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Title: Agri and food waste valorisation co-ops based on flexible multi-feedstocks biorefinery processing technologies for new high added value applications.

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Associated Task(s)	*Task 6.3. Soil assessment of crop cultivation with new waste valorisation approach*
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1 Introduction and objectives

Deliverable D6.5 comes at the very end of the production chain of the materials produced during the Agrimax project. After use as packaging/potting materials or agricultural films, bioplastics can be left in the soil and contribute to soil fertility, when a number of conditions are fulfilled. Also, after thorough characterisation of the fertilizer materials (D6.3 and D6.4), their impact on soil properties should be investigated. The present deliverable thus concerns the assessment of the impact on soil of a variety of products from the project. This includes a variety of materials namely some components used in the production of the bioplastics pots and agricultural films, the actual pots and films, and a number of novel high value fertilizers.

Within D6.5, the aim was to analyse the impact of these materials on a variety of soil processes and properties relevant for agriculture and environment. This includes agronomic parameters such as N mineralization, impact on directly available P in soil, parameters related to both agricultural and environmental aspects such as the amount of stable organic C (and thus C sequestration potential) and soil biological quality parameters, and pure environmental parameters namely N_2O emissions.

Within this D6.5 also the impact of the novel fertilizers on plant performance was assessed, first of all in pot trials, and following this also in field experiments with tomato as test plant.

This allows us to draw conclusions with respect to the values and risks of all these materials and fertilizers when they are left in the soil as residual material or when they are added to the soil as fertilizer/soil improver.

2 Assessment of effects of biocomposites and bio-fertilizers on soil processes and soil biological quality

2.1 General set-up, characterization of bioplastic compounds, bioplastics and fertilizers, and soil

In this study, 23 types of Agricultural and Food Processing Waste (AFPW) in the forms of biocomposites and bio-fertilisers were incubated with soil in a controlled environment for three months. Considering the quantity and availability of the amendments, the incubations were performed in multiple batches (Table 1). All batches were carried out with identical soil samples and the same experimental protocols.

2.1.1 Soil characterization

The samples for this trial were collected following a random pattern from an agricultural field in Kruisem, Belgium (50°56’38.5” N, 3°31’21.4” E) according to the procedure suggested by the European Commission (Tóth et al., 2013). Collected soil samples were mixed well and dried at room temperature (21°C) until air dry and sieved through ≤5.0 mm mesh. Prepared soil samples were then transferred to polyethene (PE) plastic bags and stored in a dry and cool room until being used for the incubations.

Determination of general soil properties was carried out on air-dried soil. pH-KCl was measured in 1 M KCl extracts (soil:KCl ratio of 1:2.5). Total C and N (TN) contents were measured with a CNS elemental analyser, respectively, as 1.24% and 0.14%.

Before starting the incubation process, a pre-incubation treatment applies to dry soil by adding one litre of demineralised water per 10 kg of soil (10% V:W) to reactivate the microbial communities and recreate the field conditions. Then the soil was mixed and transferred to containers, covered with paraffin-coated film, and placed in a dark room at an ambient temperature of 21°C for 10 days. For allowing air exchange, small holes were created to the parafilm cover of the containers.

2.1.2 Biopolymers and fertilizers

The materials were supplied from partners in the Agrimax project in Italy and Spain. These compounds can be categorised into two classes based on their composition and valorisation procedure;

A. BPMs: Biobased packaging materials such as biobased coatings, bio-composites and bio-active packaging.
B. BAPs: Biobased agricultural products such as bio-fertilisers and biodegradable mulching films and pots.

These materials are mainly process wastes originating from cereals (wheat bran, oat hulls, oat mill fed, oat bran), olives (olive cake, two-phase olive mill waste, olive mill wastewater, olive stone and olive leaves), potatoes (raw pieces and pulp, potato starch waste and peels), tomatoes (tomato plant and the industrial processing by-products), plant fibres and fungal mycelium by implementing several technologies such as ultrasound-assisted extraction, solvent extraction; filtration; and thermal and enzymatic treatments. Different bio-based plastics like polybutylene succinate (PBS), polylactic acid (PLA) were also included. The characteristics of the amendments, and the application rate per each soil sample is indicated in Table 1. However due to insufficient quantity being avaialble at the time of the start of these experiments, a number of parameters of some samples could not be measured.

2.1.3 General set-up

For each batch, a set of parallel aerobic incubations under controlled environmental conditions were established in this study with a mixture of soil and amendments to determine the C mineralisation and the N mineralisation, enzyme activities, microbial biomass C (MBC) and phospholipid fatty acids (PLFAs).

C and N mineralisation results were evaluated separately for BPM and BAP samples, considering the differential characteristics and potential fields of application.

For each batch of samples (Table 2), C and N mineralisation assessments were carried out separately yet following identical procedures for 12 weeks. Nevertheless, considering the sometimes limited amount of material available, and the timing of the sample delivery, some modifications were needed in terms of the application quantity and incubation duration (shortened incubation times to allow for sufficient time for analysis and interpretation). Additionally, for a comprehensive understanding of C mineralisation rates in some treatments, the incubation process was extended for a number of samples (Table 2).

In the first batch, due to insufficient materials, only four materials were considered for C mineralisation incubation (FA-P, FA-L(Chiesa group), PBSO-10 and PBSD-5-FA (Aimplas group)).

Table 1: Characteristics and application rates of the materials used in the different batches. Treatments in coloured rows represent the BPMs compounds.; (*) volumetric application in ml.; ND = Not defined (parameter could not be measured due to insufficient sample quantity)

	Assitional Materials ID	materials tag (abbreviation)	Composition	Fresh mass added (g)	fresh mass added (ton/ha) *	Dry matter (%)	Total C (% Dry matter)	Total N (% Dry matter)	C:N (% Dry matter)
Batch 1	Chiesa:FA process powder	FA-P	ferulic acid processed powder	1	6,0	ND	ND	ND	ND
	Chiesa:FA process liquid	FA-L	ferulic acid processed liquid	2 ml*	12.1	ND	ND	ND	ND
	AIMPLAS:PRO15-0377-03-11	PBS-10-PF	PBS + 10% potato fiber	2	12.1	ND	ND	ND	ND
	AIMPLAS:PRO15-0377-03-04	PBS-10-OG	PBS + 10% oligomer	1.5	9.0	ND	ND	ND	ND
	AIMPLAS:PRO15-0377-03-06	PBSZ-5-FA	PBS + 5% FA	2	12.1	ND	ND	ND	ND
	AIMPLAS:PRO15-0377-04-02	PBSO-10	PBS + 10% oligomer	1.5	9.0	99	55.3	0.02	2762.5
	AIMPLAS:PRO15-0377-04-04	PBSD-5-FA	PBS + 5% FA	2	12.1	99	55	0.02	2750
Batch 2	AIMPLAS:PRO15-0377-03-18-02	PBS-20-PF	PBS + 20% potato fibres	2	12.1	99	52.3	0.1	422
	MOGU:P01 + 20% bran	CFFM	Cotton fibres + 20% bran + fungal mycelium	2	12.1	94	39.6	1.3	30
	MOGU:P07 + 20% bran	HFFMl	Hemp fibres + 20% bran + fungal mycelium	2	12.1	94	42.3	0.9	49
	MOGU:P07 + 0% bran	HFFMu	Fungal mycelium	0.1	0.6	90	36.5	4.4	8
Batch 3	AIMPLAS:PRO15-0377-04-20-01	PBSO-5	PBS 92+ 5% oligomers	0.7	4.2	ND	54.9	0.02	2968
	AIMPLAS:PRO15-0377-04-20-02	PBSOX	PBS 92+ 5% oligomers + 10% PBAT	0.7	4.2	ND	55.6	0.02	2739
		PHYTBIOP	Tomato stalks and leaves + cull/ discarded tomatoes + corn stover + Trichoderma atroviridae + Gibellulopsis nigrescens	5	30.1	43	30.6	1.8	17
		PHYTBIOF	Tomato stalks and leaves + cull/ discarded tomatoes + corn stover + Bacillus licheniformis + Gibellulopsis nigrescens	5	30.1	56	30.6	1.8	17
		CREF	Tomato stalks and leaves + cull/ discarded tomatoes	5	30.1	81	25.3	2.0	13
Batch 4		PHBV-B	PHBV+Bran	1.5	9.0	99	55.5	0.6	87.1
		PBS-B	PBS+Bran	1.5	9.0	99	57.7	0.4	162.1
		CAC	CD+30% ATEC+10% bran	2	12.1	99	52.4	0.1	459.7
		PBSPB-10-PF	PBS + 10% Potato Fiber	2	12.1	99	58.5	0.03	2003.4

		PLA-C	PLA+11% bran	2	12.1	99	52.5	0.2	291.7
		PHBV-C	PHBV+15% bran	2	12.1	99	55.3	0.6	89.8
Batch 5		UL-1	Organic compost	5	30.1	59	17.4	1.1	15.3
		UL-2	Organic compost + PGPMs	5	30.1	45	16.2	1.0	16.8
		FERT-S	Solid fertiliser	1.5	9.0	76	32.5	7.0	4.7
		FERT-L	Liquid organic fertiliser	1.1 ml*	6.2	25	9.8	1.6	6.1

For mineral N assessments, five sampling points (with 14 days intervals) were considered for each batch. However, due to insufficient material, the incubation of the first batch was limited to 63 days, and), and only two sampling times were possible for PBSO-10, PBS-10-OG samples. Similarly, the 4th and 5th batch's sampling point was carried out on days 56 and 70 instead of day 63. The incubation period of 4th batch was prosecuted till day 84 days, while it was 70 days for the 5th batch (Table 2).

Table 2: Incubation details applied for each batch of samples; (*) modifications were applied for some samples

Batch no.	**Total samples No.**	**Incubation duration (days)**		**N min Sampling**	
		C min	N min	Sampling points	intervals (day no.)
1	7	110	63	5*	0-14-28-42-63
2	4	100	84	6	0-14-28-42-63-84
3	5	86	84	6	0-14-28-42-63-84
4	6	153	84	7	0-14-28-42-56-70-84
5	4	70	70	6	0-14-28-42-56-70

2.2 Impact on N and P dynamics in soil

2.2.1 Set-up of incubation experiments

N mineralisation was measured during aerobic incubation in controlled environmental conditions. Accordingly, variable amounts of fresh AFWCBs amendments were mixed thoroughly with soil. A set of six samples in three replications were prepared for each treatment. One additional set without any additives was also included in this set as the control. Samples were transferred carefully in PVC tubes with a radius of 2.3 cm and 18 cm in height. The content of the tubes was gently compacted using a wooden cylinder to achieve a bulk density of 1.35 $Mg.m^{-3}$. The water content of all tubes was adjusted to 50 vol% WFPS (equal to 0.18 $g.g^{-1}$ corresponding approximately to a water tension of pF=2.5) for obtaining optimum aerobic microbial activity. The moisture content was kept constant throughout the incubation by adding demineralised water as needed (based on weight loss of samples).

Sample tubes were then covered by parafilm, and the parafilm was pinholed to preserve the balance of moisture content while providing air exchange. Sample tubes were kept in an incubator at a constant temperature of 16°C, representing the average soil temperature during spring-summer (De Neve et al., 2004).

For the amount of mineral N present at t = 0, one set of samples were measured for N amount before the start of the incubation.

Measurement of mineral N was carried out according to the sampling interval (Table 2). For analysis of mineral N, the tubes were sampled destructively and soil was homogenised, and followed by extraction of mineral N with 1M KCl solution. Measurement of NO_3^--N and NH_4^+-N was performed on extracted suspensions with a continuous flow auto-analyser (Chem-lab 4, Skalar 223 Analytical, Breda, The Netherlands).

The net N release from the applied AFWCBs was calculated with the following equation and expressed as a percentage of the total N added from the AFWCBs.

$$Nmin\ net\ (\%) = \frac{Namended - Ncontrol}{Total\ added\ N} \times 100$$

in which:

$N_{min\ net}$: percentage of net cumulative N mineralised from each material (%)
$N_{amended}$: cumulative N mineralised from each material (mg.kg $^{-1}$ dry soil)
$N_{control}$: cumulative N mineralised from the control (mg.kg^{-1} dry soil)
Total added N: total nitrogen contained in the materials applied (mg.kg^{-1} dry soil)

Readily available phosphorus

Readily available phosphorous (P) was assessed on incubated soil samples with the modified $CaCl_2$ extraction method (Hylander et al., 2008) (Erp et al., 2008). 50 ml of $CaCl_2$ (0.01M) were added to 5 g of fresh soil sample (1:10 w/v) and were shaken on a reciprocating shaker for 2 hours, followed by extraction through Whatman ashless filter papers (grade 598/3). The extracts were subsequently analysed by inductively coupled plasma-atomic emission spectrometry (ICP-AES) at two different wavelengths.

2.2.2 Impact on N mineralization - N availability

BPMs

Given the lack of materials that was available at the onset of the incubation, unfortunately the exact amount of added N could not be measured, and hence for a number of samples in the first batch no N mineralization expressed as % on total N could be calculated and reported.

The application of BPM material to soil resulted in remarkable N immobilisation in most of the samples due to the low N contents of BPM materials, except PLA-C (Table 3). Because of the very low N content, the immobilization expressed in % of total N added is extremely high in a number of cases, and therefore only the cases where net N mineralization was obtained are expressed as % of total N added. The mineralisation pattern in PBS-B started decreasing in the second week of incubation. The immobilisation phase in PLA-C turned to mineralisation on day 70 and continued ascending slowly until the end of incubation. By the end of incubation on day 84, the N mineralisation was the highest in PLA-C with 23.5% (net cumulative N mineralised). Mineralisation was not observed in any other samples with the lowest (highest immobilisation) in PBSOX sample, namely -8366.4%.

Table 3: Net cumulative N mineralised from BPMs material (in mg mineral N . kg^{-1} dry soil, and percentage) on day 84. Values are means ± standard errors, and different letters show statistical differences (p <0.05). N.D; Not defined

BPMs material	Net N_{min} (mg.kg^{-1} dry soil)				Net N (%)
PHBV-B	-36.7	±	0.3	c	No net N mineralization
PBS-B	-4.5	±	5.2	a	No net N mineralization
CAC	-23.2	±	1.6	b	No net N mineralization
BPSPB-10-PF	-38.6	±	0.5	c	No net N mineralization
PLA-C	3.8	±	2.0	a	23.5 ± 12.2
PHBV-C	-36.2	±	0.5	c	No net N mineralization
PBS-20-PF	-55.3	±	0.1	d	No net N mineralization
PBSO-5	-46.3	±	1.4	cd	No net N mineralization
PBSOX	-53.0	±	1.0	d	No net N mineralization

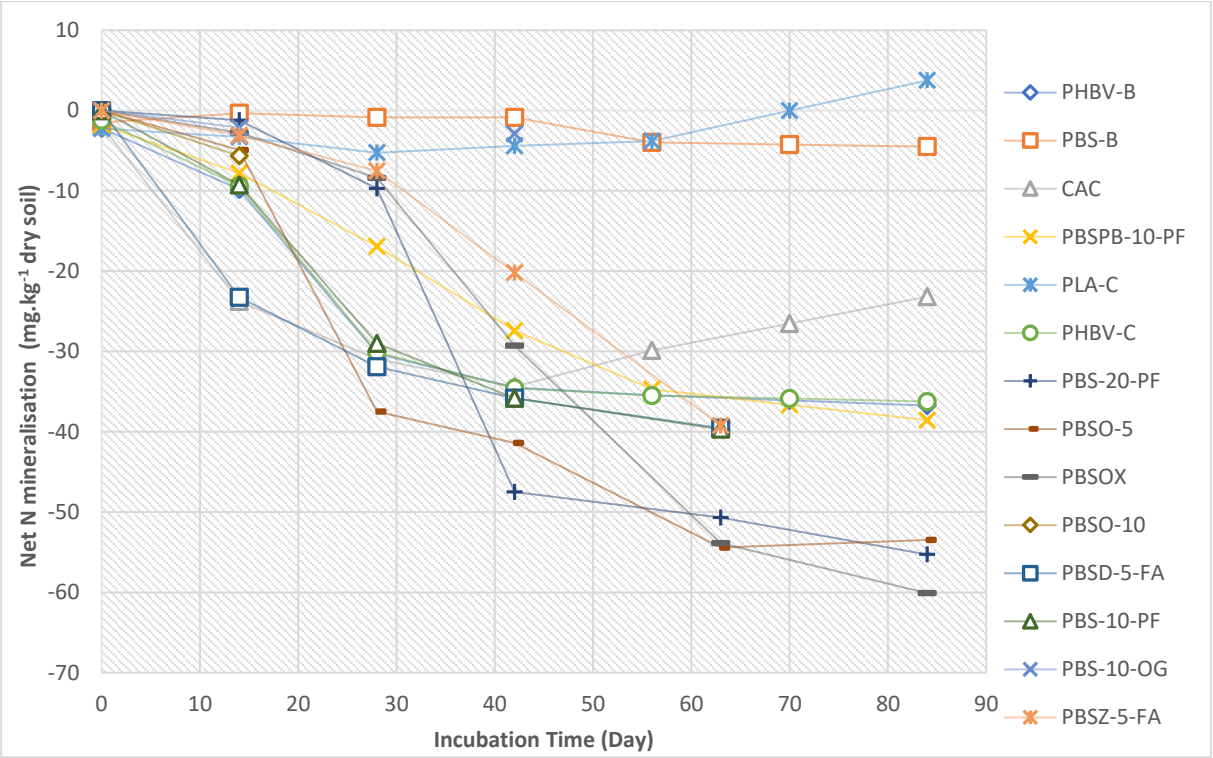

Figure 1: Net nitrogen mineralisation in soil with BPMs amendments (in mg mineral N. kg^{-1} dry soil).

BAPs

Application of BAPs resulted in both mineralisation and immobilisation of N in the examined samples, as given on Table 4. Mineralisation was the highest in Fert-S with over 55%, with a stable ascending trend throughout the incubation period. On the other hand, HFFMl revealed the highest immobilisation up to 73% among this group significantly higher than other samples ($p < 0.05$). Considering the starting patterns, FERT-L had a noticeable increase after the second week of incubation, pointing at a priming effect. While most samples were following an arch shape pattern, we observed alternating mineralisation-immobilisation in UL-1 and UL-2 samples throughout the incubation time. We noted a sudden change of mineralisation phases in HFFMu, and in FERT-L in week 6 and 8 respectively.

Table 4: Net cumulative N mineralised from BAPs material (in mg mineral N. kg^{-1} dry soil, and percentage) on day 84. Values are means ± standard errors, and different letters show statistical differences (p <0.05).

BAPs material	Net Nmin (mg.kg^{-1} dry soil)				Net N (%)			
CFFM	-48.4	±	1.8	cd	-43.6	±	1.7	d
HFFMu	7.0	±	0.7	bcd	39.5	±	3.7	a
HFFMl	-53.3	±	0.8	d	-73.3	±	1.1	e
PHYTBIOP	61.4	±	1.2	b	35.1	±	0.7	a
PHYTBIOF	61.4	±	1.6	b	27.1	±	0.7	ab
CREF	17.6	±	0.9	bc	4.9	±	0.3	bc
UL-1	-14.0	±	8.8	cd	-9.4	±	5.9	c
UL-2	-10.8	±	2.0	cd	-11.3	±	2.1	c
Fert-S	197.6	±	33.1	a	55.5	±	9.3	a
Fert-L	8.5	±	1.8	bcd	42.7	±	9.2	a

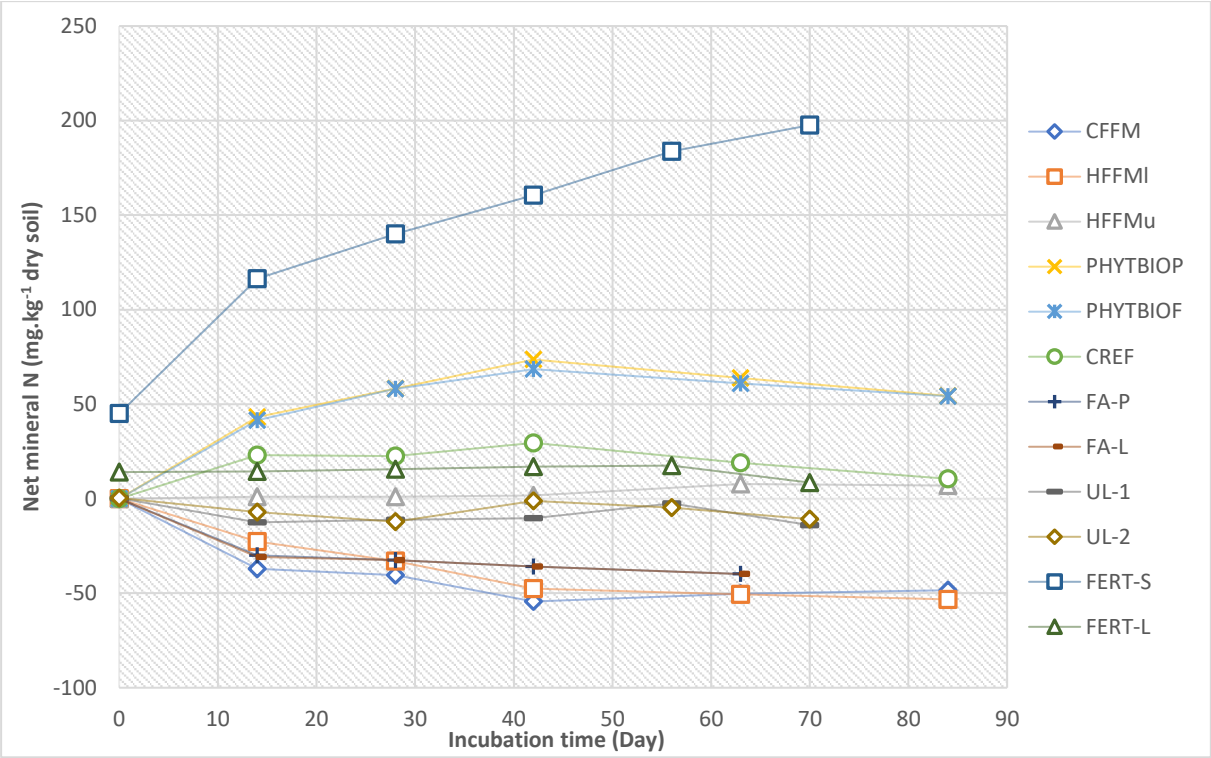

Figure 2: Net nitrogen mineralisation/immobilisation in soil with BAPs amendments expressed in mg.kg^{-1} dry soil.

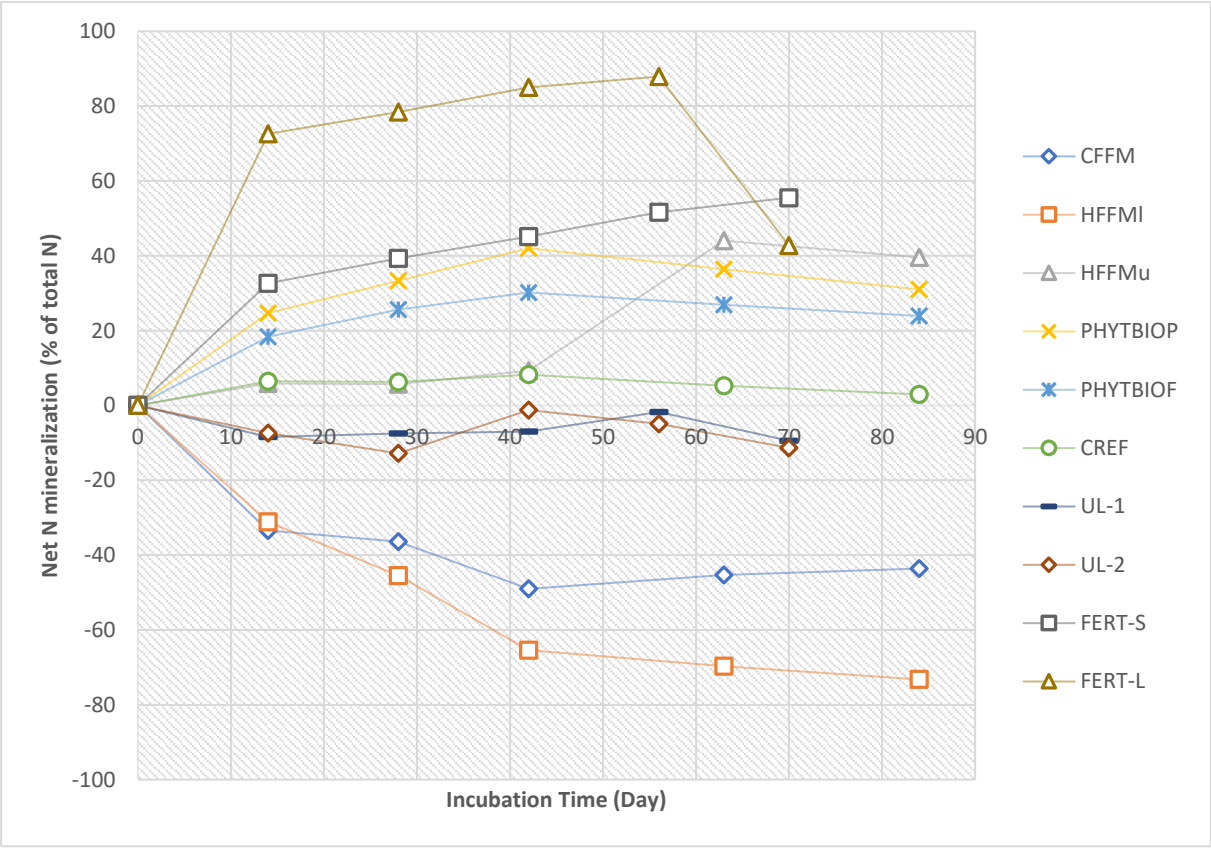

Figure 3: Net nitrogen released in soil by BAPs amendments expressed as a percentage of total nitrogen added to the soil by over 70 and 84 days of the incubation period.

2.2.3 Impact on easily available P

Assessments for extractable P were performed on both BPMs and BAPs samples, and the results were compared to related control and expressed as net values accordingly (Figure 4). However, samples from the first batch could not be examined due to insufficient materials. Results show lower values than controls for both groups throughout the experiments except for CFFM and HFFMl, with a slight increase in comparison. However, any significant increase compared to control were not observed in any of the examined samples.

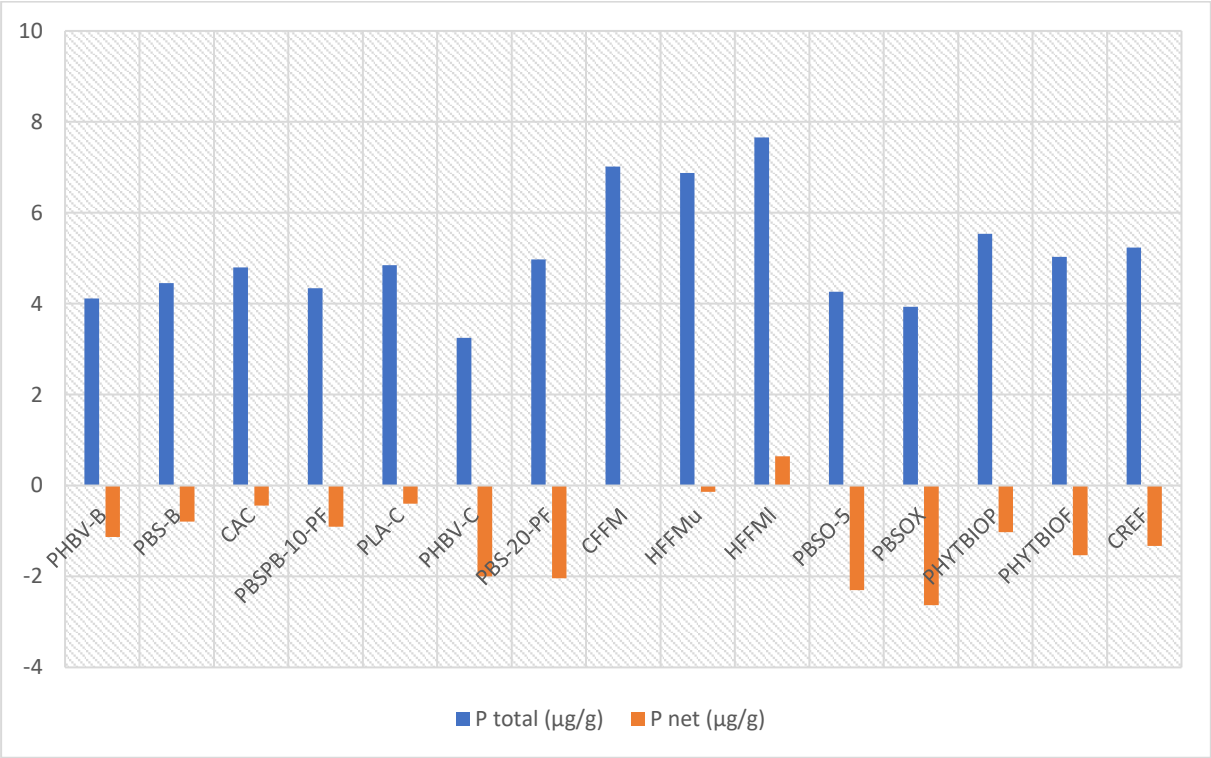

Figure 4: $CaCl_2$ extractable phosphorus (in µg $CaCl_2$-$P.g^{-1}$ dry soil) at the end of the 12-week incubation period according to the materials tested in BPMs and BAPs groups.

