imply an increase in enzymatic activity. Importadnly, samples still containing MBP residues (mostly 3% PHB samples) have lower ARS activities comparing to soils with completed PHB biodegradation. We conclude that the structure of the microorganism comunities in PHB containing soil significanltly changed, which is in line with other authors (Šerá et al., 2022). Overall, the results showed higher acquisition of soil organic sulphur and indicates negative effect on sulphur stock in SOM.

NAG activity (see Figure 7C) is analysed, because of its role in cycles of carbon and nitrogen (especially in chitin conversion to amino sugars, which are sources of mineralizable N in soils) (Ekenler and Tabatabai, 2004). As the N content in soil after metabolization of N was not changed (Table 2), NAG was analysed to clarify possible nitrogen transformation. The results from this study had a different trend than in other enzymes activities The NAG activities in chernosols decreased compared to blanks after degradation and the soil samples with 0.5% and 1% PHB addition. On the other hand, the enzymatic activity was higher in cambisol at 0.5% and 1% PHB contamination and also at 3% PHB for chernozem and phaeozem. The soil microorganisms require more nitrogen to balance carbon consumption and that's why they produce more NAG (Currey et al., 2010). With increasing concentration of PHB, the need for nitrogen rises (Brtnicky et al., 2022). The cambisol with PHB residues (3% PHB) were different. Nevertheless, addition of PHB into soil increased the C/N ratio, increased the N need for microorganisms, which immobilized the N preveented its loss and its content remained unchanged.

The other enzyme involved in nitrogen cycle is urease (Figure 7D). It was analysed because of its key role in production of bioaccesible ammoinum nitrogen during the nitrogen mineralization. Similar to the NAG, the urease activity rised with PHB concentration. In all soils, the blank sample after experiment provided the same urease activity as sample with 0.5% PHB. The remaining samples contained less nitrogen and it causes higher urease activity. Similar results were observed in previous works, where the enhanced NAG and urease activity after carbon supply from PHB was attributed to microbial N immobilization (Zhou et al., 2021).

Phosphatase (see Figure 7E) is key in the phosphorus cycle in phosphorus-deficient soils. Microorganisms produce it to extract nutrients for growth and enzyme synthesis (Sardans et al., 2006). We obsereved an increase in phospahatase activity with increasing concentration of PHB (up to 1%). Compared to original control soils, at 0.5% the activity was either higher (cambisol) or lower (chernozem and phaeozem). The results also suggest a deficit of phosphours in microbial acitivity in

chernosols with increasing PHB concentration. The results for cambisol showed lower production of phospahatase, which indicates that microorganisms had relative abundance of phosphorus in 0.5% P3HB. On the contrary, it increased in the 1% PHB samples, where the demand increased.

Implications from the obtained data

It was shown that the biodegradation of PHB in soil increased the demand for nutrients and enhanced CO_2 evolution in all investigated soils. This resulted in a decrease in total SOM content, which may be explained as a priming effect (PE), which is a term reffering to an increase in SOM decomposition rate after input of fresh organic matter (Fontaine et al., 2003). PE is induced by enhanced microbial activity caused by higher availability of carbon sourse and energy and results in the faster mineralization of SOM. Typically, the SOM mineralizaiton is determined by a measurement of CO_2 evolution or nitrogen mineralization rates, although it was suggested to seach also for new analytical approaches and ways for identification of underlying mechanisms. In particular, the information on carbon dynamics in SOM should include microbial biomass, enzymatic activity, community structure and others (Blagodatskaya and Kuzyakov, 2011a).

In this work, for the first time, we used as a supportive methods such as TG and elemental analysis. TG revealed a decrease in SOM content after 300 days of incubation induced by enhanced enzymatic activity and in particual, a decrease in stabilized SOM fraction. The different stages of degradation induced by different concentrations degraded at different periods suggested that the SOM degradation proceeds after use of labile C source and is common for three soil types.

In fact, addition of labile C to soil may induce either acceleration (positive PE) or retardation of SOM (negative PE) (Blagodatskaya and Kuzyakov, 2008a). The PE includes two components such as real and apparent priming. Real PE is induced by the lack of nutrients (mostly nitrogen), which can be supplemented by SOM decomposition or due to SOM co-metabolism in which soil microorganisms use the energy of available compounds to synthesize enzymes hydrolyzing the less available compounds (Blagodatskaya and Kuzyakov, 2008b). Apparent PE reffers to a change in the CO_2 evolution from microbial biomass turnover after the input of a labile substrate (Bastida et al., 2019a). To summarize, real PE is caused by SOM decomposition whereas apparent PE is caused by changes in microbial biomass turnover with no effects on SOM decomposition (Blagodatskaya and Kuzyakov, 2008b). The apparent and real priming is uneasy to distinguish (Bastida et al., 2019b), but in general, apparent priming tends to occur shortly after adding readily availably substrates (days to weeks), while real priming takes longer (Kuzyakov, 2010).

Therefore, the processes and changes in SOM content observed in this work suggest the occurence of real PE in all investigated soil types upon addition of PHB substrate. An unknown and unpredictible (so far) effect would have the plants, which may via root exudates and growth promoting microorganis

444 living in the close vicity of roots (rhizosphere) influence the dynamics and mechansism of SOM
445 turnover.

446 Up to now, the PE was observed for glucose, fructose, alanine, celulose, plant residues, manure or slurry
447 (Blagodatskaya et al., 2014; Blagodatskaya and Kuzyakov, 2011b; Conde et al., 2005; Hamer and
448 Marschner, 2005). The PE was also observed for thermoplastic starch (TPS)-blend (PLA-TPS blend) as
449 a substrate (Bher et al., 2019; Polman et al., 2021).

450 Therefore, for pure microbially labile chemicals or substances poor of nutrient content, added to soil,
451 the PE is a probable scenario. PHB is a substance produced by microorgansisms as a energy and C
452 reservoir. Its biodegradaiton in soil is fast, which may paradoxically represent problem due to fast
453 depletion of nutrients in the plastisfere and lead to SOM degradation and to a negative infleunce on plant
454 growth, as obseved recently in (Brtnicky et al., 2022).

455 **Conclusion**

456 This paper describes the results of a biodegradation experiment with three different soils contaminated
457 with three different concentrations of MBP PHB which was conducted according to EN ISO 17556:2012
458 using a respirometer. In soils with 0.5 and 1% PHB concentration, complete biodegradation was
459 achieved, but not all PHB carbon was captured in respirometer because some of this carbon remained
460 in the soil as MBP or it was immobilized in the soil. Therefore, this carbon could not be detected by the
461 respirometer. Therefore, all soils were analysed by thermograviemetry, which revealed part of the
462 immobilised carbon in the soil and additionally indicated a possible SOM loss (priming effect), which
463 was most noticeable in the SOM-rich soils in the samples containing initially 0.5% PHB, where
464 biodegradation was completed first. This suggests that the biodegradation has the strongest effect on
465 fertile soils with high SOM content and a longer post-bidegradation period which is usually beyond the
466 length of standard biodegradation tests. There was also a reduction in soil moisture content caused by
467 the loss of SOM. The activity of five key soil enzymes was determined in all samples to confirm a
468 possible priming effect. To sum up our results, the standard biodegradation tests focuse mostly on soil
469 respiration and overlook what remains in soil after the experiment and change in soil quality. Also the
470 fast biodegradation of bioplastics may pose a threat to fertile soils with high SOM content which may
471 result in liberate carbon from these soils in form of CO_2 and made them less fertile.

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