2.3 Stable organic C and C sequestration potential

2.3.1 Set-up of incubation experiments

C mineralisation was determined with the closed chamber respiration method, using glass jars with air-tight lids (Gebremikael et al., 2020; Sleutel et al., 2005). Soil-amendment mixtures were prepared in 3 replications and were filled in PVC tubes with a radius of 3.5 cm and 5 cm in height. The content of the tubes was compacted using a wooden cylinder (De Neve and hofman, 2000) to obtain a bulk density of 1.35 $Mg.m^{-3}$. A set of three soil samples with no amendment was also included as control. A glass vial containing 10 ml of 1 M NaOH was placed in each jar to trap emitted CO_2 during the incubation. The jars' lids were closed firmly and placed in a controlled environmentn incubator at 16°C. The amount of evolved CO_2 was measured at predetermined time intervals by titration of excess NaOH with 0.5 M HCl (Keeney, 2015) to pH 8.3 in the presence of $BaCl_2$ (Anderson, 1982). Following each

sampling event, the jars were left open for 2 hours to replenish the O_2 content, and the moisture content of samples was adjusted in addition. The jars were sealed right after refilling the glass vials filled with fresh NaOH and transferred to the incubation chamber to continue the C mineralisation process.

The following formula was used to obtain the amount of Carbon released in the form of carbon dioxide:

$$CO_2\_C = (V_{HCl,Blank} - V_{HCl,Sample}) \times [M_{HCl}] \times E$$

CO_2_C: carbon released during mineralisation (mg)
$V_{HCl,sample}$: volume of HCl used to titrate the NaOH solution in the sample (ml)
$V_{HCl,blank}$: average volume of HCl used to titrate the NaOH solution in the blanks (ml)
M_{HCl}: exact molarity of HCl (mmol.ml^{-1})
E: equivalent weight of carbon in the reaction.

The percentage of net cumulative C mineralised from the AFWCBs was calculated at each time with the following equation:

$$C_{\min net} = \frac{C_{amendment} - C_{control}}{Total\ added\ C} \times 100$$

$C_{min\ net}$: percentage of net cumulative CO_2_C mineralised from each material (%)
$C_{amendment}$: cumulative CO_2_C mineralised from each material (mg.100g^{-1} dry soil)
$C_{control}$: cumulative CO_2_C mineralised from the control (mg.100g^{-1} dry soil)
Total added C: total carbon contained in the materials applied (mg.100g^{-1} dry soil)

2.3.2 C mineralization, stable organic C and C sequestration

BPMs

C_{min} in all samples kept an ascending trend during the incubation up to week 12. However, in continued incubated batches, the mineralisation pattern in PLA-C and CAC reached a plateau phase after week 17. Mineralisation in the rest of the samples kept the ascending pattern in the continued incubation period (Figure 5). The amount of organic carbon (C_{org}) mineralisation in the BPMs amended samples, expressed as the percentage of net mineralised C (C_{min}) during 12 weeks of incubation, are given in Table 5. The mineralisation was the highest in PBSO-5 with 49% (of total C_{org} added), significantly higher than all other treatments ($p < 0.05$). The lowest mineralisation was in PLA-C, with 6.5%, significantly lower than the other samples.

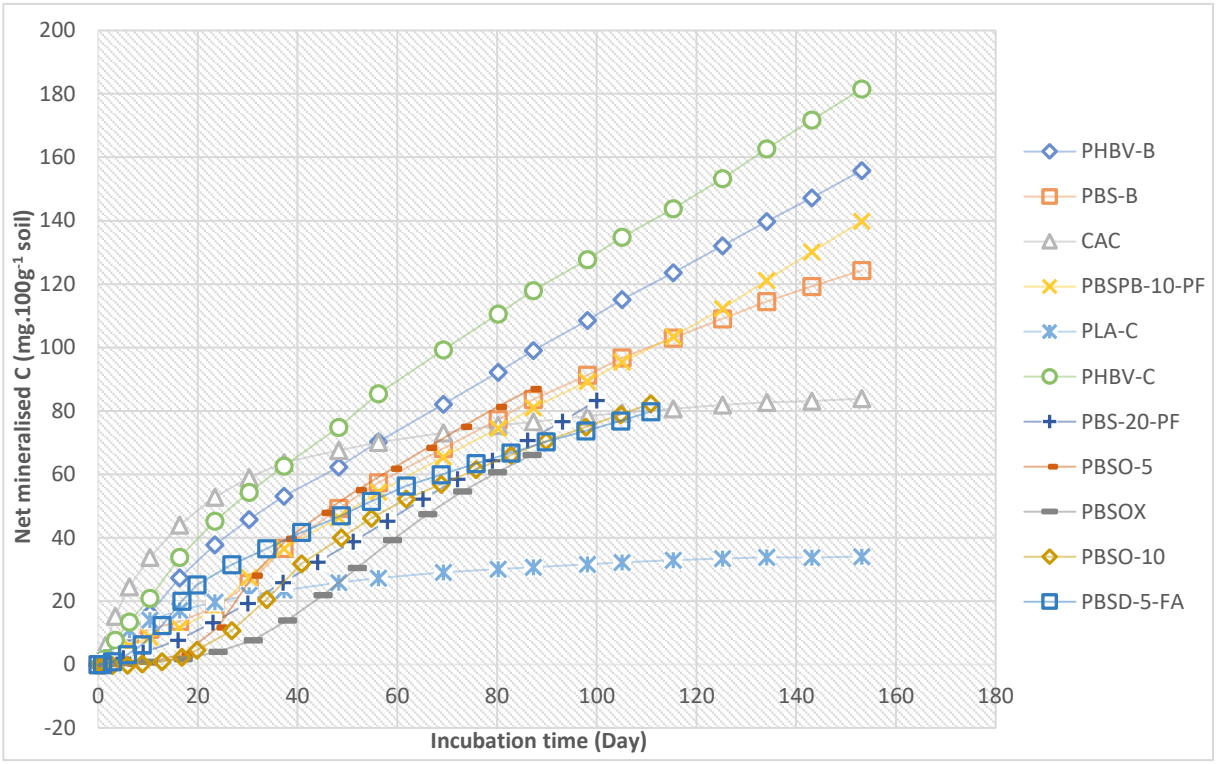

Figure 5: Evolution of the cumulative net mineralised C from the BPMs materials expressed as (mg.100 g dry soil-1) over the incubation period.

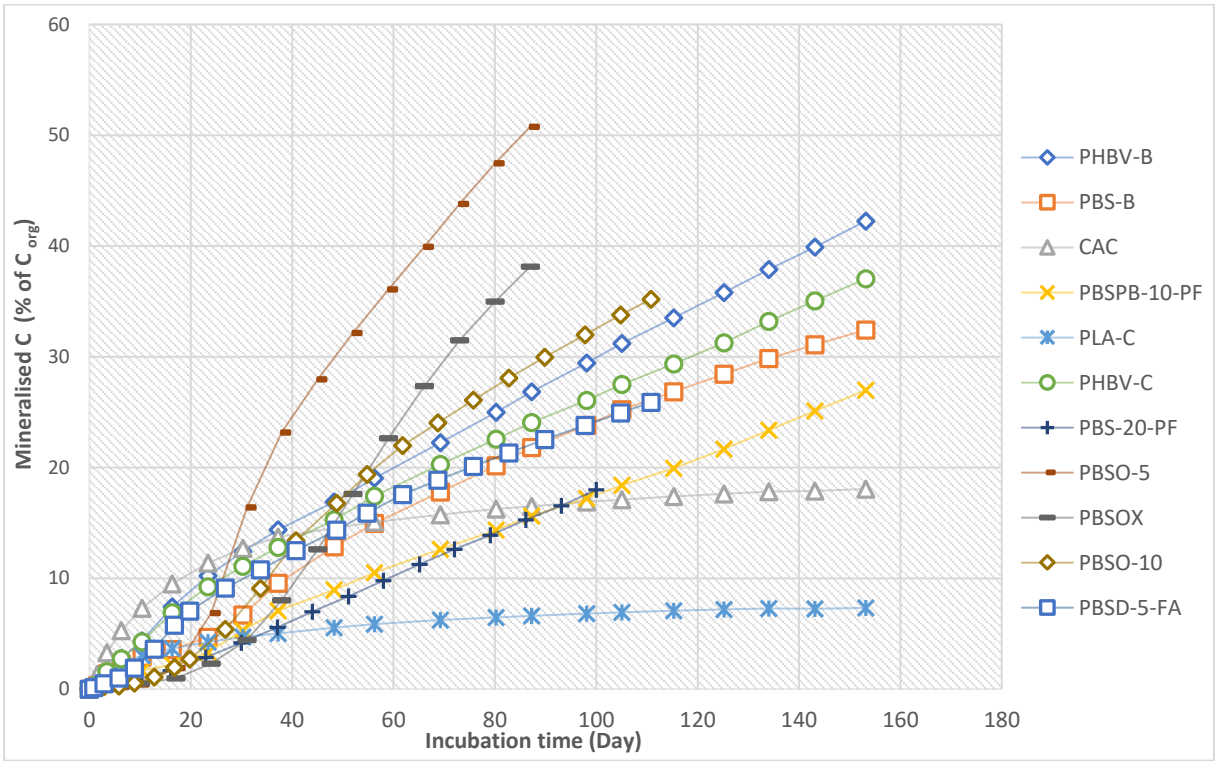

Figure 6: Evolution of the cumulative net mineralised C from the BPMs materials expressed as a percentage (%) of total Carbon added to the soil over the incubation period.

Table 5: Cumulative net mineralised C (expressed as a percentage of the total C added to the soil) of the applied BPMs materials after 12 weeks of incubation. Values represent means ± standard errors, and different letters show statistical differences (p <0.05).

Applied material	Net C_{min} (%)			
PBSO-5	49.3	±	0.4	a
PBSOX	36.8	±	4.5	b
PBSO-10	28.4	±	0.2	c
PHBV-B	26.0	±	0.3	c d
PHBV-C	23.4	±	0.1	c d e
PBSD-5-FA	21.5	±	0.8	cdef
PBS-B	21.0	±	0.2	d e f
CAC	16.4	±	0.1	e f
PBSPB-10-PF	15.0	±	0.6	f
PBS-20-PF	14.9	±	1.0	f
PLA-C	6.5	±	0.3	g

BAPs

The highest C mineralisation was observed in Fert-L with 56.2%, and the lowest in CREF, namely 3.4%, significantly different amongst each other (Figure 8). In general the tomato stalk based composts had low mineralization rates, which is normal given the fact that these are organic materials that have already gone through a stabilization process. However, this was in stark contrast to the UL composts which showed a C mineralization that was comparable to those of the non-compost materials. The fungal based pots also had high C mineralization, which was perhaps less expected, given that the fungal channel in soils is normally assumed to represent the more slowly cycling C and nutrient pools. For the fertilizer materials, the C mineralization obviously is less important, given that these materials are added to soil with the primary aim to add plant nutrients rather than adding stable organic matter.

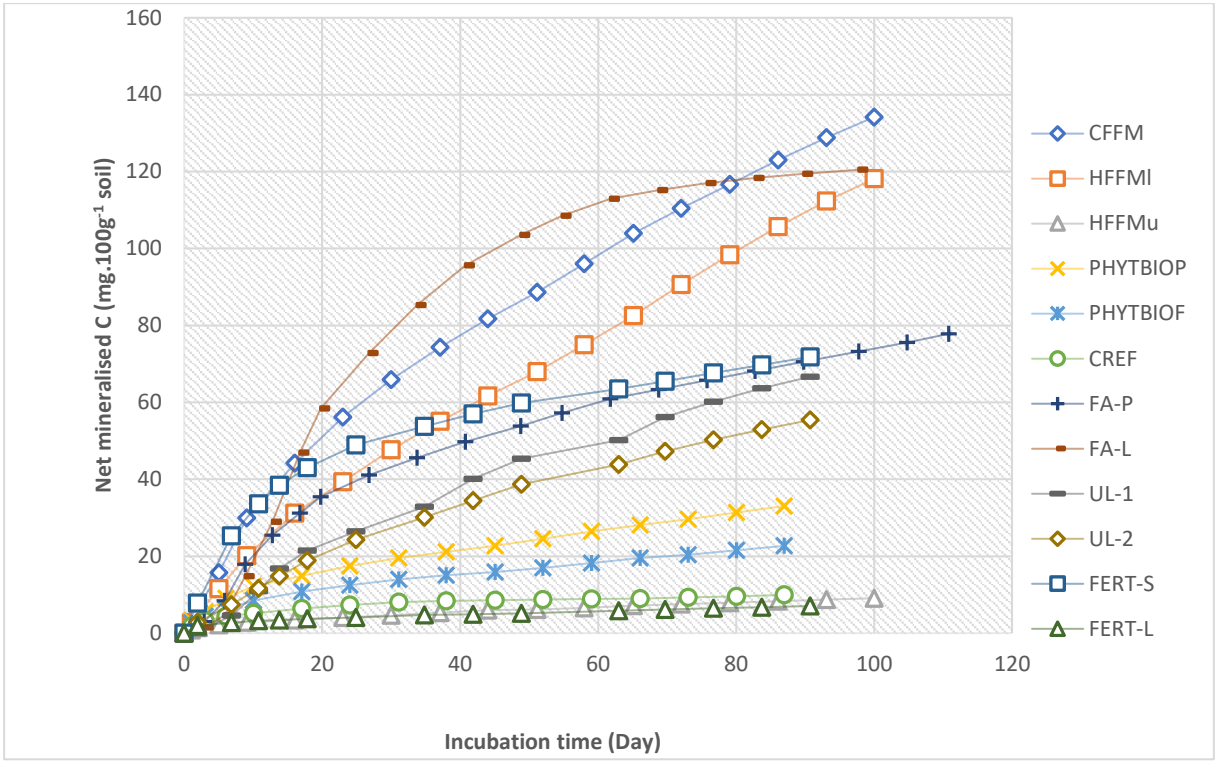

Figure 7: Evolution of the cumulative net mineralised C from the BAPs materials expressed as (mg.100 g soil^{-1}) over the incubation period.

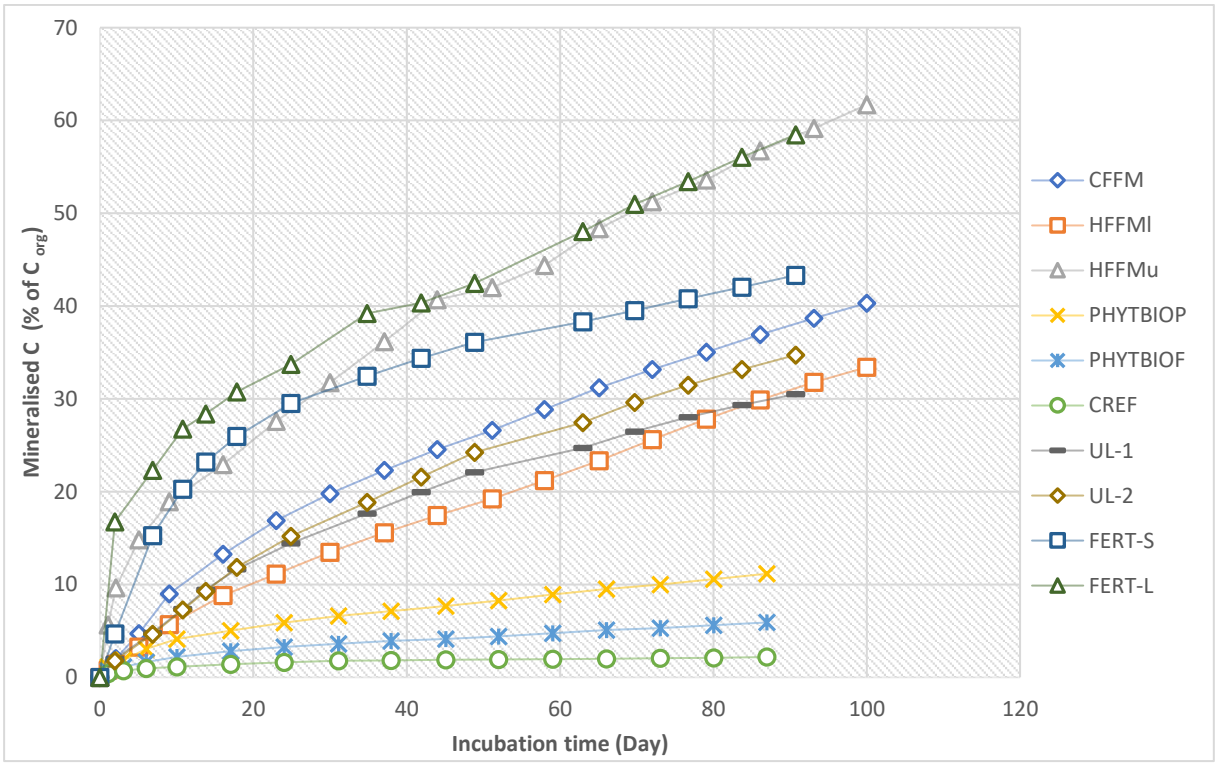

Figure 8: Evolution of the net C mineralised from the BAPs materials expressed as a percentage (%) of total Carbon added to the soil over an incubation period. % C mineralized could not be calculated for FA-L and FA-P due to the insufficient amount of material available.

Table 6: Cumulative net mineralised C (expressed as a percentage of the total C added to the soil by the substrates) of the applied BAPs materials after 12 weeks of incubation. Value s± standard errors, and different letters show statistical differences (p <0.05). ND; Not defined

Applied material	Net C_{min} (%)			
HFFMu	55.8	±	8.8	a
FERT-L	56.2	±	13.0	a
CFFM	36.4	±	0.2	ab
Fert-S	42.1	±	0.5	ab
UL-2	33.2	±	0.4	abc
HFFMl	29.3	±	1.3	bcd
UL-1	29.4	±	1.2	bcd
PHYTBIOP	10.9	±	2.1	cde
PHYTBIOF	5.8	±	0.3	de
CREF	3.4	±	1.0	e
FA-L		ND		
FA-P		ND		

The C mineralization data can then be extrapolated to a period corresponding to one year under field conditions, which is approximately equivalent to an incubation duration of 185 days (based on rescaling of temperatures to field conditions). The C mineralization extrapolated to this data can then be used to calculate the humification coefficient (hc), i.e. the amount of organic C derived from the materials that is still present in the soil one year after addition under field conditions. The humification coefficients (Table 7) are exceptionally low for some of the BAP (notably HFFMu and FERT-L) suggesting that these will not contribute to build-up of SOC. Also a number of BPM had a quite low humification coefficient (PBSO-5 and PBSOX), in the range of crop residues that are added to soil. Very high humification coefficients were found for PLA-C (very difficultly degradable), CAC and for the composts based on the tomato stalks, which will thus contribute to a very large extent to SOC build-up. It is these humification coefficients that are used in practice to advice farmers on the management of their SOC. In this way, these materials can be also incorporated in such management systems of SOC, and be directly compared to agricultural inputs (crop residues, organic manures, …) that are immediately recognizable to farmers.

Table 7: Humification coefficients (hc) determined by extrapolation of C mineralization data (to equivalent to one year under field conditions). ND: Not defined.

Applied BPM	hc	Applied BAP	hc
PBSO-5	0.18	HFFMu	0.04
PBSOX	0.26	FERT-L	0.08
PBSO-10	0.52	CFFM	0.47
PHBV-B	0.52	Fert-S	0.44
PHBV-C	0.58	UL-2	0.49
PBSD-5-FA	0.63	HFFMl	0.53
PBS-B	0.62	UL-1	0.60
CAC	0.81	PHYTBIOP	0.83
PBSPB-10-PF	0.68	PHYTBIOF	0.92
PBS-20-PF	0.67	CREF	0.96
PLA-C	0.92	FA-L	ND
		FA-P	ND

2.4 Impact on soil biological quality

2.4.1 Soil microbial biomass carbon

2.4.1.1 Methodology

Soil microbial biomass C was determined using the fumigation-extraction procedure Joergensen, 1996). Two series of fresh soil (30 g each) were prepared from each sample, namely an unfumigated control and a fumigated sample. Fumigation was done with ethanol-free chloroform in a dessicator for a period of 24h. After the fumigation, bot fumigated and unfumigated treatments were placed in 250 ml plastic beakers, and 60 ml of K_2SO_4 (0.5M) was added subsequently. The solutions were shaken on a reciprocating shaker for an hour and extracted through Whatman nr. 42 filter paper. Extracts were stored at -18 °C until analysis.

The organic carbon content of the extracts was determined with a TOC analyser (TOC-VCPN, Shimadzu Corp., Kyoto, Japan). For conversion from organic C contents in the extracts to MBC in the soil, a k_{EC} value of 0.45 was assumed.

$$C_{Biomass} = \frac{C_F - C_{NF}}{K_{EC}}$$

$C_{Biomass}$ (µg . g $^{-1}$ dry soil)
C_F: mass of total carbon extracted from fumigated samples per gram of dry soil (µg . g $^{-1}$ dry soil)
C_{NF}: mass of total carbon extracted from non-fumigated samples per gram of dry soil (µg . g $^{-1}$ dry soil)
K_{EC}: coefficient of efficiency of microbial biomass carbon extraction

The metabolic coefficient (qCO_2) which expresses the amount of CO_2_C produced per unit of biomass and time was then calculated to serve as an indicator of the physiological status of soil microorganisms (Anderson & Domsch, 1990).

Biochemical and microbial examinations were performed on different control samples for each batch, resulting in fluctuations of control samples' reference values. Therefore, the control values were subtracted to eliminate related errors in data evaluations, and the results for samples were expressed as net values. Accordingly, zero was considered as the reference level in statistical assessments, representing the control values.

2.4.1.2 Results

BPM application led to an increase in microbial biomass C in all samples compared to the control, except where the MBC was lower than in the corresponding control soil (Figure 9). MBC was the highest in PBSOX with +170 (µg C.g^{-1} dry soil) [1], and the lowest in PBSZO-10 with -30 compared to the control. Only slight differences were observed between the examined samples in our statistical evaluation.

In the BAPs group, results were in a wide range between +614 and -127 compared to control, with FA-P as the highest and FA-L as the lowest, respectively. In our statistical evaluations, only FA-P differed

[1] which will further be refered to all MBC activity results

from the control and the other samples significantly, while in other samples there were no significant differences compared to control ($p > 0.05$).

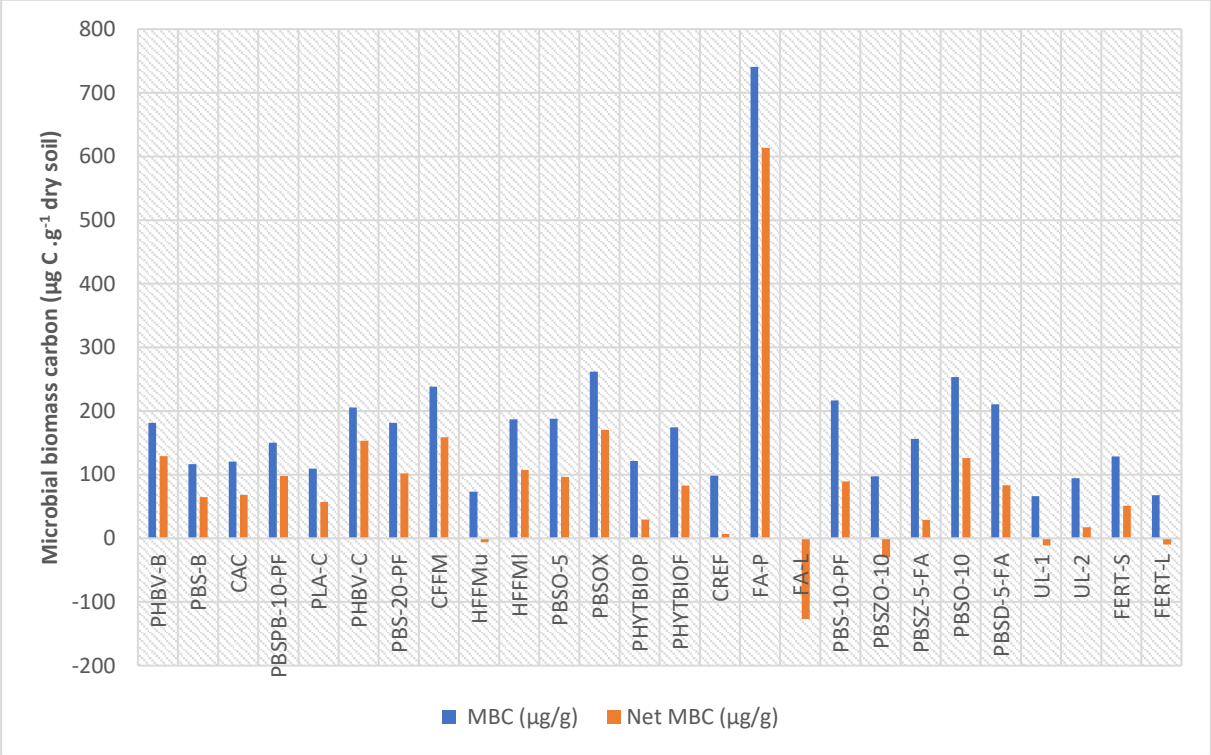

Figure 9: Microbial biomass in the different treatments, and net microbial biomass (i.e. with the respective controls subtracted).

Surprisingly, there was no strong relationship between the C mineralization rate and the MBC, despite the fact that comparable amounts of organic C were added with most of these materials, and hence C mineralization rates can be directly intercompared.

2.4.2 Enzyme activities and microbial quotient

2.4.2.1 Methodology

β-glucosidase

β-Glucosidase is involved in the last step (hydrolysis of cellobiose to glucose) in the enzymatic degradation of cellulose, the main component of plant polysaccharides in soils and is frequently used as an indicator of soil microbial activity and soil quality (Moeskops et al., 2010; Turner et al., 2002).

The activity of β-Glucosidase was assayed by the method of Eivazi and Tabatabai (1988), using the substrate analogue para-nitrophenyl-β-D-glucopyranoside (PNPG). 1.0 g of fresh soil was weighed into push-cap glass test tubes (three replicate samples per soil) and incubated for 1 h at 37 °C with 4 ml of 0.05 M modified universal buffer (pH 6.0) and 1 ml of 25 mM pNPG dissolved in buffer. After incubation, 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.2 M Tris–hydroxymethyl (aminomethane) buffer pH 12 (adjusted with NaOH) were added. For blanks, 1 ml of para-nitrophenol (pNP) solution was added immediately. Suspensions were filtrated directly through Watman (no.5) filter paper. Extracts were diluted 10 times using Tris buffer pH 10 to be assured concentrations of p-nitrophenol (pNP) fit within

the range of the standard series. The amount of released para-nitrophenol (pNP) in the soil was measured by colour intensity of extracted samples at 400 nm with a Hitachi 150-20 spectrophotometer (Hitachi Ltd., Tokyo, Japan). β -glucosidase activity is expressed as µmol PNP released g^{-1} dry soil h^{-1}.

β-glucosaminidase

Soil β-glucosaminidase activity was assayed according to the method provided by Parham & Deng (2000). Measurements for each treatment were performed in 3 replications by placing 1.0 g of soil into push-cap glass test vials and then adding 4 ml of 0.1 M acetate buffer solution (pH 5.5) and 1.0 ml of 10 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide solution (PNNAG). One blank sample was assigned per treatment, only containing the acetate buffer solution. Containers were capped, mixed thoroughly and placed in the incubator at 37 °C. After one hour of incubation, 1.0 ml of 0.5M $CaCl_2$ and 4 ml of 0.5M Tris buffer solution (pH 12) were added to stop the reaction. For blank samples, PNNAG substrate was added after the incubation. The sample vials were swirled for a few seconds and directly filtered through Whatman no.5 filter papers. The colour intensity of the extracts was measured at 405 nm with a spectrophotometer. The p-nitrophenol contents of the filtrates were then calculated by comparing the results to a standard curve for p-nitrophenol.

Dehydrogenase

The dehydrogenase enzyme activity followed the procedure as modified by Moeskops et al. (2010). Assessments were performed in duplicate for each treatment by placing 5.0 g of fresh soil into push-cap glass test vials and then adding 2 ml of 3 % Triphenyltetrazolium chloride (TTC) and 2 ml of 0.1 M Tris-hydroxymethyl(aminomethane) pH 7.8 (Tris buffer). The blank samples were prepared by adding only 4 ml of Tris buffer. Containers were sealed and incubated in the dark at 37 °C. After 24 hours, 20 ml methanol was added to each vial and were shaken for 2 hours with a linear shaker at 125 rpm. The suspension was filtered through Whatman no. 5 filter papers directly into 50 ml volumetric flasks. The remaining soil in the vials was washed twice with 4 ml methanol to collect all produced Triphenylformazan (TPF). All flasks were adjusted to 50 ml with methanol. Measurements were performed by colourimetry at 485 nm with a Hitachi 150-20 spectrophotometer, and results are expressed in µg TPF g^{-1} dry soil 24 h^{-1}.

After measuring the enzymatic activities, ratios of these activities by units of MBC were also calculated to represent the links between enzymes and soil microorganisms.

2.4.2.2 Results

β-glucosidase activity

BG activity in samples was between -5 and +31(*µg PNP.g^{-1} dry soil.h^{-1}*)[2], with a higher activity rates in the BAPs group than BPMs (Figure 10). In our study, some samples activity was lower than control samples, resulting in negative values of net BG. Accordingly, in the BPMs group, the highest was PHBV, and the lowest was PBSOX with +12 and -5, respectively. In BAPs samples CFFM had the highest BG activity and the lowest was CREF, respectively equal to +31 and -2.0.

[2] which will further be refered to all BG activity results

Only slight differences were observed in statistical assessments among samples in both BPMs and BAPs.

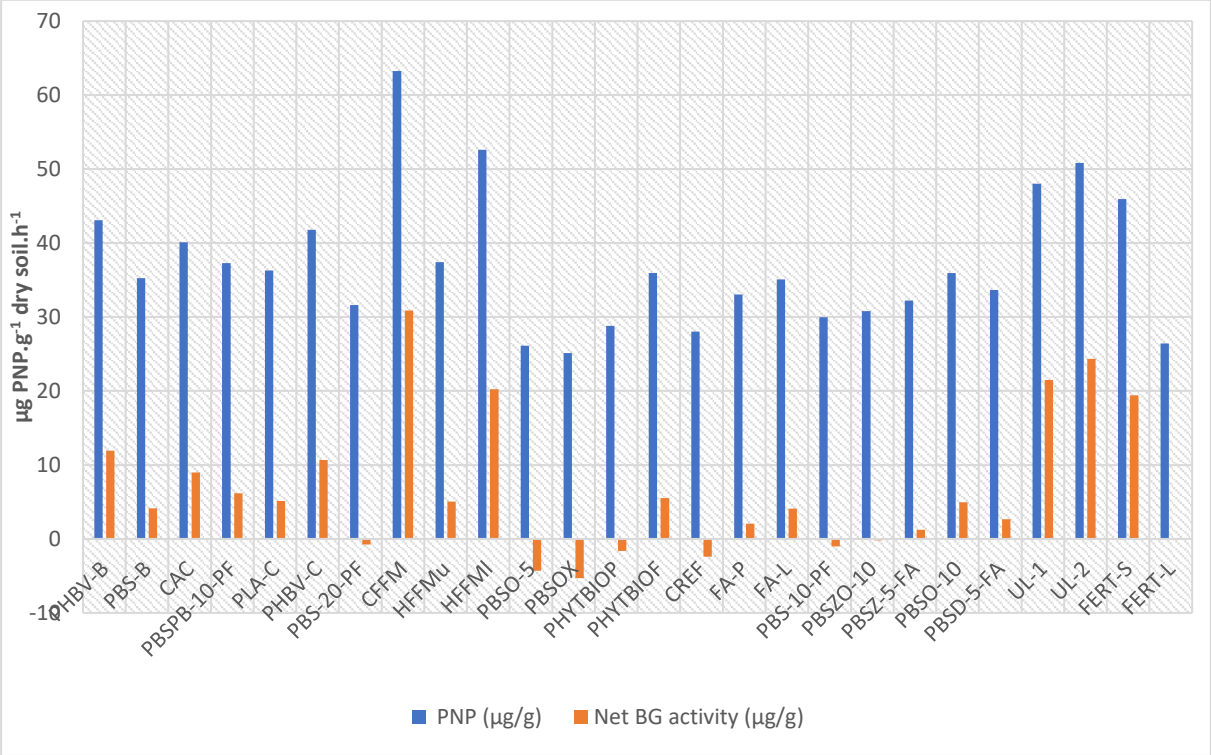

Figure 10: β-glucosidase activity in BPMs and BAPs, expressed in (µg PNP.g^{-1} dry soil.h^{-1}) for both total and net values (control values were subtracted). Values are mean).

N-acetyl-β-D-glucosaminidase activity

BGA activity for the samples of the first batch could not be performed due to insufficiently tested materials.

In BPMs samples, all tested materials showed higher BGA activity than the control samples (Figure 11). The BGA net activity was significantly the highest in PBSO-5 with +19 *(µg PNP*.g^{-1} dry soil.h^{-1}*)* [3] compared to other samples ($p < 0.05$). The lowest was PLA-C, with +2.5 *(µg PNP)* and only significantly different than BPSO-5 and BPSOX samples ($p < 0.05$). Considering the BAPs group, CFFM had the highest value of +51, significantly higher than other samples except for HFFMI and UL-1. The lowest activity was in FERT-L, only significantly different with CFFM and UL-1. Additionally, FERT-L was the only sample with less activity than control and negative BGA net value equal to -1.0.

[3] which will further be refered to all BGA activity results

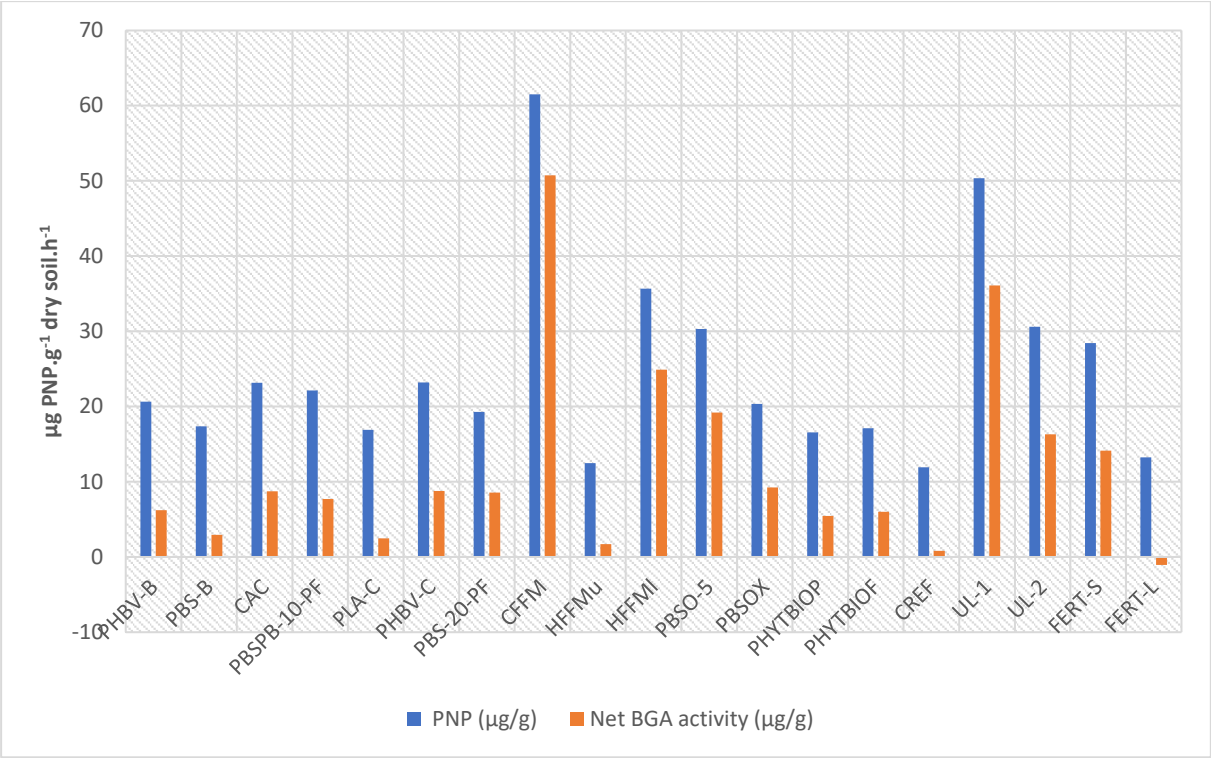

Figure 11: N-acetyl-β-D-glucosaminidase activity in BPMs and BAPs, expressed in µg PNP.g^{-1} dry soil.h^{-1} for both total and net values (control values were subtracted). Values are mean.

A) Dehydrogenase activity

Results showed significant differences in DHG activity in BPMs and BAPs within the range of -2 to +102 (*µg TPF.g^{-1} dry soil.24h^{-1})*, respectively as the lowest and highest, compared to the control sample (Figure 12).

Accordingly, in the group of BPMs, the highest activity was in PBSO-10, namely +102 , significantly higher than all other samples except for PHBV-C. The lowest activity was in PBSZO-10, significantly lower than PBSOX, PBSO-10 and PHBV-C but with no differences with the other samples. Despite the different DHG activity in the rest of the samples, only minimal statistical differences were observed compared to the reference point.

In the BAPs group, DHG activity was the highest in FA-P with +93, significantly higher than other samples ($p < 0.05$). The lowest net activity was in Fert-L equal to -2.0, lower than control and other samples in this group, yet with no significant differences compared to control or other samples except to FA-P and CFFM ($p < 0.05$).

Metabolic quotients for both BPMs and BAPs are provided in Table 9. In BPMs the highest metabolic quotient (qCO_2) was in PBS-B, up to +6 (µg CO2-C.mg^{-1} MBC.h^{-1}), significantly higher than other samples and the control ($p < 0.05$). The lowest was in PLA-C, but not significantly different from the control. In BAPs group the highest was UL-1 with up to +55 and the lowest UL-2 with -1.0 compared to the control. However, no significant differences were observed between samples and controls.

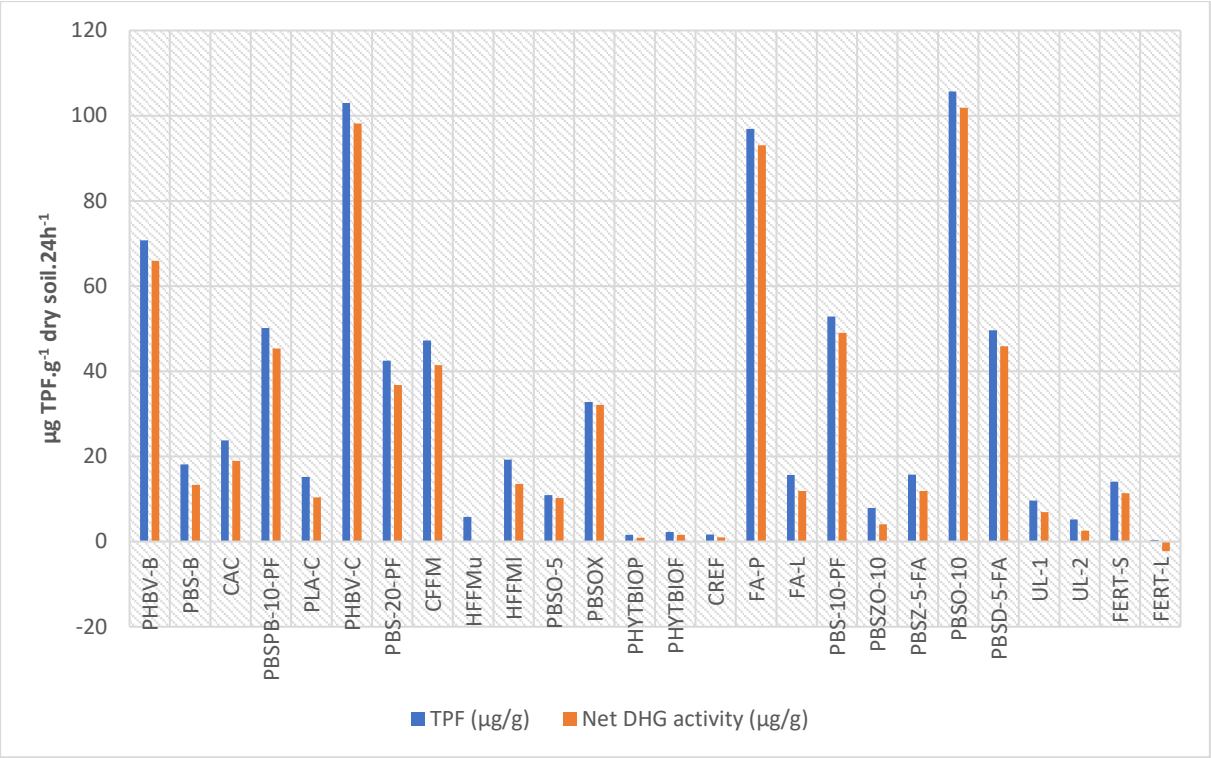

Figure 12: Dehydrogenase activity in BPMs and BAPs, expressed in (µg TPF.g^{-1} dry soil.24h^{-1}) for both total and net values (control values were subtracted). Values are mean.

Considering the BG:MBC, the highest was CAC with +0.13 (µg PNP. µg^{-1} MBC. h^{-1}) in BPMs group, significantly higher than control but on par with other samples. BPSO-5 was the lowest but without any significant differences to control. Considering the BGA:MBC ratio, samples in the first batch excluded (BGA were not performed), and among the eligible series, the BPSO-5 was the highest and PBS-B and PLA-C were both the lowest in BPMs group, with significant differences only with BPSO-5. Considering the DHG:MBC ratio, HFFMu, with the ratio of +8 (µg TPF. µg^{-1} MBC. 24h^{-1}) was significantly higher than the other samples and the control ($p < 0.05$). For the rest there were no significant increases in the DHG:MBC ratio in other samples of both groups compared to control.

Table 8: Net Enzymatic activities at the end of the 12-week incubation period (control values were subtracted). Values are means ± standard errors, and different letters show statistical differences (p <0.05). Statistical analysis for BPMs and BAPs groups was performed separately compared to reference equal to zero as control. (ND): not defined.

	Treatments	DHG (µg TPF.g^{-1} dry soil.24h^{-1})				BG (µg PNP.g^{-1} dry soil.h^{-1})				BGA (µg PNP.g^{-1} dry soil.h^{-1})				MBC (µg C.g^{-1} dry soil)			
BPMs	PHBV-B	65.9	±	5.0	b	11.9	±	2.2	a	6.2	±	0.4	bcd	129.1	±	4.0	abc
	PBS-B	13.3	±	2.3	def	4.1	±	0.6	bcde	3.0	±	0.4	bcd	64.4	±	3.0	de
	CAC	18.9	±	0.3	def	9.0	±	1.7	abc	8.7	±	1.0	bc	68.4	±	2.3	de
	PBSPB-10-PF	45.3	±	3.1	bc	6.2	±	1.1	abcd	7.7	±	0.6	bc	97.9	±	0.4	cd
	PLA-C	10.4	±	1.1	ef	5.1	±	0.9	abcde	2.5	±	0.7	cd	57.3	±	8.8	de
	PHBV-C	98.2	±	2.0	a	10.7	±	2.0	ab	8.8	±	0.6	bc	153.4	±	10.4	ab
	PBS-20-PF	36.7	±	1.6	cd	-0.8	±	2.9	defg	8.6	±	1.2	bc	101.9	±	4.3	bcd
	PBSO-5	3.8	±	0.6	f	-4.3	±	0.3	fg	19.2	±	3.4	a	96.2	±	6.8	cd
	PBSOX	32.3	±	4.8	cde	-5.3	±	0.5	g	9.2	±	1.5	b	170.2	±	20.5	a
	PBS-10-PF	49.0	±	15.4	bc	-1.0	±	0.4	efg	ND				89.3	±	15.1	cd
	PBSZO-10	4.1	±	1.2	f	-0.2	±	1.8	defg	ND				-29.9	±	5.2	g
	PBSZ-5-FA	11.9	±	1.6	def	1.2	±	0.5	defg	ND				29.2	±	12.2	ef
	PBSO-10	101.9	±	6.8	a	5.0	±	0.2	bcde	ND				126.3	±	3.4	abc
	PBSD-5-FA	45.8	±	3.5	bc	2.7	±	0.6	cdef	ND				83.2	±	21.2	cd
	Referance (control)		0.0		f		0.0		defg		0.0		d		0.0		fg
BAPs	CFFM	41.5	±	4.5	b	30.9	±	4.8	a	50.8	±	18.3	a	158.9	±	33.6	b
	HFFMu	-1.9	±	2.4	c	5.0	±	5.4	bcde	1.7	±	0.4	c	-6.3	±	6.0	bc
	HFFMl	13.5	±	4.1	c	20.2	±	5.5	abcd	24.9	±	3.3	abc	107.7	±	8.0	b
	PHYTBIOP	0.6	±	0.8	c	-1.6	±	2.0	de	5.4	±	0.9	c	29.7	±	8.0	bc
	PHYTBIOF	1.8	±	0.7	c	5.5	±	1.6	bcde	6.0	±	1.4	c	82.6	±	9.6	b
	CREF	1.2	±	1.2	c	-2.4	±	1.6	e	0.8	±	0.4	c	6.6	±	7.9	bc
	FA-P	93.1	±	10.5	a	2.1	±	0.4	bcde	ND				613.5	±	114.6	a
	FA-L	11.9	±	3.4	c	4.1	±	2.6	bcde	ND				-127.2	±	0.0	c
	UL-1	6.9	±	2.7	c	21.5	±	8.6	abc	36.1	±	3.7	ab	-11.4	±	6.9	bc
	UL-2	2.5	±	1.9	c	24.3	±	7.9	ab	16.3	±	0.9	bc	17.2	±	14.4	bc
	FERT-S	11.4	±	1.0	c	19.4	±	2.8	abcde	14.1	±	1.7	bc	51.2	±	18.2	b
	FERT-L	-2.3	±	0.4	c	-0.1	±	3.0	cde	-1.1	±	0.3	c	-9.8	±	7.6	bc
	Referance (control)		0.0		c		0.0		cde		0.0		c		0.0		bc

Table 9: Metabolic quotient (qCO2), dehydrogenase / microbial biomass carbon (DHG:MBC), β-glucosidase / microbial biomass carbon (BG:MBC) and N-acetyl-β-D-glucosaminidase / microbial biomass carbon (BGA:MBC) ratios for the different materials tested at the end of the incubation period. Values are means ± standard errors, and different letters show statistical differences (p <0.05). Reference values represent the control. ND; not defined.

	Tested materials	qCO2 (µgCO2-C.mg^{-1} MBC. h^{-1})				DHG:MBC (µg TPF. µg^{-1} MBC. 24h^{-1})				BG:MBC (µg PNP. µg^{-1} MBC. h^{-1})				BGA:MBC (µg PNP. µg^{-1} MBC. h^{-1})			
BPMs	PHBV-B	3.12	±	0.11	b	0.51	±	0.04	ab	0.09	±	0.02	ab	0.05	±	0.00	cd
	PBS-B	5.84	±	0.30	a	0.21	±	0.03	ab	0.07	±	0.01	abc	0.04	±	0.01	cd
	CAC	0.99	±	0.05	cd	0.28	±	0.01	ab	0.13	±	0.03	a	0.13	±	0.01	ab
	PBSPB-10-PF	3.95	±	0.12	ab	0.46	±	0.03	ab	0.06	±	0.01	abc	0.08	±	0.01	bc
	PLA-C	0.69	±	0.22	d	0.19	±	0.02	ab	0.09	±	0.00	ab	0.04	±	0.01	cd
	PHBV-C	2.89	±	0.20	bc	0.64	±	0.03	ab	0.07	±	0.01	abc	0.06	±	0.01	bcd
	PBS-20-PF	3.74	±	0.15	b	0.36	±	0.02	ab	-0.01	±	0.03	bc	0.08	±	0.01	bc
	PBSO-5	3.55	±	0.11	b	0.04	±	0.00	ab	-0.04	±	0.00	c	0.20	±	0.04	a
	PBSOX	2.00	±	0.21	bcd	0.21	±	0.06	ab	-0.03	±	0.01	bc	0.06	±	0.02	bcd
	PBS-10-PF		ND			0.61	±	0.23	ab	-0.02	±	0.01	bc		ND		
	PBSZO-10		ND			-0.16	±	0.08	b	0.02	±	0.08	abc		ND		
	PBSZ-5-FA		ND			0.85	±	0.57	a	0.06	±	0.02	abc		ND		
	PBSO-10	2.07	±	0.13	bcd	0.81	±	0.07	a	0.04	±	0.00	abc		ND		
	PBSD-5-FA	3.13	±	1.31	b	0.63	±	0.16	ab	0.05	±	0.03	abc		ND		
	Reference (control)	0			d	0			ab	0			bc	0			d
BAPs	CFFM	2.48	±	0.31	a	0.28	±	0.06	ab	0.21	±	0.05	a	0.30	±	0.05	a
	HFFMu	27.6	±	53.83	a	7.90	±	4.68	a	-14.95	±	7.71	b	2.79	±	4.38	a
	HFFMl	4.11	±	0.56	a	0.13	±	0.05	b	0.18	±	0.04	a	0.23	±	0.03	a
	PHYTBIOP	5.72	±	4.80	a	0.01	±	0.02	b	-0.02	±	0.09	a	0.21	±	0.05	a
	PHYTBIOF	0.84	±	0.12	a	0.02	±	0.01	b	0.07	±	0.01	a	0.07	±	0.01	a
	CREF	0.03	±	1.57	a	0.10	±	0.05	b	0.03	±	0.23	a	0.06	±	0.03	a
	FA-P	0.24	±	0.03	a	0.16	±	0.01	b	0.00	±	0.00	a		ND		
	FA-L	-0.52	±	0.06	a	-0.09	±	0.03	b	-0.03	±	0.02	a		ND		
	UL-1	54.68	±	64.72	a	2.23	±	2.59	ab	2.38	±	4.39	a	15.52	±	17.60	a
	UL-2	-0.95	±	5.79	a	-0.05	±	0.14	b	0.16	±	0.85	a	-0.20	±	0.70	a
	FERT-S	3.69	±	1.94	a	0.31	±	0.13	ab	0.52		0.24	a	0.39	±	0.18	a
	FERT-L	1.49	±	2.41	a	-0.12	±	0.24	b	-0.35		0.32	a	-0.09	±	0.14	a
	Reference (control)	0			a	0			b	0			a	0			a

2.4.3 Microbial community composition

2.4.3.1 Methodology

Fatty acid composition of the microbial membrane phospholipids was used to assess (changes in) microbial community structure, following the procedure provided by Balser (2001) as modified by Moeskops et al. (2010). Briefly, 4 g freeze-dried soil was weighed in glass tubes followed by adding 3.6 ml of 0.1 M phosphate buffer pH 7.0, 4 ml chloroform and 8 ml methanol. Sample containers were shaken for 1 hour and subsequently centrifuged at 2500 rpm for 10 min. Containings were transferred into new glass tubes, and 8 ml phosphate buffer and 8 ml chloroform were added afterwards. Suspensions were left overnight for phase separation, and the lipid fraction was transferred to new tubes. The lipid fraction mixture was dried under N_2 in a nitrogen bath. Fractionation of lipids was conducted by solid-phase extraction using silica columns (Chromabond, Macherey-Nagel GmbH, Düren, Germany) resulting in the separation of phospholipids from the neutral and glycolipids. The neutral lipid and glycolipid fractions were removed by chloroform and acetone, respectively, and the phospholipids were eluted using 5 ml of methanol. The collected methanol extract was dried under N_2. 1.0 ml methanol:toluene (1:1 v:v) solution were added to dissolve the dried phospholipids, followed by adding 1.0 ml 0.2 M methanolic KOH. Afterwards, the samples were incubated at 35 °C for 15 min, resulting in transesterification of the PLFAs to fatty acid methyl esters (FAMEs). After samples had reached room temperature, 2 ml hexane:chloroform (4:1 vol:vol), 1 ml 1 M acetic acid and 2 ml water were added. Then containers were mixed well and centrifuged at 2000 rpm for 5 min. The top layer containing hexane-methylated PLFAs were quantitatively transferred to pointed glasses and rinsed with hexane:chloroform solution to transfer residuals. Collected hexane fractions were dried with N_2. Samples were injected on a Varian capillary column CP Sil 88 (100 m × 0.25 mm i.d., 0.2 µm film thickness; Varian Inc., Palo Alto, USA) and determined by GC–MS on a Thermo Focus GC combined with a Thermo DSQ quadrupole MS (Thermo Fisher Scientific Inc., Waltham, USA) in electron ionisation mode using decanoate fatty acid (C19:0). (Moeskops et al., 2010).

The concentrations of the different PLFAs observed were then used to obtain the concentration of several biomarkers in order to characterise the microbial communities in terms of functional groups. For Gram+ bacteria, the sum of the fatty acids iC15:0, aC15:0, iC16:0, iC17:0, aC17:0 was considered, while the fatty acids C16:1ω7t, C16:1ωSc, C18:1ω7, cy17:0, cy19:0 were used to represent Gram- bacteria (Kozdrój & Elsas, 2001). The Gram+:Gram- ratio was calculated by dividing the sum of the respective biomarkers. The fatty acid C18:2ω6 was used as the signature fatty acid for the saprotrophic fungi community (Kaiser et al., 2010) and the fatty acid C16:1ω5c to represent arbuscular mycorrhizal fungi (AMF). The total bacterial community was described by the sum of Gram+, Gram- and C15:0 and C17:0 fatty acids and the ratio bacteria:fungi was calculated as the total bacterial community divided by the saprotrophic fungi marker (Frostegård & Bååth, 1996). Monounsaturated fatty acids were 35 represented by the sum of C16:1ω7c, C16:1ω5c, C18:1ω9c and C18:ω7c while saturated fatty acids were expressed as the sum of C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0 and C20:0 (Bossio & Scow, 1998). The ratio of monounsaturated to saturated PLFAs was calculated by dividing the respective sums of the corresponding biomarkers. Finally, the ratio of cyclopropyl to precursor (cy:pre) was calculated by dividing the sum of the fatty acids cy17:0 and cy19:0 by the sum of the fatty acids C16:1ω7 and C18:1ω7 (Bossio & Scow, 1998).

2.4.3.2 Results

The total concentration of PLFAs was significantly higher in soil samples containing CFFM and HFFMl compared to control samples (Table 10). No other materials tested produced significant changes in the total PLFAs concentration whereas the MBC analysis showed that the majority of the materials tested increased the soil microbial biomass. Nevertheless, the total concentration of PLFAs was significantly correlated with

Table 10: Concentrations in soils of total PLFAs and selected biomarkers after 12 weeks incubation for the different materials tested (nmol.g^{-1} dry soil). Values are means ± standard errors and different letters show statistical differences (p <0.05).

	Tested materials	Gram$^+$				Gram$^-$				Actinomycetes				Saprotrophic fungi				AMF				Total PLFA			
Concentration	PBS-20-PF	5.3	±	0.2	ac	11.8	±	0.3	d	2.0	±	0.0	ab	0.6	±	0.6	a	0.9	±	0.1	a	36.0	±	0.6	ad
	CFFM	7.8	±	0.4	c	13.3	±	1.2	d	2.8	±	0.1	b	0.0	±	0.0	a	3.1	±	0.2	b	48.8	±	2.9	d
	HFFMl	6.9	±	0.4	bc	12.8	±	0.7	d	2.5	±	0.1	ab	0.9	±	0.9	a	2.9	±	0.3	b	45.0	±	3.8	cd
	HFFMu	5.2	±	0.2	ac	6.4	±	0.5	ab	2.2	±	0.1	ab	0.5	±	0.3	a	1.2	±	0.0	a	29.6	±	1.4	ab
	PBSO-5	4.8	±	0.5	ab	9.4	±	0.6	bd	1.7	±	0.1	a	1.1	±	0.6	a	1.4	±	0.1	a	32.7	±	1.4	abc
	PBSOX	5.5	±	0.5	ac	10.9	±	1.2	cd	2.0	±	0.1	ab	1.3	±	0.7	a	1.4	±	0.2	a	37.9	±	2.7	bd
	PHYTBIOP	5.0	±	0.4	ac	7.5	±	0.6	abc	2.1	±	0.1	ab	0.0	±	0.0	a	1.1	±	0.1	a	29.1	±	2.0	ab
	PHYTBIOF	4.1	±	1.4	a	4.7	±	1.5	a	1.8	±	0.6	ab	0.6	±	0.3	a	1.1	±	0.3	a	22.7	±	7.2	a
	CREF	5.0	±	0.3	ac	7.2	±	0.5	abc	2.2	±	0.1	ab	0.3	±	0.3	a	1.0	±	0.1	a	30.4	±	1.4	abc
	Control	4.5	±	0.3	ab	6.4	±	0.3	ab	1.8	±	0.1	a	0.5	±	0.2	a	0.9	±	0.0	a	27.1	±	1.7	ab

Table 11: Ratio of PLFA biomarkers after 12 weeks incubation for the different materials tested. Values are means ± standard errors and different letters show statistical differences (p <0.05).

	Tested materials	Gram$^+$:Gram$^-$				B:F				Cy:Pre				Mono:Sat			
Ratio	PBS-20-PF	0.4	±	0.0	a	356.9	±	208.9	a	0.9	±	0.1	ab	0.8	±	0.0	bc
	CFFM	0.6	±	0.0	abc	1267.3	±	35.6	a	1.2	±	0.1	ac	0.9	±	0.0	c
	HFFMl	0.5	±	0.0	abc	1044.5	±	586.4	a	0.9	±	0.0	ab	1.0	±	0.0	c
	HFFMu	0.8	±	0.1	d	615.5	±	600.5	a	1.8	±	0.5	bc	0.6	±	0.1	a
	PBSO-5	0.5	±	0.0	abc	365.9	±	356.9	a	0.9	±	0.0	ab	0.9	±	0.0	bc
	PBSOX	0.5	±	0.0	ab	473.8	±	465.5	a	0.8	±	0.1	a	0.9	±	0.0	c
	PHYTBIOP	0.7	±	0.0	bd	1303.7	±	152.7	a	1.2	±	0.1	ac	0.7	±	0.0	ab
	PHYTBIOF	0.8	±	0.1	d	252.2	±	239.4	a	2.0	±	0.3	c	0.7	±	0.0	a
	CREF	0.7	±	0.0	bd	477.7	±	241.9	a	1.1	±	0.0	ac	0.6	±	0.0	a
	Control	0.7	±	0.0	cd	456.6	±	206.1	a	1.2	±	0.1	ac	0.6	±	0.0	a

the MBC (Figure 9). The CFFM product resulted in a significant increase in all biomarker concentrations for the different microbial functional groups studied compared to the control with the exception of the saprotrophic fungi marker. The latter group did not show any significant difference between the materials tested and the control. However, the CFFM material had little influence on the ratios of the different microbial functional groups, with only the mono:sat ratio showing a significant increase compared to the control (Table 11).

All the composts tested (PHYTBIOP, PHYTBIOF and CREF) did not demonstrate any significant difference in PLFA biomarkers between themselves or with the control. Similarly, the two films tested (PBSO and PBSOX) did not show any significant variability between them for all PLFA biomarkers. The PBSOX film nevertheless led to a significant increase in the concentration of Gram- bacteria in the soil compared to the control and thus led to a significantly lower Gram+: Gram- ratio. The mono:sat ratio was also significantly higher after incorporation of both films in the soil compared to the control. Similarly to the PBSOX product, PBSPF only significantly increased the Gram- and mono:sat biomarkers compared to the control, combined with a significant decrease in the Gram+:Gram- ratio. Concerning the HFFM material, the upper part (HFFMu) had no impact on all PLFA biomarkers, whereas the lower part (HFFMl) significantly increased the Gram-, AFM, total PLFAs and mono:sat markers compared to the control.

Furthermore, such as MBC, the total concentration of PLFAs was correlated with C:N ratio of the materials and nitrogen mineralisation (Table 12).

Table 12: Correlation coefficients (n=27) between the total amount of carbon added (TC; mg.g^{-1} dry soil), the C:N ratio, the cumulative net C mineralised (Cmin; %), the net N released (Nmin, %), the microbial biomass carbon (MBC; µg .g^{-1} dry soil), dehydrogenase activity (DHG; µg TPF.g^{-1} dry soil.24h^{-1}), ß-glucosidase activity (GLU; µg PNP.g^{-1} dry soil.h^{-1}), N-acetyl-ß-D-glucosaminidase activity (NAG; µg PNP.g^{-1} dry soil.h^{-1}), the metabolic quotient (qCO2; µg CO_2_C . mg^{-1} MBC . h^{-1}) and the total PLFAs concentration (Total PLFAs; nmol.g^{-1} dry soil) at the end of the 12-week incubation period of soil samples containing different materials tested (only samples where PLFA was analysed).

	C:N	C_{min}	N_{min}	MBC	DHG	GUC	NAG	qCO_2	Total PLFAs
TC	-0.06	-0.79***	-0.06	0.01	0.15	0.24	-0.09	0.04	0.12
C:N		0.33	-0.92***	0.76***	0.61***	-0.31	0.70***	0.51**	0.51**
C_{min}			-0.27	0.15	0.18	0.19	0.26	0.31	0.30
N_{min}				-0.74***	-0.67***	0.35	-0.59**	-0.44*	-0.60**
MBC					0.65***	0.23	0.63***	0.15	0.53**
DHG						0.40*	0.53**	0.45*	0.61***
GLU							0.64***	0.26	0.59**
NAG								0.25	0.69***
qCO_2									0.43

*p-value < 0.05; **p-value < 0.01; ***p-value < 0.001;

The PLFA biomarkers and their ratios were further analysed by a principal component analysis (Figure 13). The first principal component of the PCA was significantly negatively correlated to all biomarkers except for the Gram+:Gram- and cy:pre ratios where the correlation was positive and for the saprotrophic fungi biomarker where the relationship was not significant. Concerning PC2, it was significantly negatively correlated with the biomarkers Gram+:Gram-, cy:pre, actinomycetes and Gram+, and positively correlated with the mono:sat ratio. This PCA of the biomarkers made it possible to differentiate CFFM and HFFMl products by the PC1. It also allowed to observe in the biplot an overlap between the three materials partly made of the PBS polymer, i.e. the products PBSPF, PBSO

and PBSOX. An overlap was also observed for the composts and the HFFMu product with the control, which was expected as none of these materials show significant differences in biomarkers compared to the control.

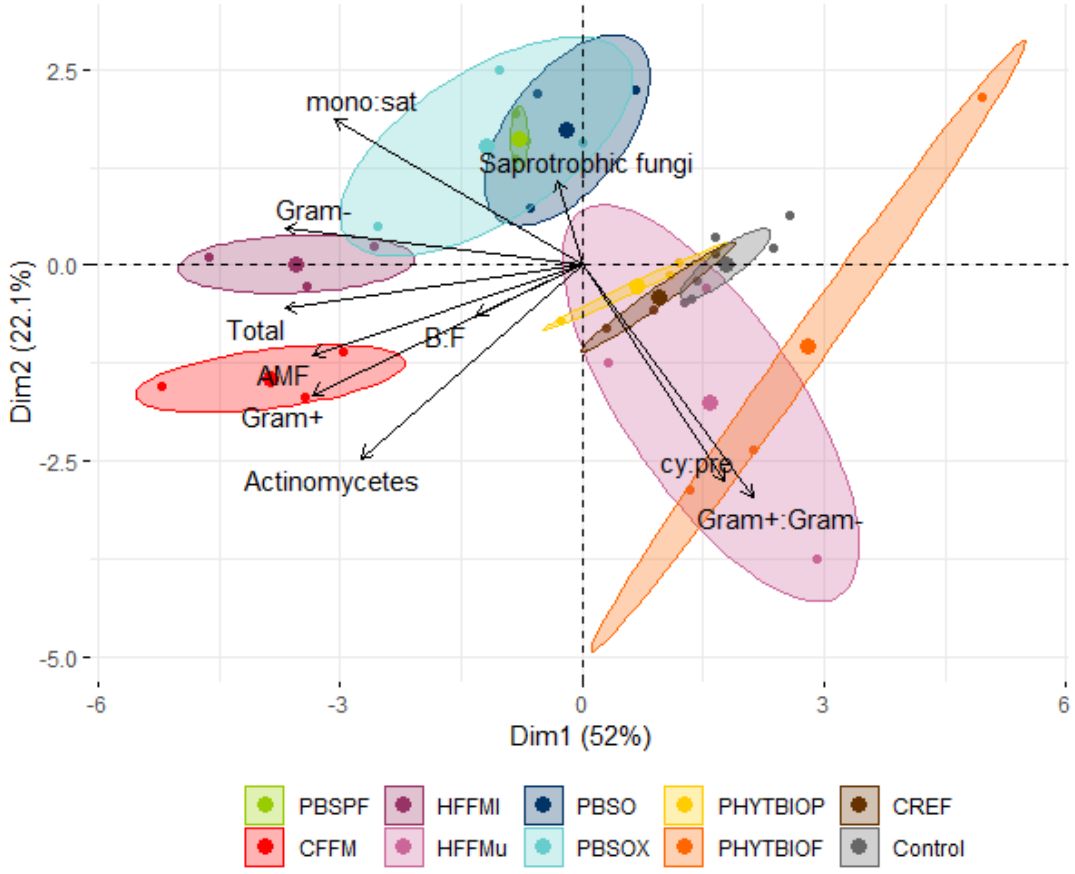

Figure 13: Principal component analysis (PCA) of PLFA biomarkers of soil microbial functional groups after 12 weeks of incubation with clustering by tested material according to P1 and PC2

2.5 Impact on N_2O emissions from soil

2.5.1 Set-up of incubation experiments

We performed a set of incubation experiments to assess the impact of soil amendment with the BPMs and BAPs on the N_2O emissions from soil. Given the late delivery of many of the materials, this was done on a selection of the BPMs and BAPs (see also Table 2), namely: PLA-C, PHBV-C, PBS-20-PF, PBSO-5, PBSOX, (in a first batch) and CFFM, HFFMu, HFFMI and FERT-S in a second batch.

This thus did not include the last biofertilizers which arrived only at the very end of the project. For these incubations, we used the same soil as was also used for the assessment of C and N mineralization and biological soil properties, for the sake of consistency. The dried soil was first remoistened and pre-incubated in the same fashion as for the C and N mineralization. Then, an amount of soil (equivalent to 246.8 g of dry soil) was thoroughly mixed with the respective materials and put into PVC tubes (diameter 6.7 cm), and the bulk density was adjusted to 1.4 Mg m^{-3} (filling height 5 cm). The amount of materials added was the same as the amounts added for the C mineralization experiment (Table 2).

Then a diluted KNO_3 solution was added to all soil cores in order to obtain a uniform nitrate concentration of 31 µg NO_3^--N g^{-1} dry soil and to bring the soil moisture to 70% water filled pore space (WFPS). These conditions are the best trade-off between having sufficiently high moisture content to create anaerobic sites in soil where N_2O is being produced, and not having too high moisture content that would further reduce most N_2O to N_2 and thus make it unmeasurable. Each soil core was put into a glass jar that could be closed airtight (with a volume of 1125 ml) and fitted with a septum to allow gas sampling (Figure 14), and incubated in the dark at a temperature of 20.5 °C. For the monitoring of N_2O emissions, the jars were closed and the headspace was sampled on the moment of closing and after 2 and 4 hours. These three measurement points per container per sampling event allows to calculate the average N_2O flux over the 4 hours sampling interval and allows also for quality control of the measurements. For the actual N_2O measurements 12 ml glass exetainers® (Labco Limited, Ceredigon, UK) were pre-evacuated three times consecutively by means of a vacuum pump and flushed with He. Headspace gas samples were collected from the closed containers using an air-tight syringe and stored in the preevacuated extainers until measurement. The containers were sampled for N_2O emissions during 14 days, which should contain the peak emissions after addition of NO_3^- which is expected in the first days of the incubations.

Problems with our former GC (Thermo Electron Trace GC Ultra gas chromatograph) caused us to purchase a new GC in 2020 (Agilent 8860 GC). This new GC had many issues during and after the installation, and measurements of N_2O emissions had to be postponed several times. Eventually, measurements could be performed using this new GC only in the final stages of the project.

Figure 14. On the left the closed sample jars with incubation cylinders inside. On the right a sample jar with sample syringe.

2.5.2 N_2O emissions

Surprisingly, the N_2O emissions were extremely low and were not distinguishable from control pots without soil, despite the conditions created in the soils (addition of NO_3^-, moisture content of 70% WFPS). A typical course of net N_2O emission rates observed is given in Figure 15 for the soil amended with PHBV-C. The emission rates could not be calculated reliably enough because in many of the samples there was no linear accumulation of N_2O over the 4 hour sampling time, with also negative N_2O emissions observed.

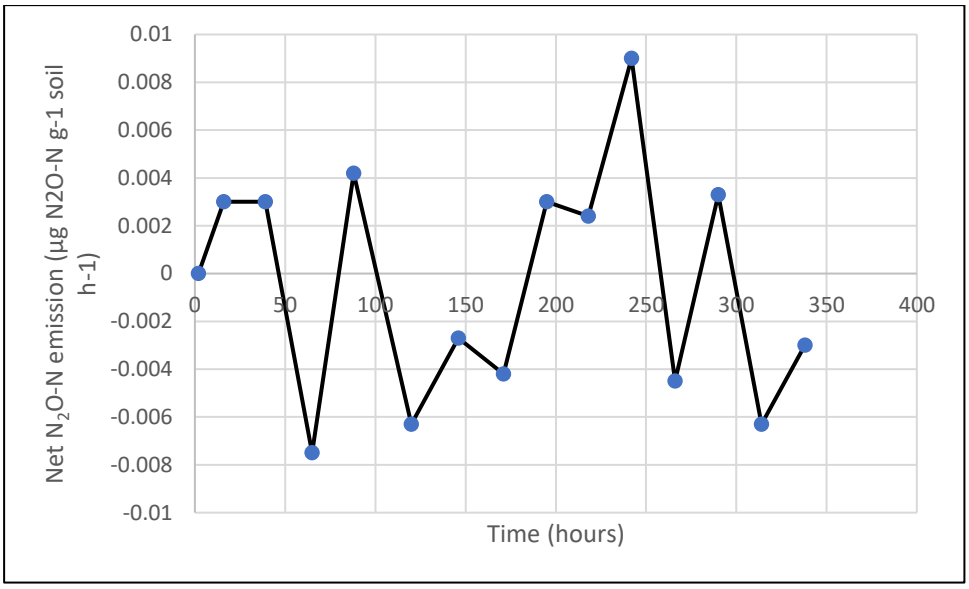

Figure 15: Pattern of N_2O emissions from soils amended with PHBV-C, monitored over a 2-week period.

The reasons for these low N_2O emissions are probably due to the nature of the materials added. Indeed, the materials that we added in these experiments mostly had a pronounced N immobilization effect, as can be seen in the graphs of net N mineralization in previous section. This N immobilization thus probably strongly reduced the NO_3^- concentrations, thus depleting the main substrate for N_2O production and emission. In the case of PLA-C there was no N immobilization nor N immobilization, but in this case the absence of N_2O emissions was probably linked to the very low degradation rate of the material (cfr. the very low C mineralization rate), leading to a lack of e^- donors for the N_2O production in soil. If indeed the N immobilization was the main cause of the low emissions, this could probably be remediated by addition of smaller amounts of these materials or/and by combining this with larger addition rates of NO_3^- (larger than the target 31 ppm NO_3^--N used in these experiments). Another option would be to further increase the soil moisture content to e.g. 75 or 80% WFPS, but this would no longer reflect realistic soil moisture contents that could be expected in the field for prolonged periods of several days. However, for at least the FERT-S we would expect higher N_2O emissions (higher N mineralization than the other materials), which were also not observed. So certainly for this specific case of the FERT-S, we are unsure of the reasons behind the low N_2O emissions.

Nevertheless, from these very low emissions obtained here we can probably safely conclude that the addition of these materials to soils will not result in increases of N_2O emissions.

2.6 Overall agricultural and environmental considerations from detailed incubation experiments

Closing the organic carbon and nutrient cycling in the biobased industries and agriculture is essential to reduce our dependence on non-renewable resources, to preserve soil quality, and to reduce greenhouse gas emissions from agriculture. The preservation of soil quality (notably soil organic carbon) is an important consideration when diverting agricultural residues towards the biobased industries with a view to extract valuable compounds or produce e.g. biocomposites, because this means automatically a reduced input or organic matter into the soil. In order to mitigate this risk of reduced organic matter inputs, the idea is to incorporate the used biobased composites from this

project (derived from agricultural wastestreams) back into soil, thus closing the organic C cycle as much as possible. Also, returning these materials, and fertilizers derived from the biobased industries, to soil will help to preserve nutrients and thus reduce the need for nutrient inputs that are fossil fuel based (N) or that are derived from finite resources (P).

2.6.1 Impact on nutrient dynamics

N dynamics

The most important potential impact on nutrient dynamics in soil was to be expected on N availability, given the specific nature of these materials (highly different C/N ratio and degradability). All BPMs immobilized N throughout the entire duration of the experiment, with the exception of PLA-C where a very small net N mineralization was observed at the very end of the incubation. Likewise, there was a consistent N immobilization from the mycelium materials CFFM and HFFMI, but (a bit surprisingly) a net N mineralization towards the end of the experiment for HFFMu. With respect to the composts, surprisingly no N mineralization (but also no net immobilization) was observed for both UL-1 and UL-2.The other composts showed very small (CREF) to considerable (relatively speaking, for composts) N release (PHYTBIOP and PHYTPBIOF). The liquid fertilizer released most of its total N already after a few weeks, making it a rapid release organic N fertilizer. The FERT-S released also a large amount of the total N it contained, but this was in a more gradual fashion, characterizing it as a more slow release organic fertilizer, somewhat comparable to liquid animal manures.

Care has to be taken with the timing of the application of these materials to soil with respect to this N release (or immobilization). The materials that lead to consistent N immobilization should not be applied before the start of the growing season, given that this could lead to a shortage of available N for the following crop. The composts could be added before the start of the growing season because there will be no mineralization or immobilization of N. The FERT-L could be added immediately before the start of the growing season. It should not be added several weeks before though, because the large N mineralization from the start could lead to N losses in case e.g. of heavy rainfall before planting/sowing or before the roots of the crops are well developed. The FERT-S could be added directly before planting/sowing or several weeks beforehand, because the relatively slow N mineralization would not pose much risks for N losses before or in the beginning of the growing season.

However, the immobilizing materials could have also a very important use later on in the season (when they would be more logically be available (pots and films expected to be available after rather than before the cropping season). Indeed, this immobilization capacity could be used to immobilize excess mineral N that is still present in the soil profile at the time of harvest. For a number of crops, such high mineral N residue at harvest is a well-known problem leading to potential high N losses (leaching, denitrification). Incorporation of these materials at the end of the growing season may thus help to mitigate these (potential) N losses.

But all these considerations have again to be placed in an overall picture taking into account the application rates. For all materials except the composts and the biofertilizers, the overall availability expressed on a per ha basis will probably be very modest, and so would also be the impact on overall N immobilization. So while in principle these materials could play a role to optimize N management in intensively grown crops, in reality this role will probably be very small, unless these materials would be diverted/concentrated in specific fields to tackle specific N management problems.

P dynamics

All BAPs had lower P-$CaCl_2$ than the unamended control soil. This result was not surprising, given that these materials contain virtually no P. So application of these materials would slightly reduce the directly available P content for crops. Surprisingly, also the application of the composts led to a slight decrease in P availability.

All this, however, has to be interpreted in light also of the initial P content of this soil, which was very high. Unfortunately it is very difficult, if not impossible to find agricultural soils in North of Belgium with low P content, hence the choice for this soil. Certainly for this specific situation, the overall P availability (always > 3 mg $CaCl_2$ extractable P kg^{-1} soil) still qualifies as (very) high so no problems with P availability are to be expected. Given that P is an environmental problem in many intensive agricultural fields (especially in areas of intensieve livestock production such as North of Belgium, the Netherlands, Denmark, Brittany in France, …), these materials that reduce P availability could perhaps even be considered as an additional measure to reduce directly available P in soil, in order to reduce the risk for P leaching.

2.6.2 Impact on soil organic carbon

To evaluate the impact of addition of these biobased materials to soils, we use the humification coefficients that can be derived from the C mineralization experiments carried out under controlled conditions. High humification coefficients are associated with stable organic materials that mineralize slowly, and thus contribute much to the organic matter content of the soils.

From the experiments carried out here, the humification coefficients (hc) calculated varied widely between the different BPM used in this study. For CAC and PLA-C, the hc was very high (around 0.8-0.9), comparable to hc's for stable composted materials. These materials will thus contribute significantly to additional SOM build up. However, the other BPMs had relatively low hc's (ranging from 0.68 to 0.18, Table 7), and surprisingly the C mineralization was relatively linear even after more than 100 days of incubation, which increases the uncertainty on the calculation of hc. For some of these materials, especially the PBSO materials, relatively little contribution to SOM buildup can be expected. To put things in perspective, this can be compared to hc's obtained for "classical" organic matter inputs in agriculture, such as straw, which has an hc of approximately 0.3.

For the BAPs, there was a big difference between the different types of composts added to soil. For the PHYTBIOP and PHYTBIOF and the CREF hc's were as expected for composted materials (and even in the high end of this range), from 0.83 to 0.96. Especially the CREF is extremely stable and will contribute enormously to SOM build up. The reason for this difference is not clear, and it is unlikely that the addition of specific microorganisms to these composts would be able to explain this. However, the UL-1 and UL-2 composts had much lower hc's of 0.60 and 0.49, and thus contribute potentially much less to SOM build up. The mycelium based materials HFFMI and CFFM also have relatively high HC's, whereas the HFFMu was extremely rapidly mineralizable in soil. As expected, the hc's of the biobased fertilizers FERT-S and FERT-L was rather limited, but the purpose of these materials is more in the addition of nutrients than in the addition of organic carbon.

It must also be stressed that the determination of the hc's is based on emission of non-labeled CO_2, and therefore it cannot be excluded that the actual hc of some of these materials is overestimated (in case that priming of native SOC would have occurred), but this could only be analysed using stable isotope labeled biomaterials, which was outside of the scope of this project.

However, apart from the intrinsic stability of the organic carbon added, also the absolute amount of these materials that would be added to soil in practical situations needs to be taken into consideration.

Clearly, the BPMs, the mycelium based materials and also the biofertilizers would be added probably in rather modest quantities, given that it concerns potting materials and agriculturals films. So even with a very high hc, these materials would only add marginally to the stable SOM pool. It is mostly the composts that would be added in large quantities (order of tons to tens of tons ha^{-1}) and that therefore have the largest potential to contribute to increasing or maintaining SOC levels.

2.6.3 Impact on soil biological properties

In general, all the amendments had positive to strongly positive effects on the soil biological properties, given that net microbial biomass C and net enzyme activities were higher than in the corresponding control soils. Thus they seem to contribute to soil (biological) quality, at least at the level of detail assessed in these experiments (which included MBC, microbial activities and microbial community composition). These materials can thus be applied without a concern for a depressive or negative effect on the soil microbial community.

2.6.4 Assessment of overall effects

The materials (both BPMs and BAPs) from the Agrimax project can be safely applied to soil without risks for increased nutrient losses of N and P, and with a (modest) contribution to build-up or maintenance of the SOC stock. If abundantly available, they can even be used to reduce potential N leaching risks at the end of the growing season. Also, these products will not increase N_2O emissions from soil. They can also stimulate microbial activity, as was evidenced by the increased enzyme activities upon application to soil.

The composts and organic fertilizers from the Agrimax project derived from residual streams from the biobased industries can positively affect the C and nutrient budget and dynamics in soil.

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3 Impacts of improved composts on crop yields in pot experiments

3.1 Set-up of pot experiments

3.1.1 Improved compost supplemented with microorganisms: experiments with melon

The improved composts (CBIO1 and CBIO2), prepared by supplementation with microorganisms as described in D.6.4., were used as substrate for growing melon plants (*Cucumis melo*), in a 3:1 ratio, i.e., three parts compost (Table 13) to one part vermiculite. After germination and growth to a seedling stage, it was possible to evaluate their effect on different plant growth parameters, related to crop yields, (CBIO1 and CBIO2) and, the incidence of the disease caused by the phytopathogenic fungus *Fusarium oxysporum* f.sp. *melonis* (FOM) (only CBIO1), causative agent of *Damping-off* or wilting (Figure 16). In both cases, the microbial biomass of compost constituted 1% (w/w) and the final moisture content was 60%. All components were mixed to ensure maximum homogeneity.

Figure 16: Foliar yellowing and systemic decay of melon seedlings affected by Damping-off.

The substrate mixtures corresponding to CBIO1, CBIO2 and control (CBIO0) were incorporated in trays intended for seed sowing, at a rate of 10 g of substrate per alveolus, to reach a total of 30 alveoli per treatment (30 x CBIO1 and 30 x CBIO2) for the study of phytostimulant efficacy and 20 alveoli for the study of biopesticidal efficacy (20 x CBIO1).

Table 13: Improved compost composition.

Nomenclature	Compost	Fungus 4425	Fungus 4563	Bacteria 1343	Water
CBIO0 (control)	1 kg	0 g	0 g	0 g	500 mL
CBIO1	990 g	5 g	5 g	0 g	500 mL

CBIO2	396 g	0 g	2 g	2 g	200 mL

After the germination phase (5 days in darkness), the plants were subjected to a controlled photoperiod of 12 h of light and 12 h of darkness, at a temperature of 25 °C, and to periodic irrigation to maintain adequate humidity until the seedling stage was reached (appearance of the first non-cotyledonary leaves). This period lasted approximately 30 days.

After that, the phytostimulant / biofertilizer efficacy of the compost, both for CBIO1 and CBIO2, was evaluated by comparing seedlings germinated in bioaugmented compost with seedlings germinated in compost without inoculum (control). For this purpose, once the substrate was removed and the roots were cleaned, the plants were placed on filter paper (Figure 17) to take the following measurements: stem diameter (mm); stem and root length (mm), fresh and dry weight (g), and true leaf count. In addition, with the calculation of lengths, the root : stem ratio was calculated.

Figure 17: Arrangement of seedlings on filter paper prepared for measurement of production parameters.

To evaluate the biopesticidal activity, seedlings with CBIO 1 were infected with the selected phytopathogenic fungus. For this, FOM was previously cultured in flasks containing 50 mL of PDB that were incubated at 30 °C under agitation for 5 days. After the incubation time, the contents of the flasks were homogenized and filtered under sterile conditions to separate the fungal biomass from the liquid culture médium containing spores that were used as pathogen inoculum at a concentration of 10^5 spores/mL to carry out infection. The seedlings were inoculated with 1 mL of the fungal cell suspension applied directly to the substrate, around the stem. Two weeks after infection, during which time the plants were kept in the same culture conditions as previously described, the above measures were taken. Additionally, the incidence of disease caused by the phytopathogen was qualitatively evaluated, applying the following scale with values from 0 to 4, depending on the severity of the typical symptoms of the disease (Table 14).

Table 14: Range of values according to the infection shown by the melon seedling.

Severity	Symptoms
0	No sign of infection
1	Displays 1 true yellow leaf
2	Displays more than 1 true yellow leaf
3	Displays 1 true yellow leaf and 1 withered leaf
4	Displays more than 1 true yellow leaf and more than 1 withered leaf

3.1.2 Hydrocompost: experiments with tomato

Hydrocompost was applied on tomato plants to assess its effect on plant development and productivity. Plants were grown in two different substrates (natural soil and inert substrate - perlite), and grouped into three sections. In one of the three sections a commercial fertiliser obtained from Leonardite was applied, in another section Hydrocompost obtained as described in D6.4 was used, and the last section was called Control, to which no fertiliser was added. Commercial fertiliser and Hydrocompost were applied once a week from the beginning to the end of the trial (56 days), according to the application rates indicated in Table 15. In all cases, the humic substances were tested at a concentration of 0.7% since a beneficial effect on germination was demonstrated at concentrations of less than 1% (previous experiments, D6.4). Fertiliser was not applied into Control plants. The mineral irrigation solution was the same for the whole trial and for all plants grown (Table 16).

Table 15: Weekly programme of humic substances supply

Week	Water consumption L·m^{-2}	L·plant $^{-1}$		Humic input cc/plant
		Inert Substrate	Natural soil	
1	1.63	0.8	0.4	6
2	2.29	1.2	0.6	4
3	2.95	1.5	0.75	7

4	3.32	1.7	0.85	8
5	3.68	1.8	0.9	9
6	3.74	1.9	0.95	9
7	3.80	1.9	0.95	10
8	4.00	2.0	1.0	10
9	4.21	2.1	1.05	11
10	4.20	2.1	1.05	11

Table 16: Mineral irrigation solution (mEq/L)

NO_3^-	$H_2PO_4^-$	SO_4^{2-}	Cl^-	NH_4^+	K^+	Ca^{2+}	Mg^{2+}	$K^+/(Ca^{2+} + Mg^{2+})$
15	2	6	2	1.5	7.5	12	4	0.47

During the trial period, growth parameters were periodically analysed by direct measurements on the plant. These variables were stem length (cm), stem diameter (mm), number of leaves, inflorescences and fruits (Figure 18). In parallel, other destructive parameters were analysed every 20 days, such as fresh and dry biomass (g) of roots, stems, leaves, inflorescences and fruits (Figure 19).

Figure 18: Analysis of growth parameters in the greenhouse

Figure 19: Sample prepared for destructive analysis

3.2 Crop yield effects

3.2.1 Improved compost supplemented with microorganisms as substrate for melon crop

Morphometric measurements of melon seedlings grown in the presence of the two improved composts (CBIO1 & CBIO2) and non-supplemented control compost (CBIO0) revealed the higher quality of CBIO1 as substrate for melon growth (Figure 20). Most of the parameters evaluated were significantly higher in seedlings with CBIO1. In fact, CBIO2 did provide any significant improvement over the control compost. This effect was especially evidenced in the increase of the root system of the plants, which is an important factor in determining the plant growth promoting potential of the microorganisms used.

The same parameters measured in the CBIO1 biopesticidal potential assay (Figure 21), resulted in statistically significant differences with respect to the controls used, especially in stem length and root:stem ratio. These results showed an improvement of the root system, already observed previously, as well as a greater vigor of the plant (Figure 22), probably caused by the defense strategy developed against the phytopathogen by the microorganism used as inoculant.

Regarding disease severity (Figure 23), it was lower in plants germinated from CBIO1 than those grown on compost without microorganisms supplementation. In general, the symptomatology of the *Damping-off* disease presented by the seedlings that used CBIO1 as substrate were rated with values close to or less than 2, in contrast to the seedlings with control Compost, which were rated with values close to 3.

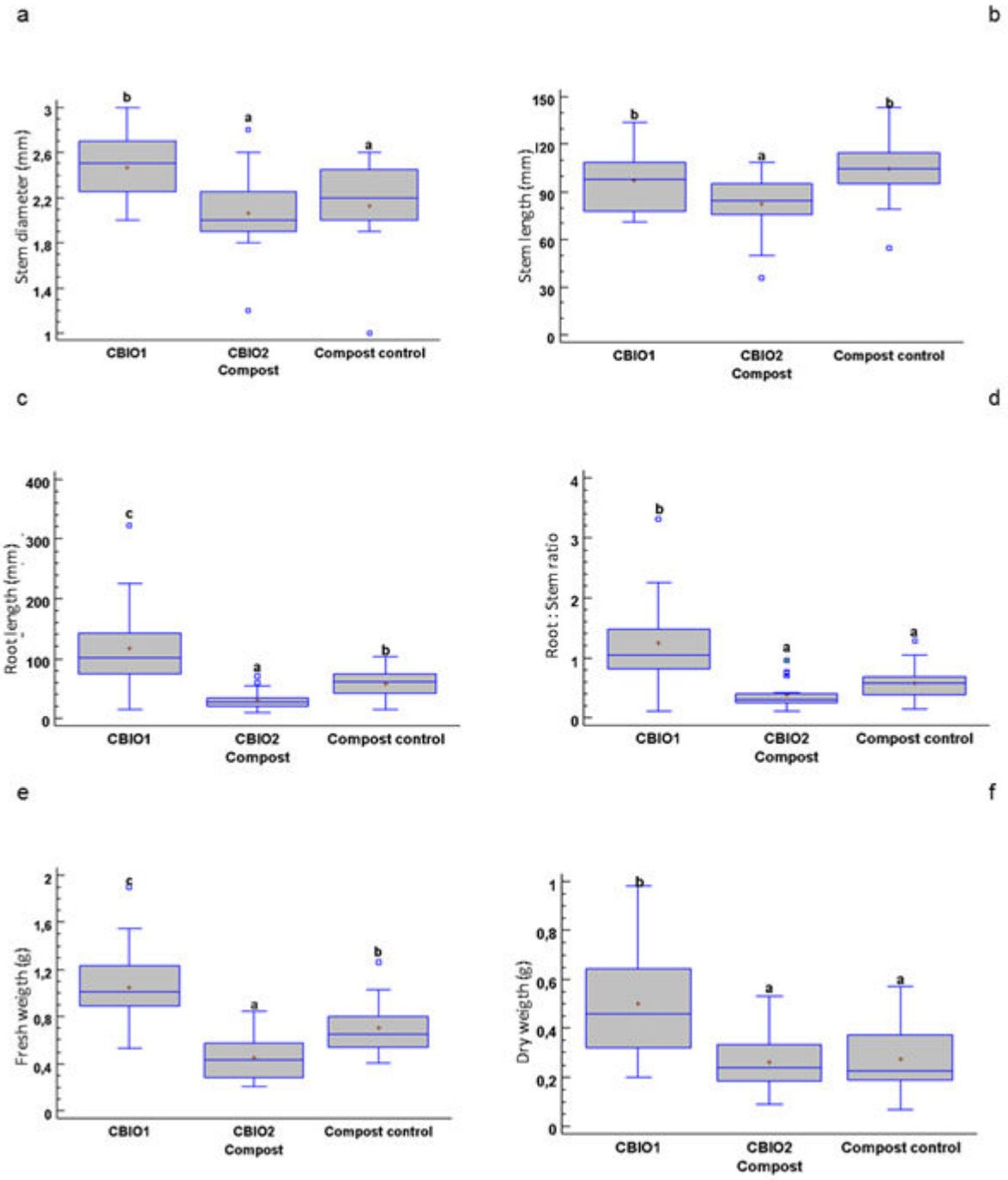


Figure 20: Vigorousness parameters of melon seedlings with CBIO1, CBIO2 and CBIO0 (control compost): stem diameter and length (a and b, respectively), root length (c), root/stem ratio (d) and plant fresh and dry weight (e and f, respectively).

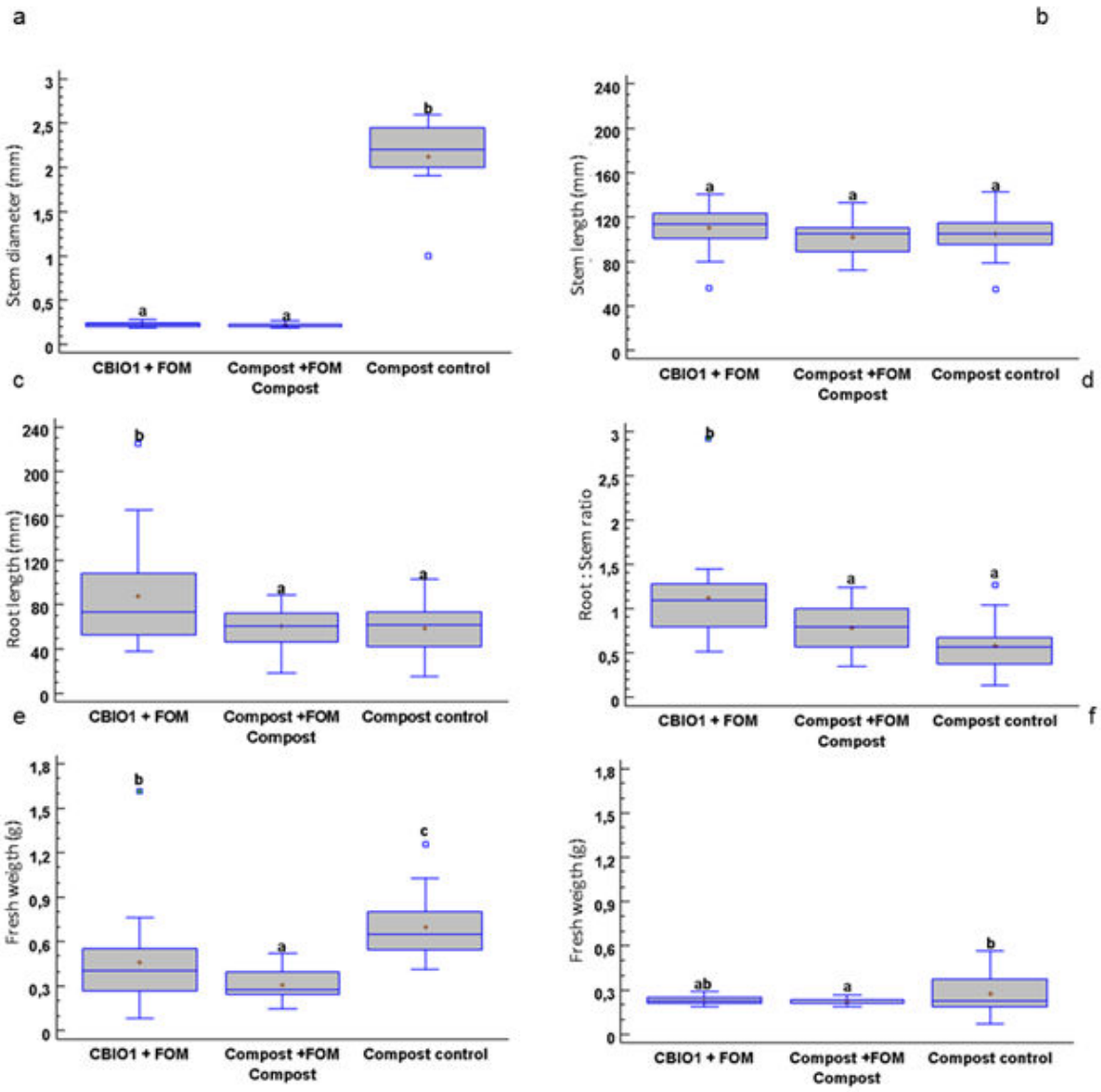


Figure 21: Evolution of the protective effect against Fusarium oxysporum *f.sp.* melonis *(FOM) of melon seedlings with CBIO1+FOM, Compost+FOM and CBIO0 (control compost) where data are collected for stem diameter and length (mm) (a and b, respectively), root length (mm) (c), root/stem ratio (d) and plant fresh and dry weight (g) (e and f, respectively).*

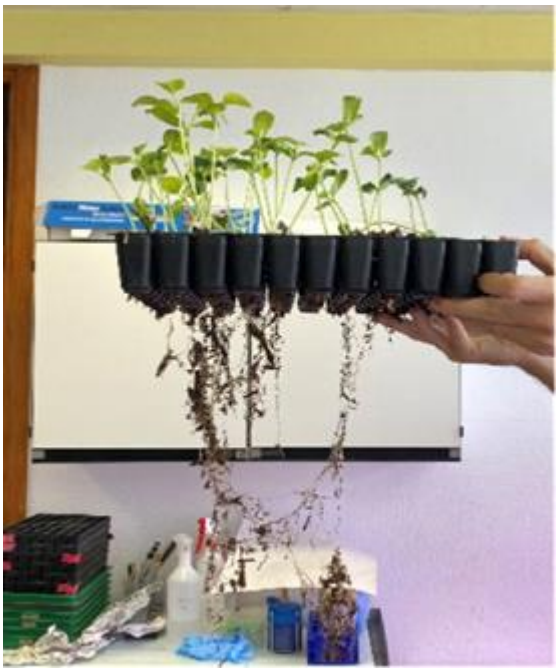


Figure 22: Melon seedlings grown from CBIO1 bioaugmented compost with evident root system improvement.

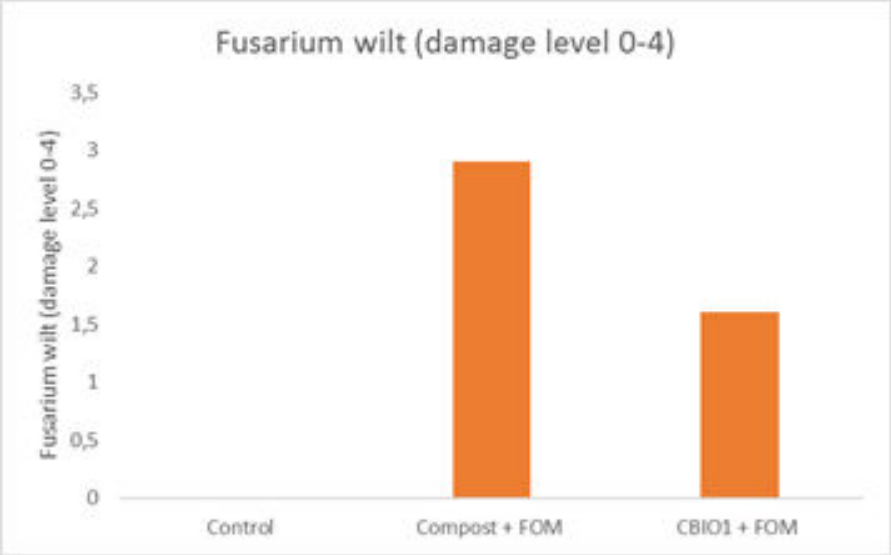


Figure 23: Disease severity against Fusarium oxysporum *f.sp.* melonis *(FOM) infection of melon seedlings with CBIO0 (control compost), Compost+FOM and CBIO1+FOM. Disease severity was evaluated according to the criteria given in Table 14.*

3.2.2 Hydrocompost experiments with tomato

After the application of the humic compounds (commercial product and hydrocompost), some very positive results could be detected in terms of plant growth. Taking into account the plant growth parameters, the results were different depending on the phenological stage of the plant. Thus, the most notable effect of hydrocompost application was detected 40 days after transplanting (dat) at phenological stage P5 (fifth pod). In this case, a slight increase in the size of the plants treated with

hydrocompost (length and thickness) was observed, as well as an increase in the number of fruits (Figure 24).

On the other hand, a significant increase in root dry biomass in hydrocompost-treated samples is worth mentioning. This increase was most noticeable 38 days after transplantation (Figure 25).

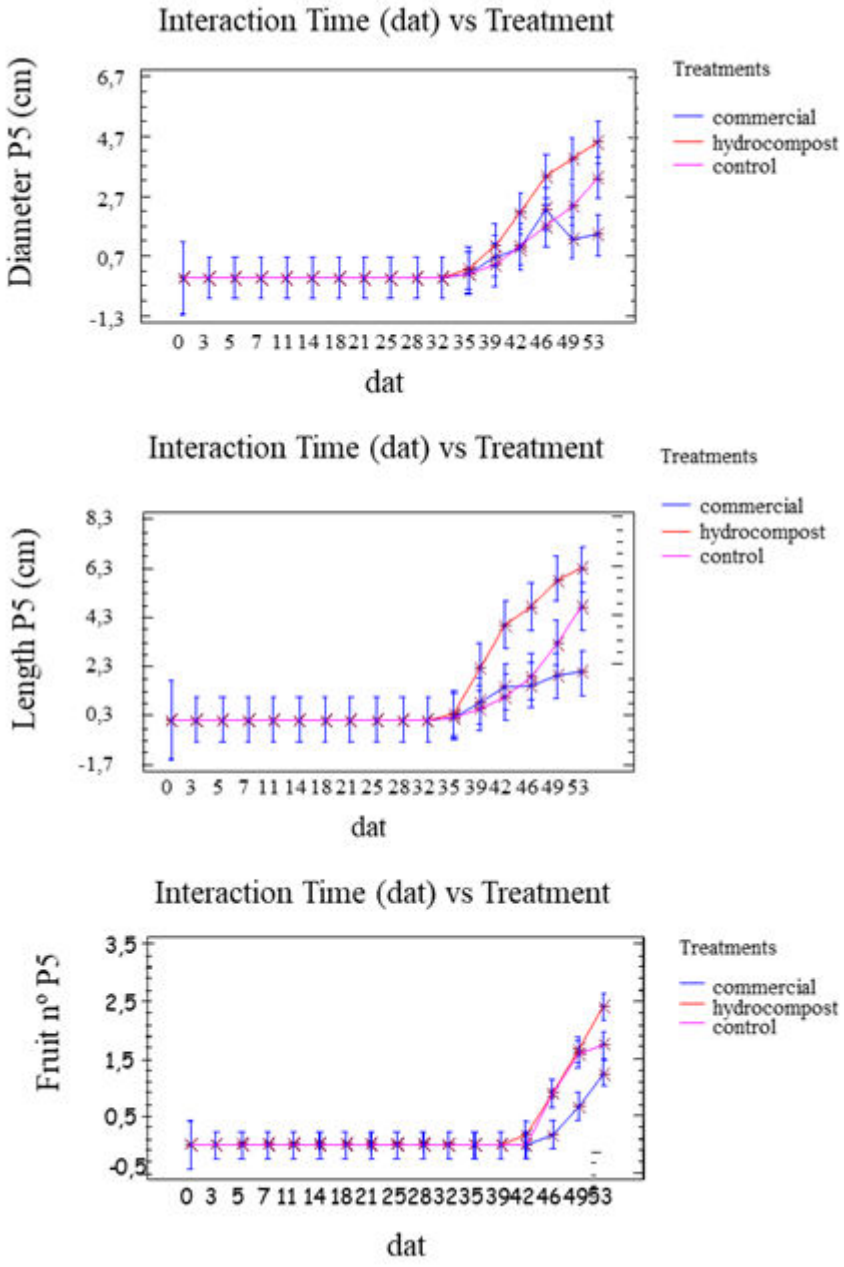


Figure 24: Effect of "time and treatment" factors on the Diameter, Length and Fruit number in the phenological stage P5 (dat: days after transplantation; P5: fifth podium)

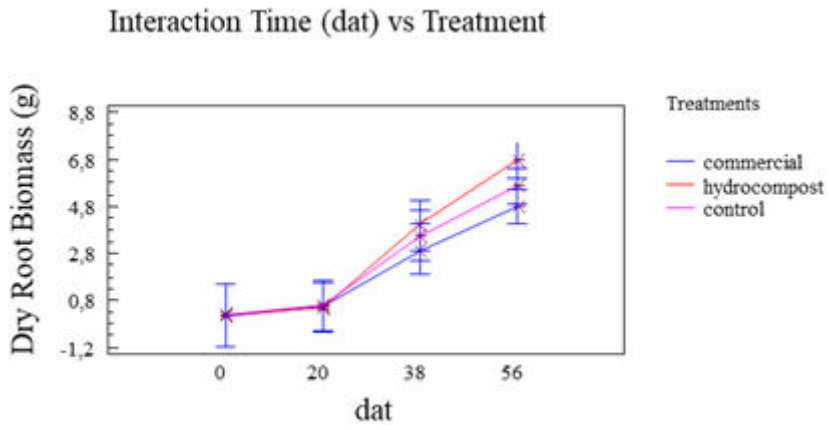


Figure 25: Effect of "time and treatment" factors on the Dry Root Biomass (dat: days after transplantation)

4 Effects of bio-fertilizers on tomato plants in field trials

4.1 Experimental design

The effect of Agrimax biofertilizers on tomato plants was evaluated at the field level by Fertinagro.

On May 20, 2021, soil is sampled within the area where the field experiment will be carried out. The development of the entire experiment is carried out on the "Los Baños" farm (Teruel), where the treatments corresponding to this trial have been applied.

Some data on the crop are pointed out, which are considered of interest:

Farmer: Tervalis Foundation
Crop: Tomato
Variety: Optima
Location: Los Baños (Teruel)
Sowing date: 07/06/2021

The treatments defined were the following:
Treatment 1:

- Solid organic biofertilizer (5-2-3,5), 1,6 % aa, 30 % OM. Application type: Bottom fertilization with fertilizer spreader, 1500 kg/ha.
- Liquid organic biofertilizer (2,5-1-2), 8 % aa, 12% OM. Application type: Fertirrigation like biostimulant 2 kg/ha in 6 moments of the crop between planting and fruit setting.

Control:

- Solid organic fertilizer (6-3-3), 43% OM. Application type: Bottom fertilization with fertilizer spreader, 1250 kg/ha.
- Liquid organic biofertilizer (2,5-1-2), 8 % aa, 12% OM. Application type: Fertirrigation like biostimulant 2 kg/ha in 6 moments of the crop between planting and fruit setting.

The field test was divided as follows in Figure 26: Field test scheme, with a total area of 300 m^2, 298 m^2 of experimental treatment (of which only 20 m^2 were to catch samples) and an area of 2 m^2 of control.

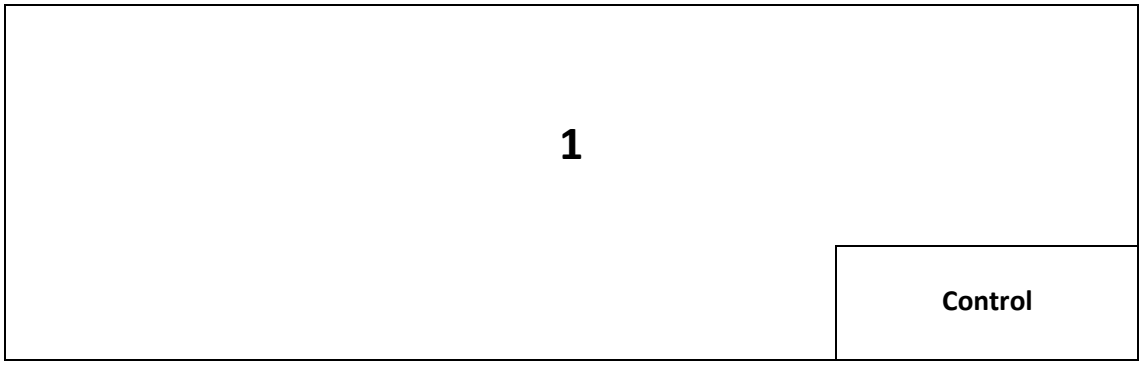


Figure 26: Field test scheme

On May 21, 2021, the solid organic biofertilizer (**1**) and solid organic (**control**) were aplicated on bottom fertilization and on May 28, 2021, the sowing was done.

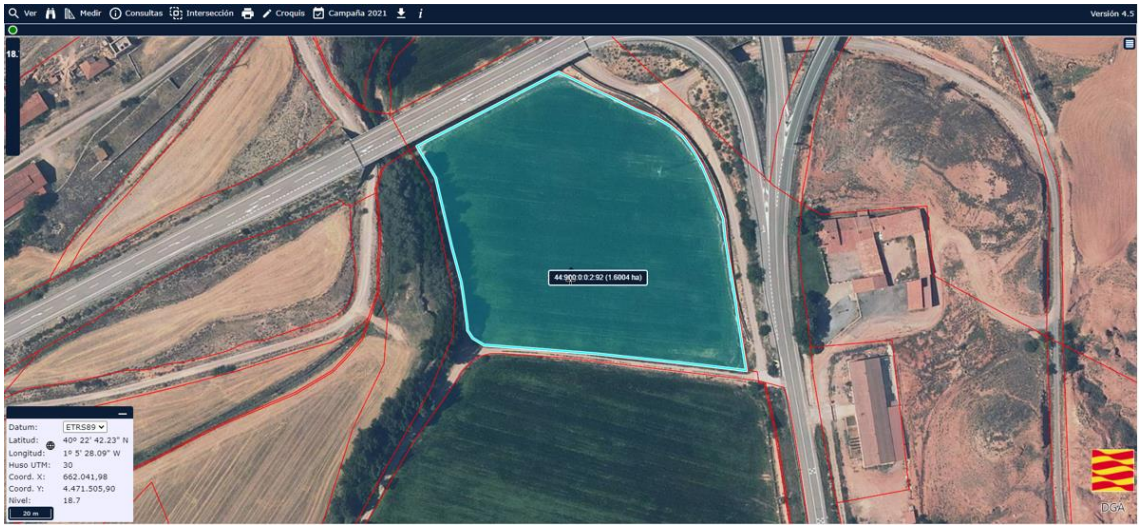


Figure 27. Photos of the test performed.

July 6, 2021

In the following figure X, a pPanoramic view of the trial (the control treatment between the wooden boards) is shown:

Figure 28: Panoramic view of the trial 45 days after sowing.

Figure 29: ***Panoramic view of the plantation frame 45 days after sowing.***

Control treatment:

Figure 30: Control treatment 45 days after sowing

1 treatment:

Figure 31a,b: Experimental treatment 45 days after sowing

August 18, 2021:

Panoramic view of the trial (the control treatment between the wooden boards):

Figure 32: Panoramic view of the trial 88 days after sowing.

Control treatment:

Figure 33: Image of the control treatment 88 days after sowing.

1 treatment:

Figure 34: Experimental treatment 88 days after sowing.

Figure 35: Experimental treatment 88 days after sowing.

September 20, 2021:

Control treatment:

Figure 36: Control treatment 117 days after sowing.

Figure 37: Control treatment 117 days after sowing.

1 treatment:

Figure 38: Experimental treatment 117 days after sowing.

Figure 39: Experimental treatment 117 days after sowing.

Figure 40: Experimental treatment 117 days after sowing.

4.2 Results

4.2.1 Impact on vegetative development and yield parameters of tomato plants

The effect of the new organic fertilizer on tomato plant development and fruit production and quality was determined.

- First sampling date: 06/07/2021. 45 days after sowing.

Table 17: Data collected in the first sampling of the experiment: vegetable height and number of fruits.

Date 06/07/2021	45 days after sowing												
1 TREATMENT	Vegetable height (cm)	42	45	47	49	42	44	48	40	47	38	48	33
	Number of fruits	2	3	2	1	2	2	1	2	3	1	1	2
CONTROL TREATMENT	Vegetable height (cm)	46	52	30	41	45	41	43	35	53	53	38	49
	Number of fruits	1	1	1	1	0	2	0	1	0	0	0	0

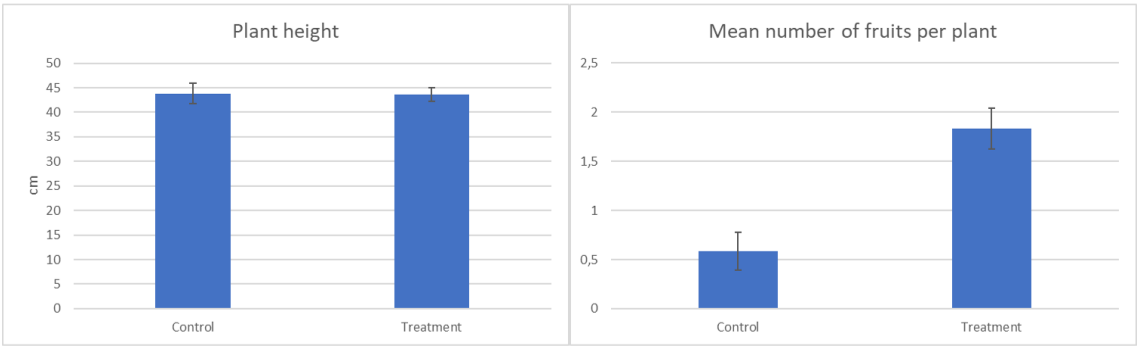


Figure 41: Mean plant height and number of fruits per plant at 45 days after sowing.

After the first sampling date, evident increase in mean number of fruits per plant under the treatment with the new fertilizer was observed.

- Second sampling date: 18/07/2021. 88 days after sowing.

Table 18: Data collected in the second sampling of the experiment: number of fruits.

Date 18/08/2021	88 days after sowing												
1 TREATMENT	Number of fruits	5	9	5	4	1	2	7	5	4	4	6	8
CONTROL TREATMENT	Number of fruits	10	6	5	9	5	9	0	11	6	5	4	6

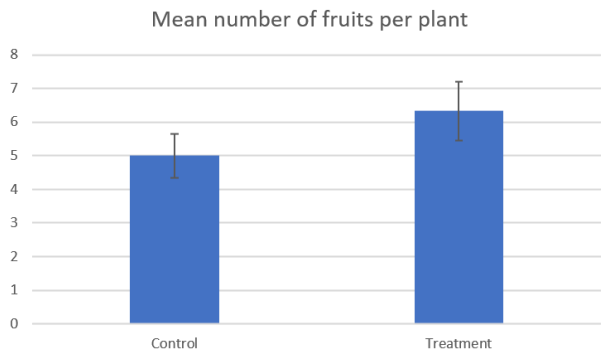


Figure 42: Mean number of fruits per plant at 88 days after sowing.

In the second sampling date, the mean number of plants was higher in plants grown in the area fertilized with the new organic fertilizer.

- Third sampling date: 20/09/2021. 117 days after sowing.

Table 19: Data collected in the second sampling of the experiment: number of fruits.

Date 20/09/2021	**117 days after sowing**												
1 TREATMENT	**Number of fruits**	6	7	8	5	7	9	6	8	5	6	6	8
CONTROL TREATMENT	**Number of fruits**	7	11	10	12	9	8	8	10	7	8	8	10

The third sampling was the last one. Twelve plants per treatment were harvested and plant fresh and dry weight was measured. Plants grown in the area fertilized with the new organic fertilizer had higher fresh and dry weight, thus showing that the new product allows higher plant biomass production.

Figure 43: Tomato plants were processed in the laboratory for biomass and nutrient content measurements.

Dry biomass of tomato plants.

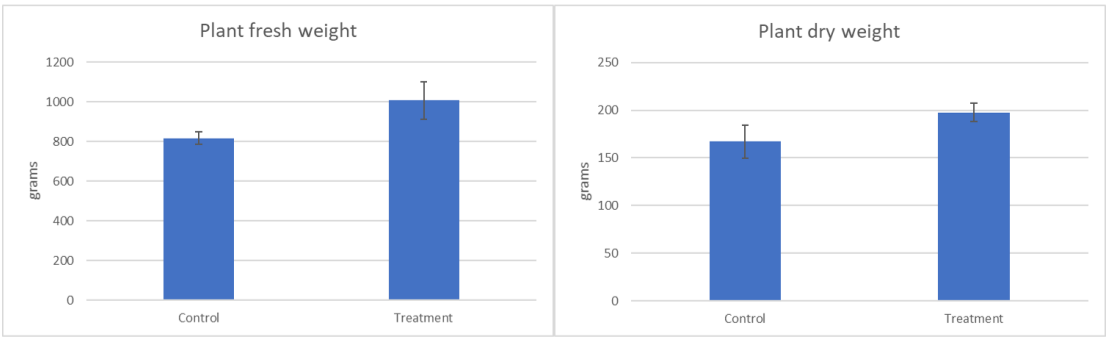


Figure 44: Mean plant fresh and dry weight of tomato plants at the end of the experiment

Elemental content of the plant biomass was measured in order to assess nutrient uptake. Plants fertilized with the new organic fertilizer showed higher content of most of the nutrients.

Table 20: Nutrient content in tomato tissues.

	Control	Tratamiento	
N	1,76	1,64	%
Ca	2,63	3,10	%
K	3,04	3,64	%
Mg	0,49	0,59	%
Na	0,10	0,13	%
P	0,29	0,28	%
S	0,36	0,34	%
B	25,68	30,70	mg/kg
Cu	83,90	78,85	mg/kg
Fe	170,14	308,18	mg/kg
Mn	23,51	46,50	mg/kg
Mo	11,43	33,88	mg/kg
Zn	88,69	90,60	mg/kg

The fruits of each of the twelve plants were harvested. Fruit weight, diameter and brix grade were higher in the plants fertilized with the new organic fertilizer than in the control.

Figure 45: Crop yield and tomato quality parameters were assessed.

Parameter measurement of tomato fruits.

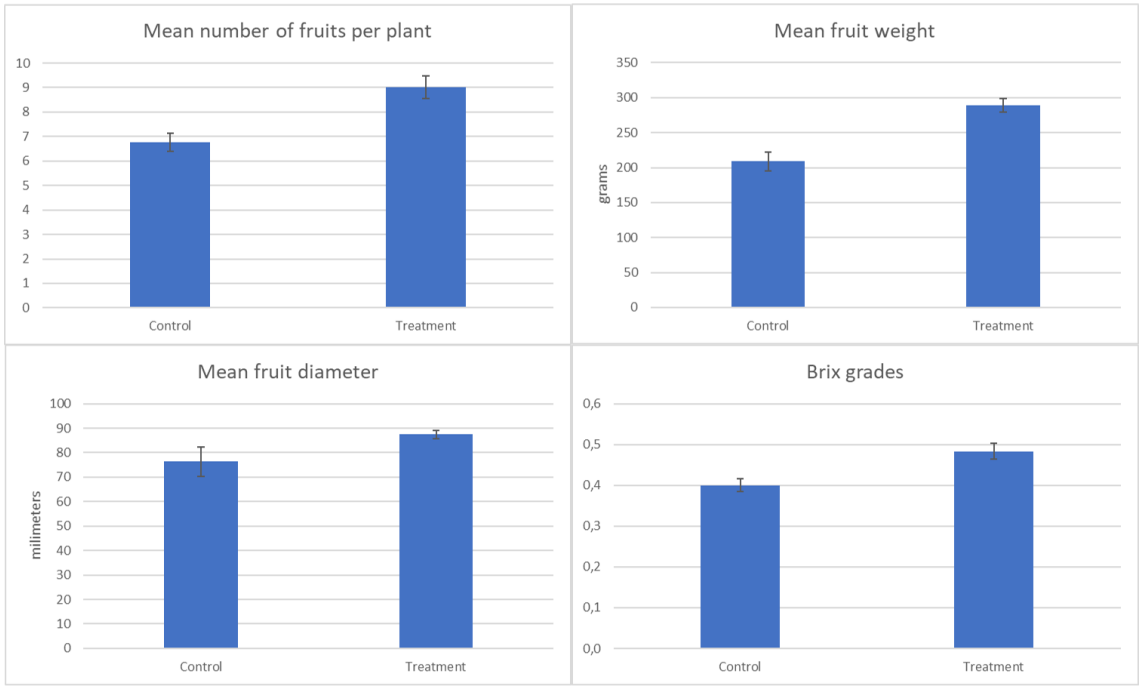


Figure 46: Mean values of parameters related to tomato yield and fruit quality

4.2.2 Impact on physicochemical soil properties

Physicochemical properties of the soil were fist analyzed before sowing. Here, main physicochemical parameters of the soil at the beginning of the trials are shown (Table 21).

Table 21: Initial properties of the soil in the field employed for field trials.

Parameter	Control	
pH	8,2	
Conductivity at20ºC	0,671	dS/m
Organic matter	2,4	%
Organic carbon	1,4	%

Macronutrients	Control	
Total N	0,117	%
Available P2O5	47,1	mg/kg
Available K2O	532,3	mg/kg
Available CaO	2062,1	mg/kg
Available MgO	492,2	mg/kg

Micronutrients	Control	
Iron	2,3	mg/kg
Manganese	1,4	mg/kg
Copper	0,5	mg/kg
Zinc	1,8	mg/kg
Boron	0,4	mg/kg

Texture: loamy (23% clay, 36% silt, 42% sand)

Most physicochemical parameters and nutrient content values were enhanced under the treatment with the new organic fertilizer when compared with the control treatment, thus indicating that the new fertilizer may improve soil physicochemical properties.

Table 22: Soil properties at the end of the experiment. Comparison between control and treatment 1 conditions.

Parameter	Control	Treatment 1	
pH	8,2	8,3	
Conductivity at20ºC	0,436	0,397	dS/m
C/N ratio	12,4	11,4	
CEC	23,9	24,5	meq/100 g
Organic matter	2,39	2,42	%
Organic carbon	1,39	1,41	%

Macronutrients	Control	Treatment 1	
Total N	0,112	0,123	%
Mineral N	16,8	18,45	mg/kg
Organic N	1103,2	1211,6	mg/kg
Available P2O5	53	25,3	mg/kg
Available K2O	532,6	675,9	mg/kg
Available CaO	3290,6	2715,4	mg/kg
Available MgO	535,8	717,5	mg/kg

Ratios	Value		Ideal
Ca/Mg	3,7	2,3	2 - 10
K/Mg	0,3	0,3	0,1 - 0,5
Ca / K	12,1	7,8	5,0 - 25,0
(Ca+Mg)/K	15,3	11,3	10,0 - 40,0

Micronutrient	Control	Treatment 1	
Iron	7,0	10,7	mg/kg
Manganese	2,0	3,3	mg/kg
Copper	0,6	1,0	mg/kg
Zinc	1,6	2,1	mg/kg
Boron	0,1	0,1	mg/kg

	Control	Treatment 1	
Exchangeable sodium	2,8	2,4	meq/100g
ESP (Exch. Sod. %)	11,5	9,6	

4.2.3 Impact on soil microbial properties

Microbial activity of the soil was determined by measuring soil respiration. Soil treated with the new fertilizer showed enhanced metabolic activity compared to the control soil.

Table 23: Soil biological activity under treatment and control conditions.

Soil basal values			
Biologic activity			
	Control	Treatment	
Basal respiration at 20ºC	1,5	1,8	µg C-CO2/kg soil*min
OM consumption at field capacity	5.193	5.865	kg MO/ha*year
Basal power at 20ºC	6	7	kW/ha
Microbial biomass	1.822	2.191	kg/ha
Organic matter	92.157	93.483	kg/ha
OM stock at field capacity	17,7	15,9	years
Genetic information content			
	Control	Treatment	
Nucleic acids	84	101	kg/ha

Microbial composition of the soil was studied by performing 16S metagenomic analysis with an Illumina MiSeq sequencer. The treatment with the new organic fertilizer enhanced relative abundance of plant beneficial bacteria in the soil, as the functional indexes show.

Table 24: Dominant taxa in the soil

Taxon	Control	Treatment
Phylum	Planctomycetes	Planctomycetes
Class	Planctomycetacia	Planctomycetacia
Order	Pirellulales	Pirellulales
Family	Pirellulaceae	Pirellulaceae
Genus	*Pirellula*	*Pirellula*
Species	*Clostridium butyricum*	*uncultivated soil bacterium clone C112*

Relative distribution of dominant phyla

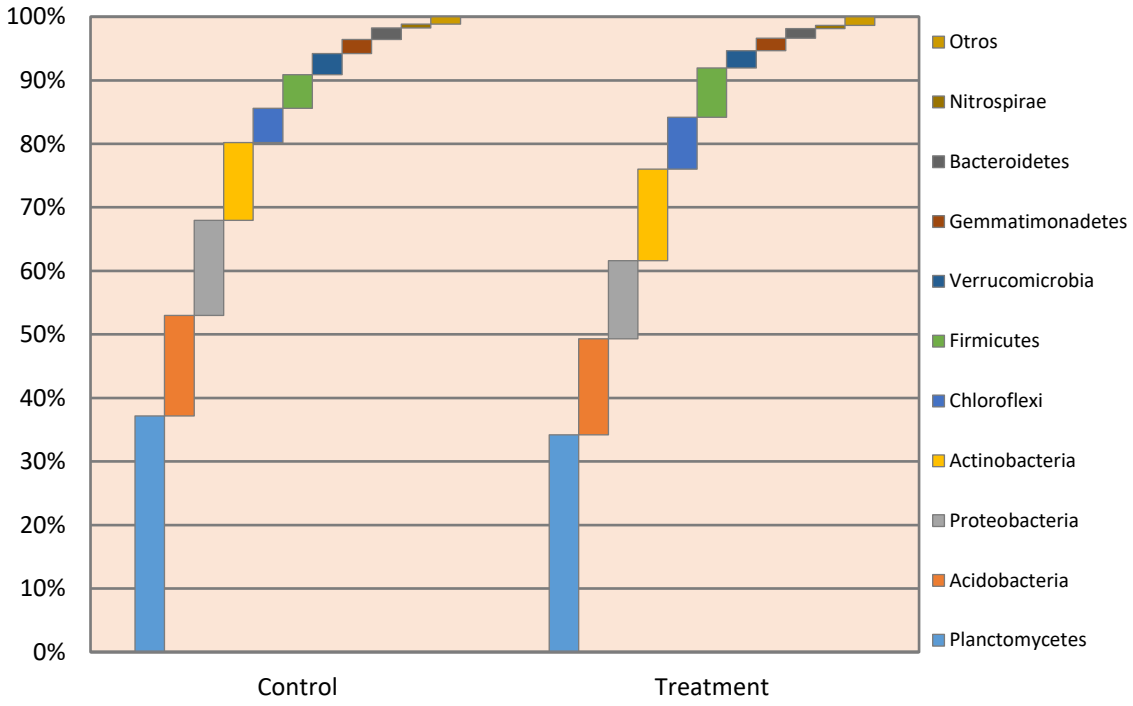


Figure 47: Dominant phyla in the soil.

Functional indexes

- Carbon cycle

Table 25: Relative abundance of copiotrophic bacteria.

	Control	Treatment
Copiotrophic index	7,99	9,01
Dominant taxon	Gammaproteobacteria	Gammaproteobacteria

- Phosphorus cycle

Table 26: Relative abundance of P-cycle bacteria

	Control	Treatment
Índice fosfoactivos	1,82	2,3
Género dominante	*Bacillus*	*Bacillus*

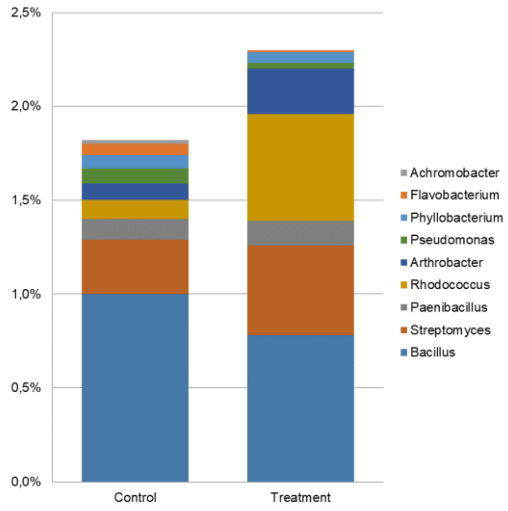
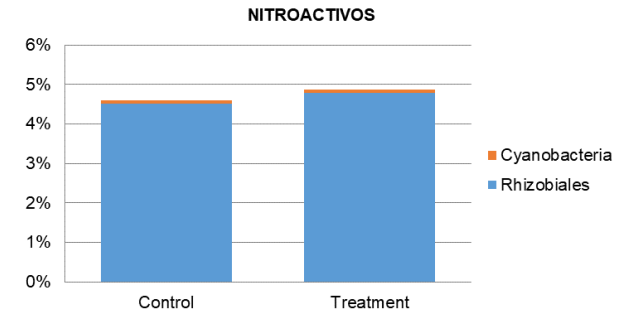


Figure 48: Relative abundance of bacteria related to P-cycle

- Nitrogen cycle

Table 27: Relative abundance of bacteria involved in N-cycle

	Control	Treatment
Índice Nitroactivos	4,6	4,87
Grupo dominante	Rhizobiales	Rhizobiales
Índice de MicroNitroactivos	2,25	2,42
Grupo dominante	*Bacillus*	*Bacillus*



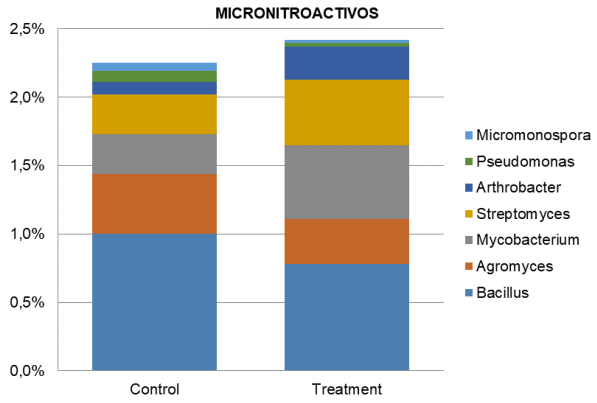


Figure 49: Relative abundance of bacteria involved in N-cycle

- Plant Growth Promoting Rhizobacteria

Table 28: Relative abundance of bacteria involved in Plant growth promoting rhizobacteria.

	Control	Treatment
Índice PGPR	1,64	1,62
Grupo dominante	*Bacillus*	*Bacillus*
Índice ACCD	1,68	1,77

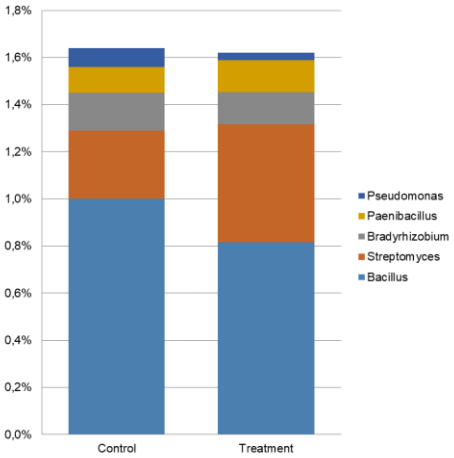


Figure 50: Relative abundance of bacteria involved in Plant growth promoting rhizobacteria.

5 Conclusions

- The biocomposites from the Agrimax project resulted in net N immobilization and reduced P availability, which means that they have to be applied to soil at the right time (not directly before sowing/planting). The biofertilizers increased crop N availability.
- Most biocomposites were degraded relatively rapidly in soil, hence contributing only modestly to soil organic matter build-up, with some notable exceptions (e.g. PLA-C and CAC). The composts had highly variable contributions to soil organic matter build-up.
- Almost all materials from the Agrimax project significantly increased soil microbial biomass and soil microbial (enzymes) activity, thus hinting at a positive impact on (biological) soil quality.
- N_2O emissions were extremely low and it appears that (for the selection of materials tested for N_2O emissions) there will be no increase in emissions upon application of these materials to soils.

- Improved compost supplemented with microorganisms CBIO1 is postulated as a compost with the capacity to be applied as an agricultural substrate not only for nutritional and plant growth promotion purposes, but also for bioprotection against the development of diseases.
- The application rate of hydrocompost on tomato plants was selected taking into account the previously observed non-phytotoxic effect on the germination rate. Thus, it was possible to observe beneficial effects after hydrocompost application on tomato seedlings, which were reflected in the aerial growth of the plant as well as in root weight. This effect was not immediate, it was most noticeable 40 days after transplanting, starting at phenological stage P5.

- Application of FERT-S led to an increase in tomato development (biomass and nutrient content in plant tissues), as demonstrated in field trials performed in Spain. Moreover, FERT-S improved crop yield (higher number of fruits and higher weight) and quality-related properties (enhanced fruit diameter and brix grades). Additionally, application of the solid organic fertilizer FERT-S reduced soil electrical conductivity and improved content of soil organic matter, and nutrient such as N, K, Ca, Mg and micronutrients. This fertilizer had a positive impact on soil microbial respiration and increased microbial communities related to plant growth promotion (PGPRs). These results show coherence with experiments performed in Activity 2 (Assessment of effects of biocomposites and bio-fertilizerson soil processes and soil biological quality, where a higher release of N under application of FERT-S is shown).

6 Subpart on biodegradability and organic recycling (originally foreseen in D6.2)

Within AGRIMAX produced compounds were evaluated for biodegradability under aerobic conditions of composting and soil and under high-solids anaerobic conditions. Moreover, the disintegration in these environments was evaluated for developed pots and films in order to evaluate if organic recycling (composting and anaerobic digestion) would be a suitable end-of-life option (circular economy) and if the products can be used for agricultural applications without causing micro-plastics accumulation.

For biodegradation testing the material is previously (cryogenically) milled or added as powder to ensure a high surface ratio and optimize biodegradation rate. Disintegration is performed on the final product (pot, film) as the thickness has an important influence on the rate of falling apart. In all biodegradation tests, cellulose was taken along as a reference material to evaluate the validate the test. For all tests, the validity criteria were met. Under composting and high-solids anaerobic conditions a biodegradation of 70% should be achieved for the reference material, while in soil the biodegradation must be more than 60%.

6.1 Biodegradation under industrial composting conditions

The controlled composting biodegradation test is an optimized simulation of an intensive aerobic composting process where the biodegradability of a test item under dry, aerobic conditions is determined. The inoculum normally consists of stabilized and mature compost derived from the organic fraction of municipal solid waste (MSW). The test item is mixed with the inoculum and introduced into a static reactor vessel where it is intensively composted under optimal oxygen, temperature (58°C) and moisture conditions.

During the aerobic biodegradation of organic materials, a mixture of gases, principally carbon dioxide and water, are the final decomposition products while part of the organic material will be assimilated for cell growth. The carbon dioxide production is monitored at regular intervals and integrated to determine the carbon dioxide production rate and the cumulative carbon dioxide production. After determining the carbon content of the test compound, the percentage of biodegradation can be calculated as the percentage of solid carbon of the test compound which has been converted to gaseous, mineral C under the form of CO_2. Also the kinetics of the biodegradation can be established.

Standard followed:

- ISO 14855-1 *Determination of the ultimate aerobic biodegradability of plastic materials under controlled composting conditions – Method by analysis of evolved carbon dioxide* (2012)

6.1.1 PBS, PBS+5%PF (potato fibre) and PBS+10%PF

The biodegradation of compounds PBS, PBS+5%PF (potato fiber) and PBS+10%PF, received from AIMPLAS, was examined according to ISO 14855-1. This method allows to determine if a material will be biodegradable under industrial composting conditions and is as such suitable for applications where industrial composting is a relevant and environmentally friendly end-of-life option. After 151 days a reinoculation with 20% fresh VGF (Vegetable, Garden and Fruit waste) was performed in order to renew the microbial population and to supply extra nutrients (see Figure 51).

According to the European standard EN 13432 *Requirements for packaging recoverable through composting and biodegradation – Test scheme and evaluation criteria for the final acceptance of*

packaging (2000) a material can only be called biodegradable when the percentage of biodegradation is at least 90% in total or 90% of the maximum degradation of a suitable reference item after a plateau has been reached for both reference and test item. The maximum allowed test duration is 180 days. From the results (see Table 29) it can be concluded that test items PBS and PBS + 10% PF fulfilled the biodegradation requirement of EN 13432 (2000) within 40 (PBS + 10% PF) days and 151 days (PBS) of testing under the given aerobic conditions and are suitable for applications for which industrial composting is a useful end-of-life option such as for plant pots. The addition of 10% potato fiber increased the biodegradation rate considerably. However, test item PBS + 5% PF showed a lower biodegradation rate, but at the end the 90% biodegradation requirement of EN 13432 (2000) was also fulfilled within 180 days of testing.

Table 29: Biodegradation percentages after 40, 151 and 195 days

Test series	TOC (%)	Net CO_2 production (mg/g test item)	Biodegradation (%)		
			AVG	SD	REL
After 40 days					
PBS + 10% PF	54.9	1974	98.1	6.9	97.6
After 151 days					
PBS	56.6	1979	95.3	7.4	89.1
After 195 days					
Cellulose	42.7	1635	104.4	0.9	100.0
PBS + 5% PF	56.0	1813	88.3	2.3	84.6

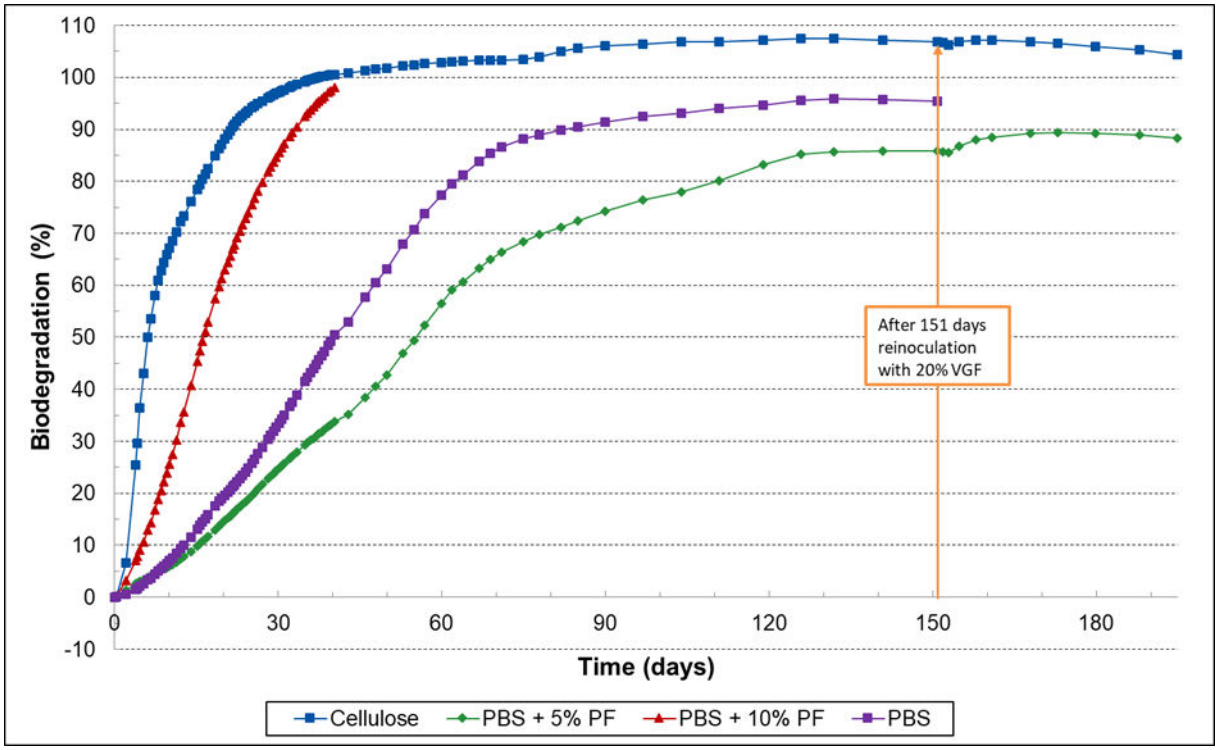


Figure 51: Evolution of the biodegradation percentage of reference and test items

6.1.2 Cellulose diacetate + 30% ATEC + 10% Bran

The result of the biodegradation of Cellulose diacetate + 30% ATEC + 10% Bran (= wheat bran), developed by FEMTO, under industrial composing conditions is given in Table 30 and Figure 52. A biodegradation of 94.9% was obtained after 150 days. The material fulfilled the 90% biodegradation requirement of EN 13432 (2000) within 180 days.

Table 30: Biodegradation percentages after 150 days

Test series	TOC (%)	Net CO_2 production (mg/g test item)	Biodegradation (%)		
			AVG	SD	REL
Cellulose	42.7	1406	89.8	11.0	100.0
Cellulose diacetate + 30% ATEC + 10% Bran	49.7	1729	94.9	0.1	105.6

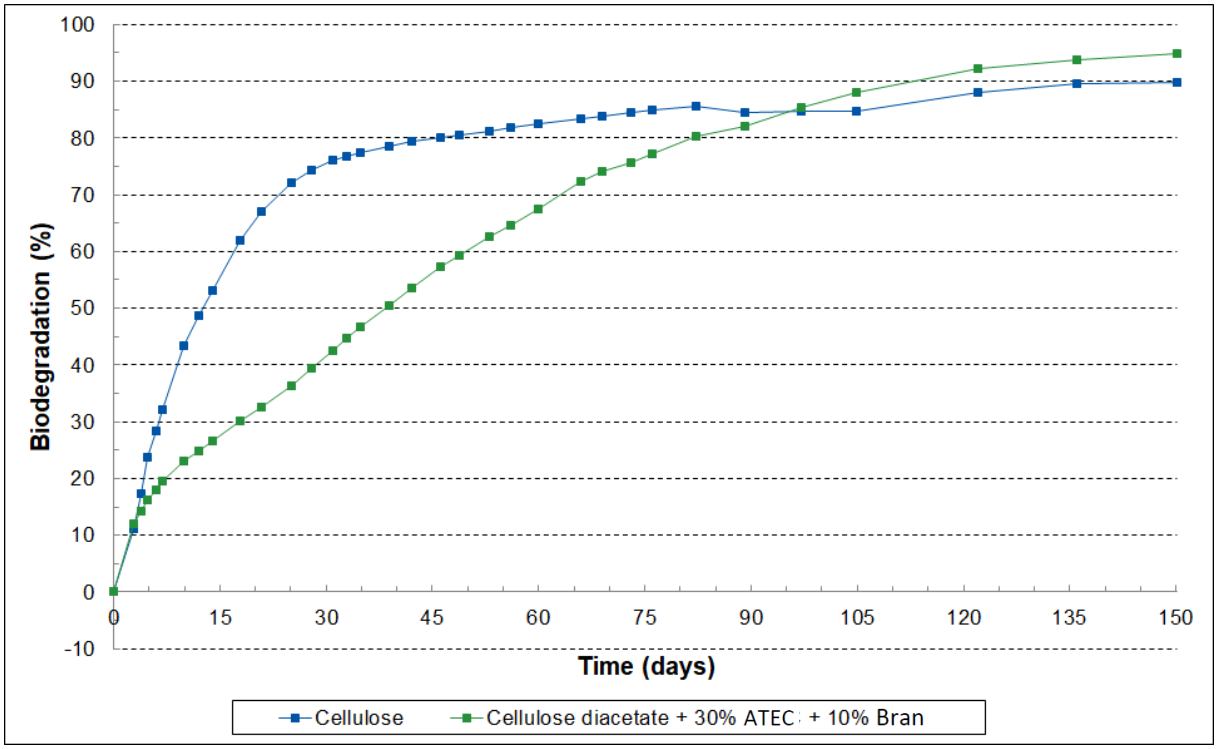


Figure 52: Evolution of the biodegradation percentage of reference and test item

6.2 Standard soil biodegradation test

The test item is directly mixed with soil and incubated in the dark at ambient room temperature (25°C). Biodegradation is taking place through microbial activity. During the aerobic biodegradation in soil a mixture of gases, principally carbon dioxide and water, is produced. The CO_2 is captured in KOH and the CO_2 production is regularly determined by titration, which allows calculating the cumulative CO_2 production. The percentage of biodegradation can be calculated as the percentage of solid carbon of the test item, which has been converted to gaseous, mineral C under the form of CO_2. Also, the kinetics of the biodegradation is established.

Standard followed:

- ISO 17556 *Plastics - Determination of the ultimate aerobic biodegradability in soil by measuring the oxygen demand in a respirometer or the amount of carbon dioxide evolved* (2019).

6.2.1 PBS, PBS+5%PF (potato fibre) and PBS+10%PF

For test items PBS and PBS + 5% PF no real breakthrough in biodegradation was seen within 180 days (Figure 53). The biodegradation of PBS + 10% PF proceeded slowly throughout the test and reached a plateau around 10%. Probably mainly the potato fiber was degraded. These results are very different compared to the biodegradation results in compost, indicating that these materials need a more aggressive environment and/or a higher temperature to obtain good biodegradation.

Table 31: Biodegradation percentages after 180 days

Test series	TOC (%)	Net CO_2 production (mg/g test item)	Biodegradation (%)		
			AVG	SD	REL
Cellulose	42.7	1125	71.8	1.6	100.0
PBS	56.6	49	2.4	1.3	3.3
PBS + 5% PF	56.0	94	4.6	0.3	6.4
PBS + 10% PF	54.9	242	12.0	2.5	16.8

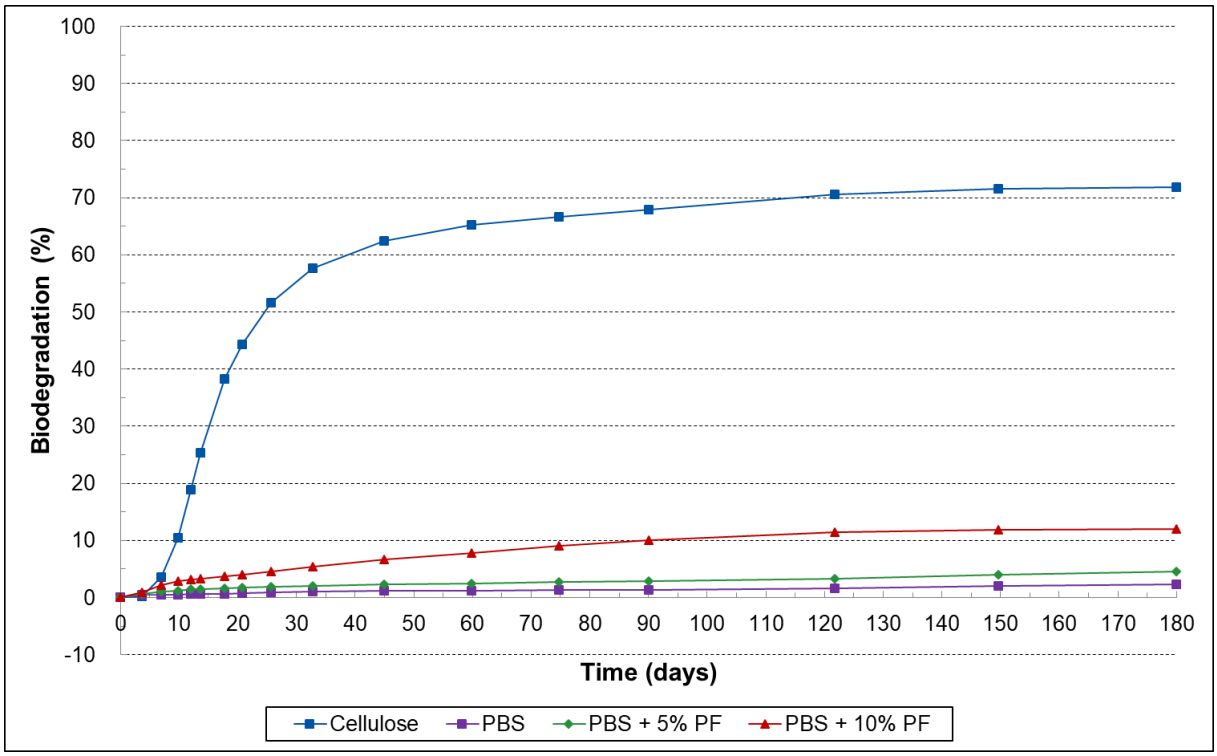


Figure 53: Evolution of the biodegradation percentage of reference and test items

6.2.2 PBSA + 5% OLIG – RE1, PBSA+0.5%AO and PBSA+5%AO

The biodegradation of PBSA + 5% OLIG – RE1, PBSA+0.5%AO and PBSA+5%AO, compounds developed by AIMPLAS and containing oligomer (OLIG) and anti-oxidant (AO) from the project, proceeded at a good rate throughout the test (*Figure 54*). All samples reached 90% relative biodegradation in soil within 1 year (*Table 32*). The OK biodegradable SOIL certification scheme of TÜV AUSTRIA Belgium and the DIN-Geprüft biodegradable in soil conformity mark of DIN CERTCO stipulate a biodegradation of at least 90% in total or 90% of the maximum degradation of a suitable reference item after a plateau has been reached for both reference and test item. The 90% limit must be reached within 2 years and the test should be executed at ambient temperature. These criteria are also applied in EN 17033 *Plastics* -

Biodegradable mulch films for use in agriculture and horticulture - Requirements and test methods (2018). This means that these compounds do fulfill these requirements and can be used for production of biodegradable mulch films.

Table 32: Biodegradation percentages after 360 days

Test series	TOC (%)	Net CO_2 production (mg/g test item)	Biodegradation (%)		
			AVG	SD	REL
Cellulose	42.7	1320	84.3	1.8	100.0
PBSA + 5% OLIG – RE1	57.5	1908	90.5	5.1	107.3
PBSA+0.5%AO	57.1	1627	77.7	3.2	92.2
PBSA+5%AO	57.0	1706	81.6	1.3	96.8

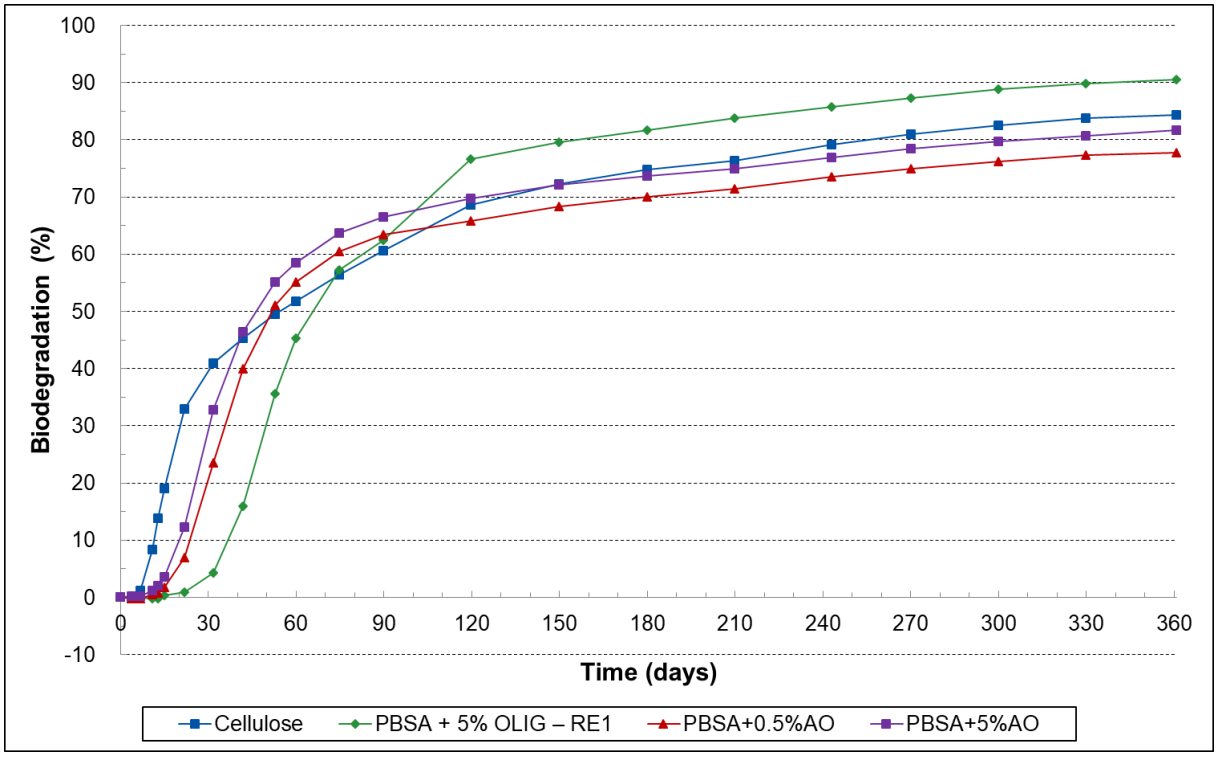


Figure 54: Evolution of the biodegradation percentage of reference and test items

6.2.3 PBSA + 5% oligomer and Film PBSA + 5% oligomer + 10% PBAT

The soil biodegradation test on samples PBSA + 5% oligomer and Film PBSA + 5% oligomer + 10% PBAT, produced by AIMPLAS, is still running. The biodegradation is proceeding at a slow rate (*Figure 55*) and much slower compared to the PBSA + 5% OLIG. Still, at the current speed it is still possible to reach 90% relative biodegradation within 2 years for both samples. The addition of PBAT decreased the biodegradation rate and might result in insufficient biodegradation.

Table 33: Biodegradation percentages after 524 days

Test series	TOC (%)	Net CO_2 production (mg/g test item)	Biodegradation (%)		
			AVG	SD	REL
Cellulose	42.7	1297	82.8	3.1	100.0
PBSA + 5% oligomer	57.8	1477	70.8	8.2	85.5
Film PBSA + 5% oligomer + 10% PBAT	57.6	1199	58.0	8.8	70.1

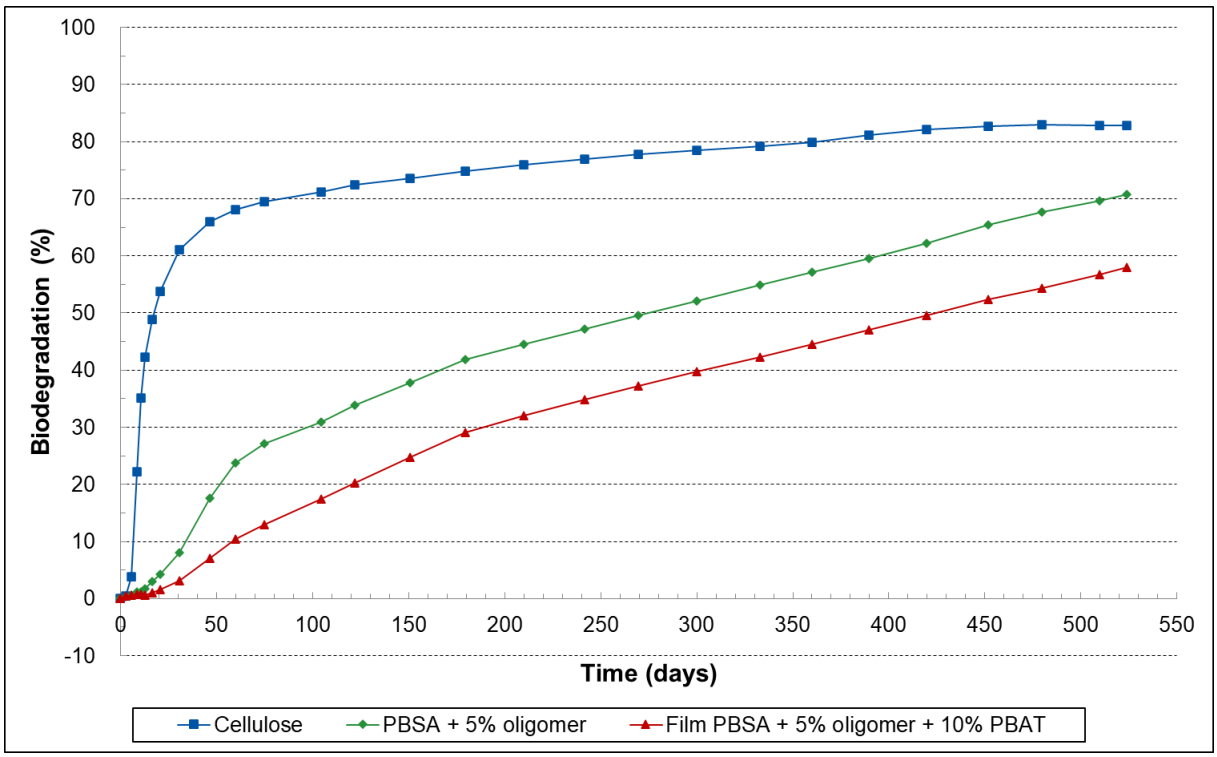


Figure 55: Evolution of the biodegradation percentage of reference and test items

6.3 High-solids anaerobic biodegradation test

The biodegradability of products in a sanitary landfill or in a solid-state anaerobic digestion system is determined through high-rate dry anaerobic batch fermentation. The HSAD method simulates a solid-state anaerobic digestion system (e.g. a landfill) because it is a stationary (no mixing) and dry (>20% solids) fermentation. It accelerates the biodegradation process because of the optimal conditions provided, including optimum temperature and high inoculation. The incubation temperature was 52°C ± 2°C.

A small amount of test item is added to a large amount of highly active inoculum that has been stabilised prior to the start of the digestion period. Optimal conditions for pH, nutrients, volatile fatty acids, etc. are provided and the mixture is left to ferment batch-wise. Likewise, biodegradation is not influenced by other factors than those inherent to the test item itself.

During the anaerobic biodegradation of organic materials, a mixture of gases, principally methane and carbon dioxide, are the final decomposition products, while some of the organic material will be assimilated for cell growth. The volume of the biogas produced is measured and the amount of CH_4 and CO_2 produced per weight unit of test item is calculated. If the carbon content of the test item is

known, the percentage of biodegradation can be calculated as the percentage of solid carbon of the test item that has been converted to gaseous, mineral C.
Standards followed:

- ASTM D5511 *Standard Test Method for Determining Anaerobic Biodegradation of Plastic Materials Under High-Solids Anaerobic-Digestion Conditions* (2018);
- ISO 15985 *Plastics – Determination of the ultimate anaerobic biodegradation and disintegration under high-solids anaerobic-digestion conditions - Method by analysis of released biogas* (2014).

6.3.1 Cellulose diacetate + 30% ATEC+ 10% Bran and PBS FZ71 + 10% potato fibres

The biodegradation of Cellulose diacetate + 30% ATEC + 10% Bran started immediately at a good rate and after 8 days already, a biodegradation percentage of 65.9% was reached. From then on, biodegradation rate slowed down to form a plateau and at the end of the test (63 days) a biodegradation percentage of 81.2% ± 0.7% or 101.7% relative to cellulose was reached. No real breakthrough in biodegradation was observed for PBS FZ71 PB + 10% potato fibers. After 6 days a plateau was formed and at the end of the test a biodegradation percentage of 6.7% ± 0.7% or 8.4% relative to cellulose was reached. It seems that PBS FZ71 PB is not degradable under anaerobic conditions.

In general, a test item has shown a satisfactory level of biodegradation when 90% absolute or relative biodegradation is reached. Therefore, Cellulose diacetate + 30% ATEC + 10% Bran can be considered as biodegradable under high-solids, thermophilic, anaerobic conditions within 15 days, while PBS FZ71 PB + 10% potato fibers cannot be considered as biodegradable under these conditions within 63 days.

Table 34: Biodegradation percentages at the end of the test (63 days)

Test series	TOC (%)	Net biogas production (Nl/kg test item)	Biodegradation (%)		
			AVG	SD	REL
Cellulose	43.6	650.1	79.8	8.1	100.0
Cellulose diacetate + 30% ATEC+ 10% Bran	48.6	737.7	81.2	0.7	101.7
PBS FZ71 PB + 10% potato fibres	54.7	68.9	6.7	0.7	8.4

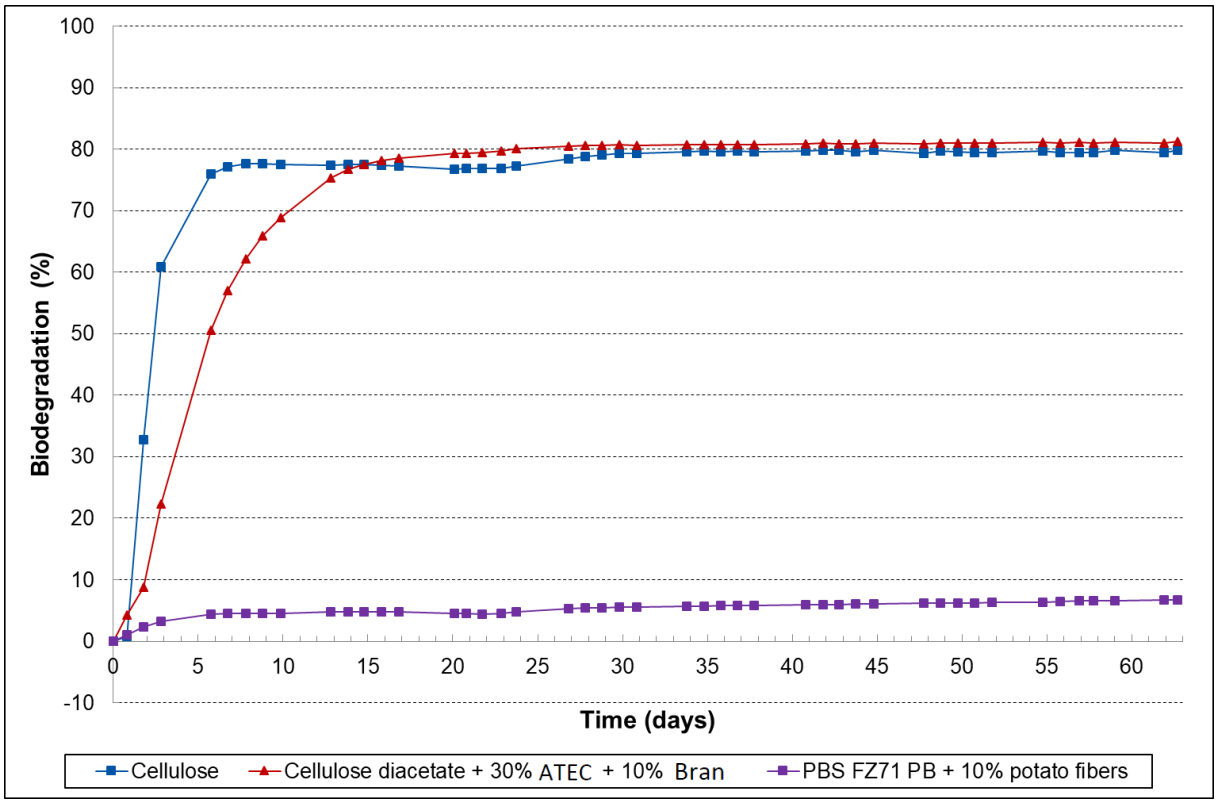


Figure 56: Evolution of the average biodegradation percentage of reference and test items

6.3.2 PLA + 11% Bran and PHBV + 15% Bran

The biodegradation of PLA + 11% Bran and PHBV + 15% Bran, produced by FEMTO, started after a lag phase of approximately 3 days and proceeded at a slow to moderate rate for PLA + 11% Bran and at a good rate for PHBV + 15% Bran. The biodegradation rate slowed down to form a plateau and after 20 days a biodegradation of 74.3% was measured for PHBV + 15% Bran (101.2% relative to cellulose). After 90 days a biodegradation percentage of 59.9% ± 5.0% and 74.9% ± 1.9% was reached. Relative to cellulose, a biodegradation percentage of 75.6% and 94.5% was calculated.

In general, a test item has shown a satisfactory level of biodegradation when 90% absolute or relative biodegradation is reached. Therefore, PHBV + 15% Bran can be considered as biodegradable under high-solids, thermophilic, anaerobic conditions within 20 days, while PLA + 11% Bran cannot be considered as completely biodegradable under these conditions within 90 days. Only partial biodegradation was obtained.

Table 35: Biodegradation percentages after 90 days

Test series	TOC (%)	Net biogas production (Nl/kg test item)	Biodegradation (%)		
			AVG	SD	REL
Cellulose	43.6	645.9	79.3	1.8	100.0
PLA + 11% Bran	48.5	542.4	59.9	5.0	75.6
PHBV + 15% Bran	52.5	735.1	74.9	1.9	94.5

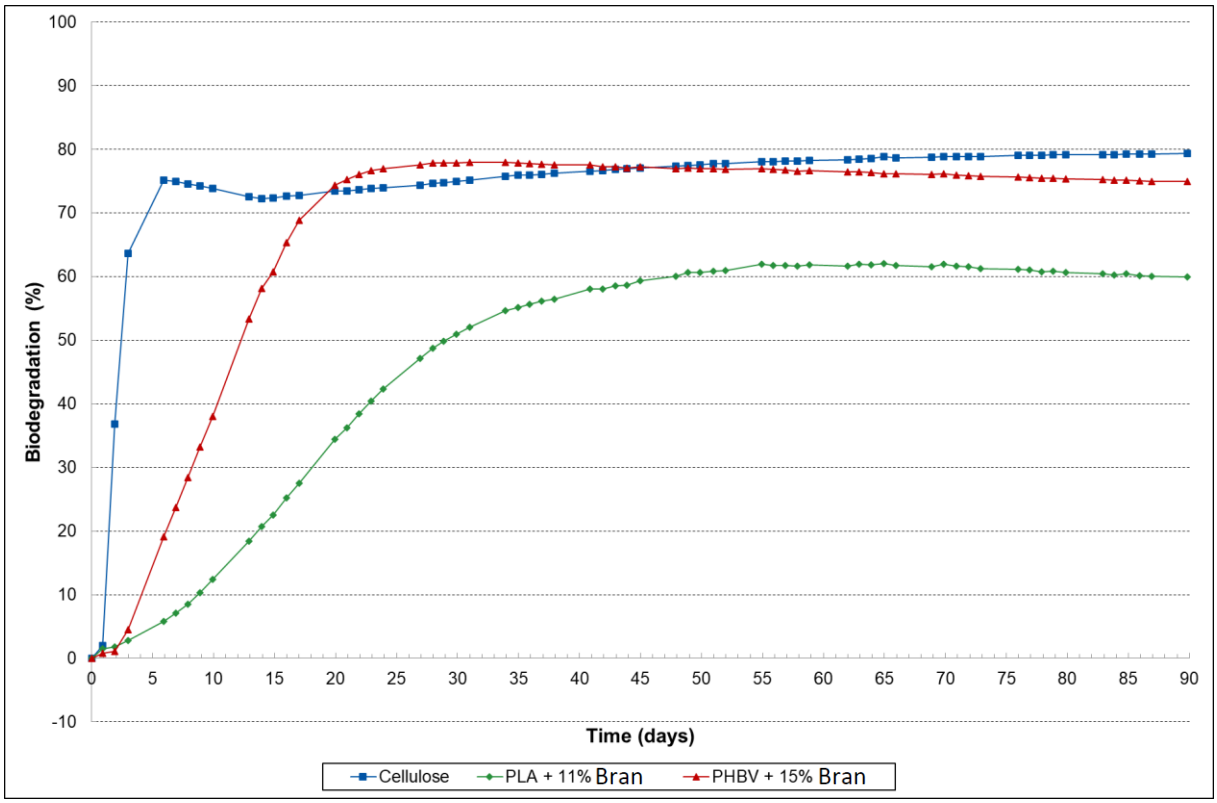


Figure 57: Evolution of the average biodegradation percentage of reference and test items

6.4 Disintegration simulating industrial composting conditions

The pilot-scale aerobic composting test simulates as closely as possible a real and complete composting process in composting bins of 200 l. The test item is mixed with the organic fraction of fresh, pretreated municipal solid waste (biowaste) and introduced in an insulated composting bin after which composting spontaneously starts. Like in full-scale composting, inoculation and temperature increase happen spontaneously. The test is considered valid only if the maximum temperature during composting is above 60°C and below 75°C, and if the daily temperature remains above 40°C during at least 4 weeks. The composting process is directed through air flow and moisture content. The temperature and exhaust gas composition are regularly monitored. The composting process is continued till fully stabilized compost is obtained (3 months). During composting, the contents of the vessels are turned manually, at which time test item can be retrieved and visually evaluated. The applied test method is based on ISO 16929 (2019). The maximum allowed duration is 12 weeks and disintegration is defined as a size reduction till < 2mm.

Standard followed:

- ISO 16929 *Plastics – Determination of the Degree of Disintegration of Plastics Materials under Defined Composting Conditions in a Pilot-Scale Test* (2019)

6.4.1 Plant pots: PBS + 5% potato fibre and PBS +10% potato fibre

The disintegration under industrial composting conditions of injected plants pots from compound PBS + 5% potato fibre (thickness: ± 0.71 mm (body), ± 0.80 mm (bottom) and ± 1.3 mm (edge)) and compound PBS + 10% potato fibre (thickness: ± 0.68 mm (body), ± 0.76 mm (bottom) and ± 1.3 mm (edge)), produced by AIMPLAS, proceeded significantly (*Table 37*). After 12 weeks the major part of the material was disappeared. Only some few small pieces were retrieved. This test was performed qualitatively, which means that it is a visual evaluation of the disintegration, but the results

demonstrated that high disintegration levels can be obtained and that these plants have the potential to reach the 90% disintegration requirement as stipulated by EN 13432 (2000) when tested quantitatively.

6.4.2 Plant pots: PBS + 15% potato fibre and PBS + 20% potato fibre

Plant Pot PBS + 15% potato fibre (thickness: 0.61 mm (body), 0.75 mm (bottom) and 1.36 mm (edge)) and Pot PBS + 20% potato fibre (thickness: 0.64 mm (body), 0.97 mm (bottom) and 1.54 mm (edge)), produced by AIMPLAS, were tested quantitatively in line with ISO 16929 involving a 1% addition and thorough mass balance at the end of the test. Disintegration proceeded fairly well, but remained insufficient (*Table 38*). After carefully selecting all fractions (2-5 mm, 5-10 mm, > 10 mm) at the end of the test, several small pieces of Pot PBS + 15% potato fibre and Pot PBS + 20% potato fibre could be retrieved in the different fractions (*Figure 58*). A disintegration percentage of 80.3% and 86.0% was obtained for Pot PBS + 15% potato fibre and Pot PBS + 20% potato fibre, respectively (*Table 36*). This means that in spite of the high potato fibre content, which was expected to facilitate the disintegration, the 90% disintegration requirement was just not fulfilled. However, it was observed that the production of Pot PBS + 15% potato fibre and Pot PBS + 20% potato fibre was optimized compared to the PBS pots with 5% and 10% potato fiber. Moreover, when decreasing somewhat the thickness of the thicker parts (bottom and especially edge), sufficient disintegration can be obtained.

Table 36: Disintegration of Pot PBS + 15% potato fibre and Pot PBS + 20% potato fibre after 12 weeks of composting

Test item	Remaining sample > 2 mm (%)	Disintegration (%)			Remaining sample > 10 mm (%)
		< 2 mm	< 5 mm	< 10 mm	
Pot PBS + 15% potato fibre	19.7	**80.3**	82.3	88.6	11.4
Pot PBS + 20% potato fibre	14.0	**86.0**	88.2	95.6	4.4

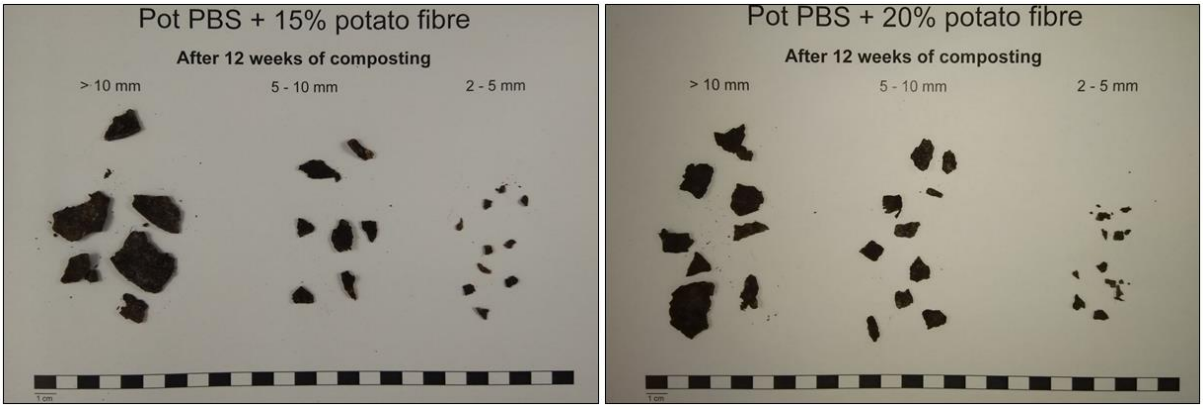


Figure 58: Visual presentation of some of the retrieved pieces of Pot PBS + 15% potato fibre (left) and Pot PBS + 20% potato fibre (right) in the different fractions at the end of the test

6.4.3 Plant pots: Cellulose diacetate + 30% ATEC+ 10% Bran, PBS FZ71 PB + 10% potato fibers, PLA + 11% Bran and PHBV + 15% Bran

Pots Cellulose diacetate + 30% ATEC + 10% Bran (thickness: ± 1.6 mm (body), ± 1.9 mm (bottom) and ± 1.5 mm (edge)), PBS FZ71 PB + 10% potato fibers (thickness: ± 1.5 mm (body), ± 1.7 mm (bottom) and ± 1.5 mm (edge)), PLA + 11% Bran (thickness: ± 1.5 mm (body), ± 1.8 mm (bottom) and ± 1.6 mm (edge)) and PHBV + 15% Bran (thickness: ± 1.5 mm (body), ± 1.8 mm (bottom) and ± 1.5 mm (edge)), produced by FEMTO, were tested qualitatively for disintegration under industrial composting conditions. The disintegration of PLA + 11% Bran and PHBV + 15% Bran proceeded well, while the

disintegration of Cellulose diacetate + 30% ATEC + 10% Bran and PBS FZ71 PB + 10% potato fibers was insufficient (*Table 39*). Based on the results it can be concluded that test items Cellulose diacetate + 30% ATEC + 10% Bran and PBS FZ71 PB + 10% potato fibers will not have the potential to reach the 90% pass level, while PLA + 11% Bran and PHBV + 15% Bran will reach the 90% disintegration requirement as stipulated by EN 13432 (2000) when tested quantitatively. By reducing the thickness of pots made of PBS FZ71 PB + 10% potato fibers, the 90% criterion can still be reached. For the Cellulose diacetate pots the reduction in thickness should be significantly larger, resulting possibly in too low mechanical properties for plant pots.

6.4.4 Mogu plant pot

Mogu plant pot (thickness: ± 15.6 mm (edge), ± 18.8 mm (wall), ± 28.0 mm (bottom); 13.9 mm (bottom middle)) was tested qualitatively for disintegration under industrial composting conditions. The disintegration proceeded well (Table 40). After 4 weeks of composting all test material had fallen apart into large fragments and 4 weeks later only some small test item pieces were retrieved from the bin. At the end of the test (= 12 weeks) all pieces had completely disintegrated. Based on the results it can be concluded that test item Mogu plant pot will reach the 90% disintegration requirement as stipulated by EN 13432 (2000).

Table 37: Visual presentation of the disintegration of plant pots made of PBS + 5% potato fibre (thickness: ± 0.71 mm (body), ± 0.80 mm (bottom) and ± 1.3 mm (edge)) and PBS + 10% potato fibre (thickness: ± 0.68 mm (body), ± 0.76 mm (bottom) and ± 1.3 mm (edge)) during 12 weeks of composting

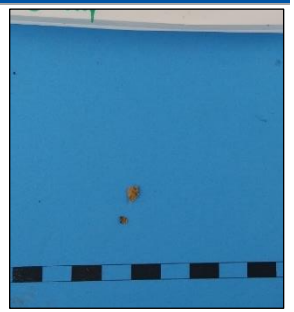
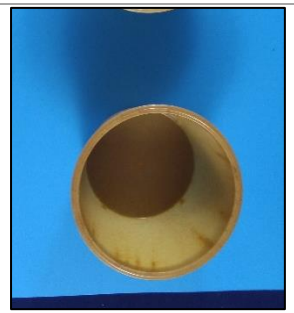
Test items	At start	2 weeks	4 weeks	6 weeks	10 weeks	12 weeks
Injected pots Compound PBS + 5% potato fibre						
Injected pots Compound PBS + 10% potato fibre						

Table 38: Visual presentation of the disintegration of Pot PBS + 15% potato fibre (thickness: 0.61 mm (body), 0.75 mm (bottom) and 1.36 mm (edge)) and Pot PBS + 20% potato fibre (thickness: 0.64 mm (body), 0.97 mm (bottom) and 1.54 mm (edge)) during 12 weeks of composting

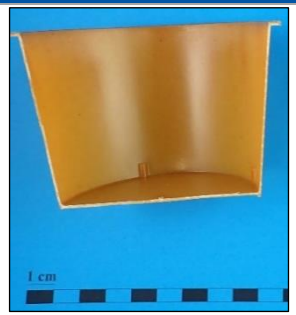
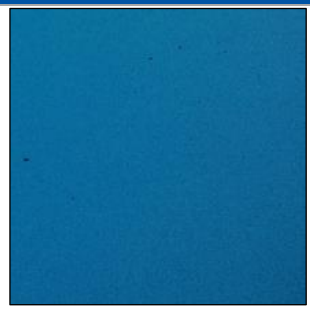
Test items	At start	1 week	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
Pot PBS + 15% potato fibre							
Pot PBS + 20% potato fibre							

Table 39: Visual presentation of the disintegration of Cellulose diacetate + 30% ATEC + 10% Bran, PBS FZ71 PB + 10% potato fibers, PLA + 11% Bran and PHBV + 15% Bran during 12 weeks of composting (thickness: ± 1.5-1.6 mm (body), ± 1.7-1.9 mm (bottom) and ± 1.5-1.6 mm (edge))

Test item	At start	1 Week	2 Weeks	3 Weeks	4 Weeks	6 Weeks	8 Weeks	10 Weeks	12 Weeks
Cellulose diacetate + 30% ATEC + 10% Bran									
PBS FZ71 PB + 10% potato fibers									
PLA + 11% Bran									
PHBV + 15% Bran									

Table 40: Visual presentation of the disintegration of Mogu plant pot (thickness: ± 15.6 mm (edge), ± 18.8 mm (wall), ± 28.0 mm (bottom); 13.9 mm (bottom middle)) during 12 weeks of composting

At start	1 week	2 weeks	4 weeks	8 weeks	12 weeks
					

6.5 Disintegration tests in soil

The purpose of this test is to evaluate the disintegration of a material at ambient temperature in soil. Regularly the moisture content is verified and adjusted when needed. At the same time the soil is manually stirred and the test item is visually monitored. The test conditions are based on the international standard ISO 17556. Disintegration is defined as a size reduction till < 2 mm.

Standard followed:

- ISO 17556 *Plastics - Determination of the ultimate aerobic biodegradability of plastic materials in soil by measuring the oxygen demand in a respirometer or the amount of carbon dioxide evolved* (2019)

6.5.1 Plant pots: PBS + 5% potato fibre and PBS +10% potato fibre

Injected pots Compound PBS + 5% potato fibre (thickness: ± 0.71 mm (body), ± 0.80 mm (bottom) and ± 1.3 mm (edge)) and Injected pots Compound PBS + 10% potato fibre (thickness: ± 0.68 mm (body), ± 0.76 mm (bottom) and ± 1.3 mm (edge)) were qualitatively tested for disintegration in soil. After 4 weeks already considerable disintegration was observed as the pots were falling apart. After 20 weeks the pots were fallen apart in big pieces and after 52 weeks only some small test item pieces could be retrieved for one replicate of Compound PBS + 5% potato fibre and both replicates of Compound PBS + 10% potato fibre (*Table 41*). For the other replicate of Compound PBS + 5% potato fibre a significant lower disintegration was observed. This might be related to impurities during production. The disintegration under industrial composting conditions proceeded much faster.

6.5.2 Films: PBSA + 5% oligomer and Film PBSA + 5% oligomer + 10% PBAT

Film PBSA + 5% oligomer (90 µm) and Film PBSA + 5% oligomer + 10% PBAT (95 µm) were qualitatively tested for disintegration as 5 cm x 5 cm pieces. Part of the pieces was placed on top of the soil in order to simulate the exposure of a mulching film on the land, while the other part was mixed in the soil. The disintegration proceeded well for Film PBSA + 5% oligomer. After 4 weeks of incubation small tears were noticed in the pieces in the soil, while the pieces on top of the soil remained intact. Four weeks later the pieces in the soil had started to fall apart into small pieces, while the pieces on top of the soil started to degrade. The test material showed complete disintegration after 16 weeks of incubation for both the PBSA + 5% oligomer film (90 µm) on top of the soil as the material incubated in soil (*Table 42*). In fact the disintegration rate might be too fast as a mulching film must often remain intact for several months. The introduction of PBAT in the compound reduced the disintegration rate. After 16 weeks all pieces of Film PBSA + 5% oligomer + 10% PBAT in the soil had completely disintegrated, however, the pieces on top of the soil were still rather intact. After 52 weeks of incubation still large pieces of test item were retrieved on top of the soil (*Table 42*). The PBAT content decreases the disintegration rate considerably. By tuning with the amount of PBAT suitable disintegration rates in line with the harvest periods might be obtained. However, it must be demonstrated that the applied PBAT also biodegrade completely in soil.

6.5.3 Trilayer films

The disintegration of films Trilayer film A/B/C; A=B=C= PBSA + 10% PBAT (49 µm), Trilayer film A/B/C; A and B = PBSA +10%PBAT, C= PBSA+5%oligomer + 10% PBAT (45 µm) and Trilayer film A/B/C; A and B = PBSA +10%PBAT; C= PBSA+5%oligomer (45 µm), produced by AIMPLAS, in soil was qualitatively evaluated. The disintegration in soil proceeded well for all three samples. Already after 4 weeks considerable disintegration was observed. Test material Trilayer film A/B/C; A and B = PBSA +10%PBAT,

C= PBSA+5%oligomer + 10% PBAT (45 µm) showed complete disintegration within 16 weeks of incubation in soil (Figure 61), while complete disintegration was obtained within 22 weeks for test materials Trilayer film A/B/C; A=B=C= PBSA + 10% PBAT (49 µm; Figure 59) and Trilayer film A/B/C; A and B = PBSA +10%PBAT; C= PBSA+5%oligomer (45 µm; Figure 61).

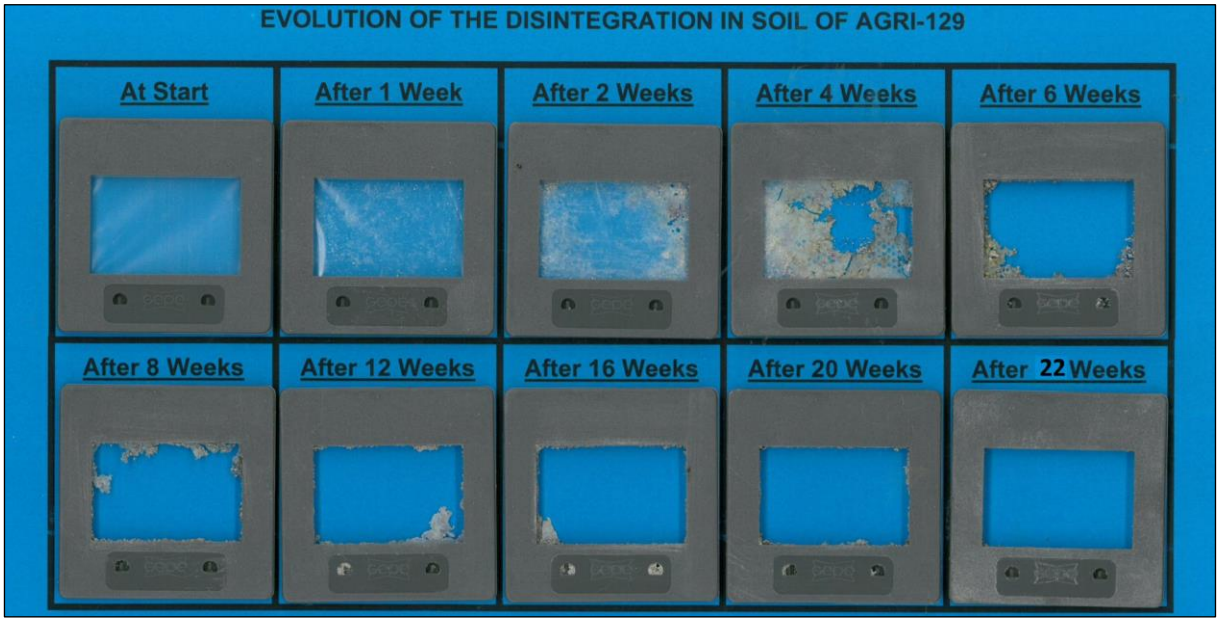


Figure 59: Visual presentation of the evolution of the disintegration of test material Trilayer film A/B/C; A=B=C= PBSA + 10% PBAT (49 µm), put into slide frames, during the incubation process in soil

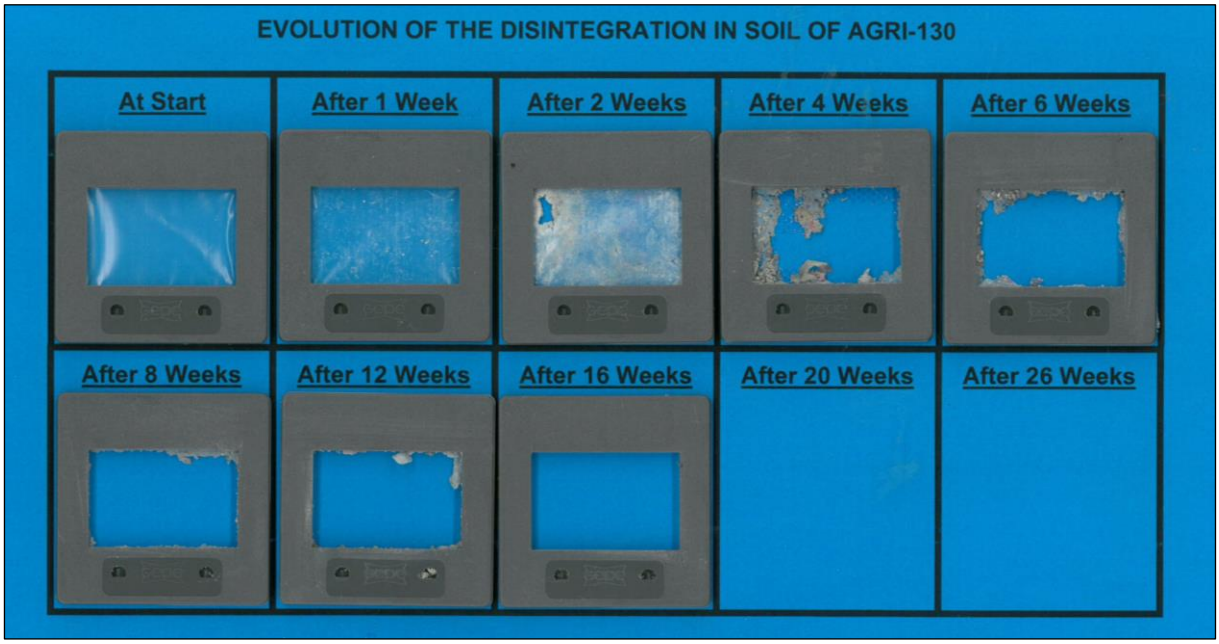


Figure 60: Visual presentation of the evolution of the disintegration of test material Trilayer film A/B/C; A and B = PBSA +10%PBAT, C= PBSA+5%oligomer + 10% PBAT (45 µm), put into slide frames, during the incubation process in soil

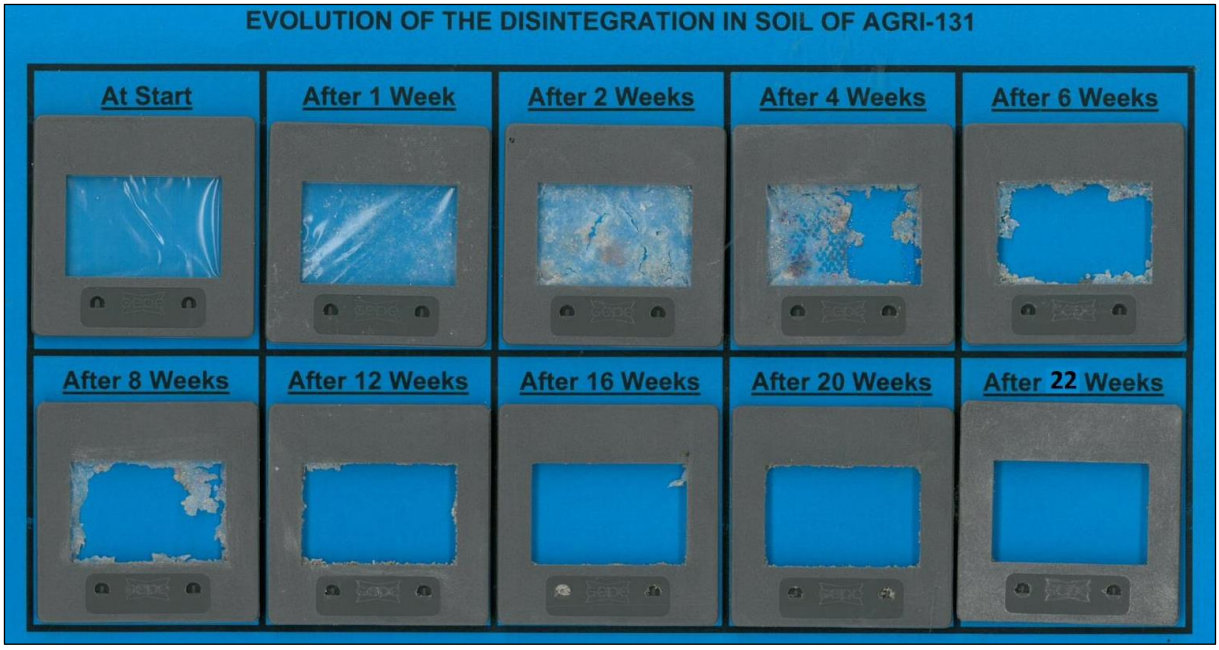


Figure 61: Visual presentation of the evolution of the disintegration of test material Trilayer film A/B/C; A and B = PBSA +10%PBAT; C= PBSA+5%oligomer (45 µm), put into slide frames, during the incubation process in soil.

6.5.4 Plant pots: Cellulose diacetate + 30% ATEC + 10% Bran, PBS FZ71 PB + 10% potato fibers, PLA + 11% Bran and PHBV + 15% Bran

Pots Cellulose diacetate + 30% ATEC + 10% Bran (thickness: ± 1.6 mm (body), ± 1.9 mm (bottom) and ± 1.5 mm (edge)), PBS FZ71 PB + 10% potato fibers (thickness: ± 1.5 mm (body), ± 1.7 mm (bottom) and ± 1.5 mm (edge)), PLA + 11% Bran (thickness: ± 1.5 mm (body), ± 1.8 mm (bottom) and ± 1.6 mm (edge)) and PHBV + 15% Bran (thickness: ± 1.5 mm (body), ± 1.8 mm (bottom) and ± 1.5 mm (edge)) were qualitatively tested for disintegration in soil. The edge of the pots was added as pieces of approximately 2.5 cm x 2.5 cm. The disintegration of PBS FZ71 PB + 10% potato fibers and PHBV + 15% Bran proceeded quite well. Only some small pieces could be retrieved after 26 weeks, while after 36 weeks the material was completely disappeared. However, cellulose diacetate + 30% ATEC + 10% Bran and PLA + 11% Bran remained largely intact during 52 weeks of incubation (*Table 43*). The disintegration of PLA + 11% Bran proceeded much slower in soil when compared to industrial composting conditions. It is known that PLA needs a thermal trigger (> 55°C) before the biodegradation starts. These higher temperatures are not established in soil.

Table 41: Visual presentation of the disintegration of Injected pots Compound PBS + 5% potato fibre (thickness: ± 0.71 mm (body), ± 0.80 mm (bottom) and ± 1.3 mm (edge)) and Injected pots Compound PBS + 10% potato fibre (thickness: ± 0.68 mm (body), ± 0.76 mm (bottom) and ± 1.3 mm (edge)) during 52 weeks of incubation in soil

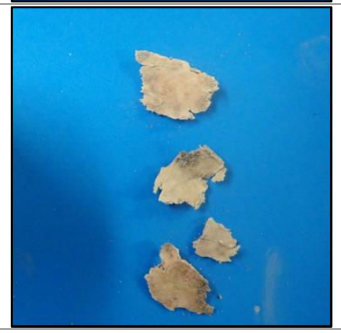
Test items	At start	4 weeks	10 weeks	20 weeks	52 weeks
Injected pots Compound PBS + 5% potato fibre					
Injected pots Compound PBS + 10% potato fibre					

Table 42: Visual presentation of the disintegration of Film PBSA + 5% oligomer (90 µm) and Film PBSA + 5% oligomer + 10% PBAT (95 µm) during 52 weeks of incubation on the soil surface.

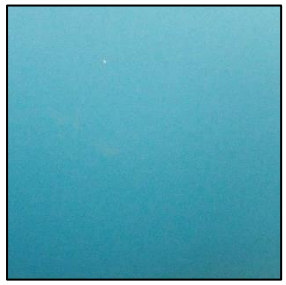
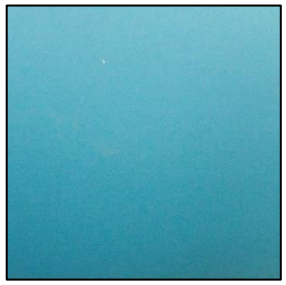
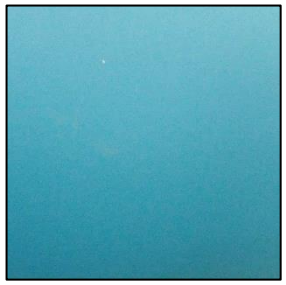
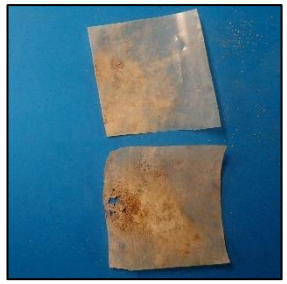
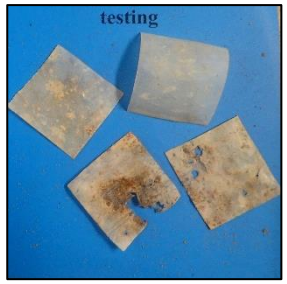
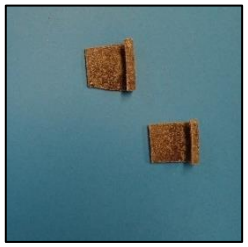
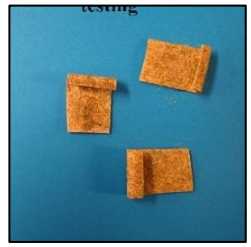
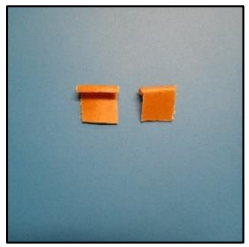
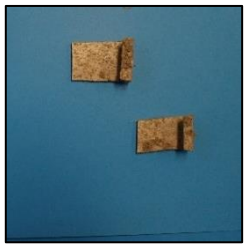
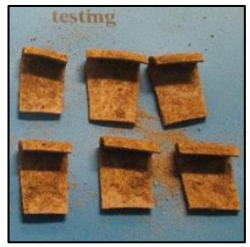
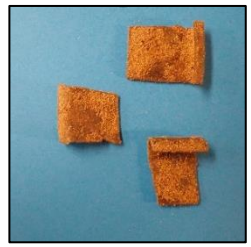
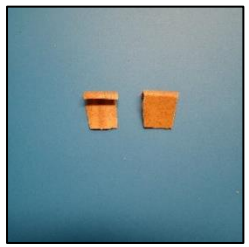
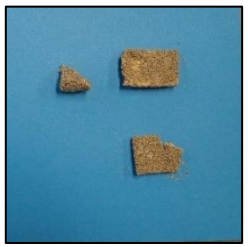
Test items	At start	6 weeks	12 weeks	20 weeks	50 weeks	52 weeks
Film PBSA + 5% oligomer						
Film PBSA + 5% oligomer + 10% PBAT						

Table 43: Visual presentation of the disintegration of Cellulose diacetate + 30% ATEC + 10% Bran, PBS FZ71 PB + 10% potato fibers, PLA + 11% Bran and PHBV + 15% Bran during 52 weeks of incubation in soil (thickness: ± 1.5-1.6 mm (body), ± 1.7-1.9 mm (bottom) and ± 1.5-1.6 mm (edge))

Test items	At start	4 weeks	14 weeks	26 weeks	36 weeks	46 weeks	52 weeks
Cellulose diacetate + 30% ATEC+ 10% Bran							
PBS FZ71 PB + 10% potato fibers							
PLA + 11% Bran							
PHBV + 15% Bran							

6.6 High-solids anaerobic disintegration test

The disintegration of products in a sanitary landfill or in a solid-state anaerobic digestion system is determined through high-rate dry anaerobic batch fermentation. This method simulates and accelerates the disintegration process that takes place in a landfill because it is a stationary (no mixing) and dry fermentation under optimal conditions. Moreover, it is representative for solid-state anaerobic digestion systems (biogas plants). The incubation temperature was 52°C ± 2°C.

A number of recognisable pieces of test item are added to a sufficient amount (to completely cover the test items) of highly active inoculum (stabilised prior to the start of the digestion period). Optimal conditions with regard to pH, nutrients, volatile fatty acids, etc. are provided and the mixture is left to ferment batch-wise. Likewise, disintegration is not influenced by other factors than those inherent to the test item itself. Every week, one piece of test item is removed from each reactor. The test item is carefully rinsed with tap water and air dried. The disintegration of test item is observed visually.

Standards followed:

- ASTM D5511 *Standard Test Method for Determining Anaerobic Biodegradation of Plastic Materials Under High-Solids Anaerobic-Digestion Conditions* (2018);
- ISO 15985 *Plastics – Determination of the ultimate anaerobic biodegradation and disintegration under high-solids anaerobic-digestion conditions - Method by analysis of released biogas* (2014).

6.6.1 Plant pots: Cellulose diacetate + 30% ATEC + 10% Bran, PBS FZ71 PB + 10% potato fibers, PLA + 11% Bran and PHBV + 15% Bran

Pots Cellulose diacetate + 30% ATEC + 10% Bran (thickness: ± 1.6 mm (body), ± 1.9 mm (bottom) and ± 1.5 mm (edge)), PBS FZ71 PB + 10% potato fibers (thickness: ± 1.5 mm (body), ± 1.7 mm (bottom) and ± 1.5 mm (edge)), PLA + 11% Bran (thickness: ± 1.5 mm (body), ± 1.8 mm (bottom) and ± 1.6 mm (edge)) and PHBV + 15% Bran (thickness: ± 1.5 mm (body), ± 1.8 mm (bottom) and ± 1.5 mm (edge)) were qualitatively tested as pieces of approximately 2 cm x 2 cm. The disintegration of Cellulose diacetate + 30% ATEC+ 10% Bran, PLA + 11% Bran and PHBV + 15% Bran proceeded well. PHBV + 15% Bran had already completely disintegrated after 3 weeks, while at that moment Cellulose diacetate + 30% ATEC + 10% Bran and PLA + 11% Bran showed the first sings of disintegration. One week later only a thin piece of Cellulose diacetate + 30% ATEC + 10% Bran was left. After 6 weeks complete disintegration was obtained for Cellulose diacetate + 30% ATEC+ 10% Bran, while PLA + 11% Bran easily fell apart into smaller pieces. Two weeks later some remnants of PLA + 11% Bran were retrieved, however, it was impossible to separate them from the inoculum. Therefore, it can be concluded that the test item was completely disintegrated after 8 weeks. The disintegration of PBS FZ71 PB + 10% potato fibers did not proceed well. No significant signs of disintegration were observed, except for some discoloration. Taking into account that the normal retention time in 'dry', thermophilic anaerobic plants is typically 3 weeks, the PHBV + 15% Bran plant pot can be treated in these systems. Cellulose diacetate + 30% ATEC + 10% Bran, and PLA + 11% Bran pot will not completely degrade during this process, but can be further degraded during a possible post-composting, which is often applied. The plant pot produced from PBS FZ71 PB + 10% potato fibers seems not suitable to be treated in anaerobic digestion plants.

Table 44: Visual representation of the disintegration of Cellulose diacetate + 30% ATEC + 10% Bran (thickness: ± 1.6 mm (body), ± 1.9 mm (bottom) and ± 1.5 mm (edge))

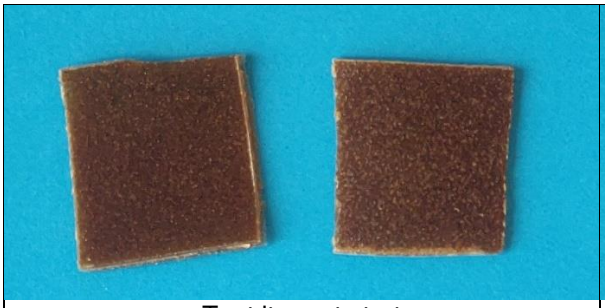
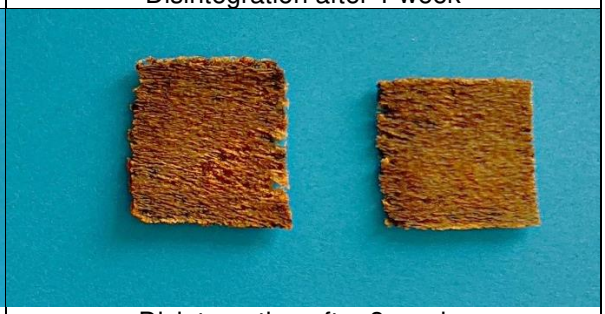
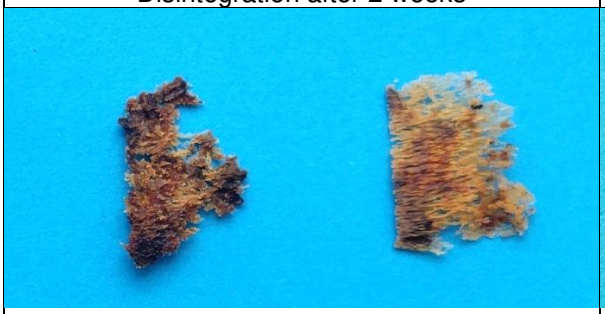
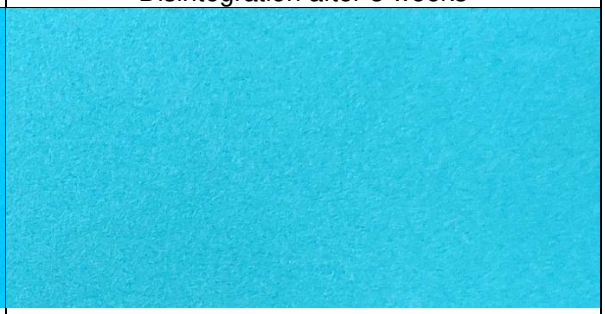
	
Test item at start	Disintegration after 1 week
	
Disintegration after 2 weeks	Disintegration after 3 weeks
	
Disintegration after 4 weeks	Disintegration after 6 weeks

Table 45: Visual representation of the disintegration of PBS FZ71 PB + 10% potato fibers (thickness: ± 1.5 mm (body), ± 1.7 mm (bottom) and ± 1.5 mm (edge))

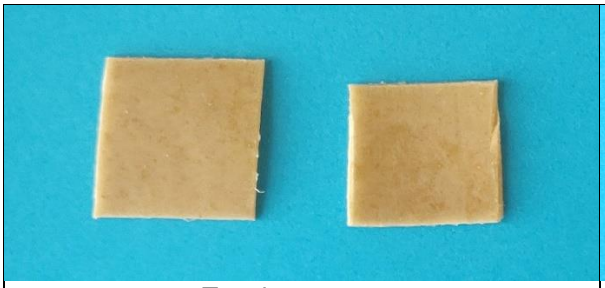
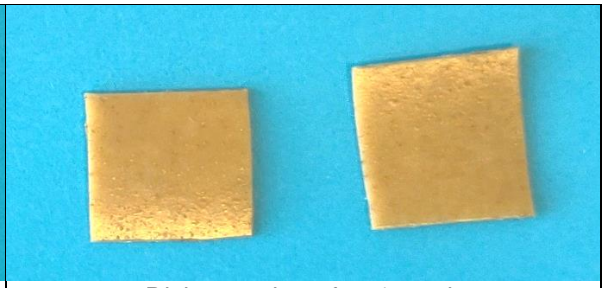
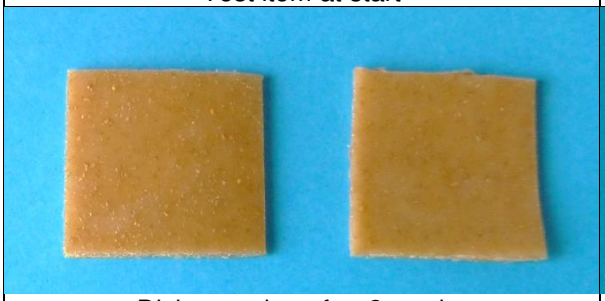
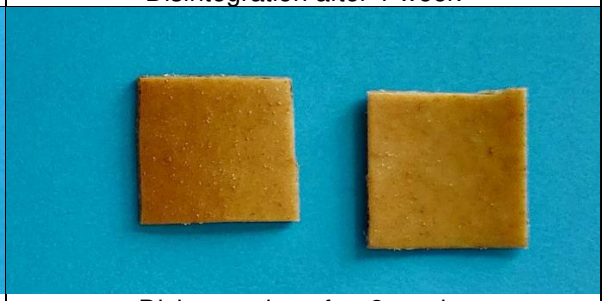
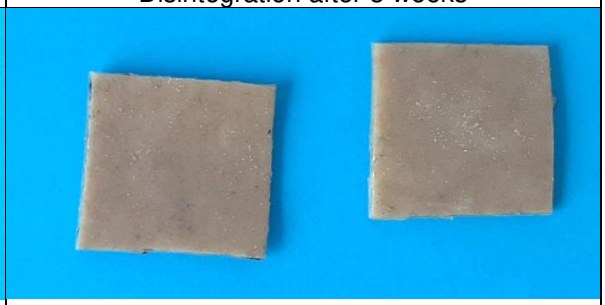
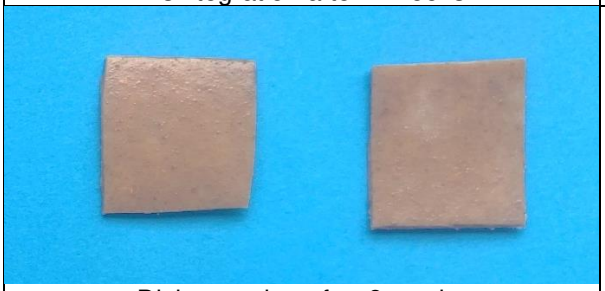
	
Test item at start	Disintegration after 1 week
	
Disintegration after 2 weeks	Disintegration after 3 weeks
	
Disintegration after 4 weeks	Disintegration after 6 weeks
	
Disintegration after 8 weeks	

Table 46: Visual representation of the disintegration of PLA + 11% Bran (thickness: ± 1.5 mm (body), ± 1.8 mm (bottom) and ± 1.6 mm (edge))

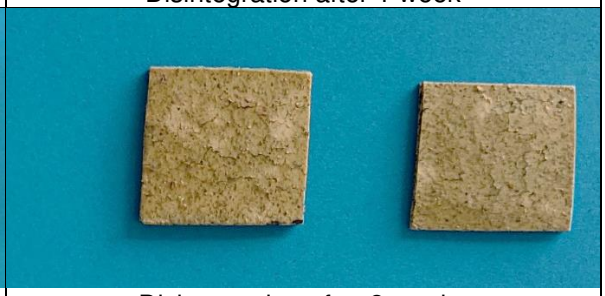
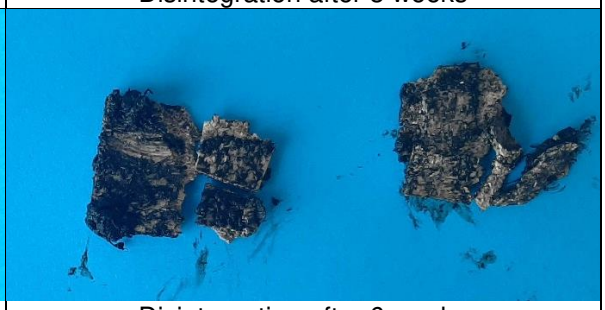
 Test item at start	 Disintegration after 1 week
 Disintegration after 2 weeks	 Disintegration after 3 weeks
 Disintegration after 4 weeks	 Disintegration after 6 weeks
 Disintegration after 8 weeks	

Table 47: Visual representation of the disintegration of PHBV + 15% Bran (thickness: ± 1.5 mm (body), ± 1.8 mm (bottom) and ± 1.5 mm (edge))

	
Test item at start	Disintegration after 1 week
	
Disintegration after 2 weeks	Disintegration after 3 weeks

6.7 Conclusions

The in WP6 developed films and plant pots for agricultural applications were evaluated for biodegradation and disintegration in relevant environments such as soil, industrial composting conditions and biogas production plants.

The European norm EN 17033 *Plastics - Biodegradable mulch films for use in agriculture and horticulture - Requirements and test methods* (2018) defines criteria on material characteristics (limit on heavy metals and substances of very high concern), biodegradation and ecotoxicity. Biodegradation is often the most difficult hurdle and therefore focus was given to this parameter. According to the standard more than 90% biodegradation (absolute or relative to a reference material such as cellulose) must be obtained within 2 years. Disintegration, which is the physically falling apart of a material in small particles, is not a requirement of this standard as this is depending on the actual use. However, this parameter is essential for producers and end-users to define the application. The disintegration should not be too quick to ensure that the crops are protected during the growth period, but it might also not be too slow in order not to hamper next growth season or to prevent accumulation of plastics in the soil.

Several films, based on PBSA and produced by AIMPLAS, were evaluated for biodegradation in soil. The 90% threshold value for complete biodegradation was obtained for PBSA + 5% OI – RE1, PBSA+0.5%AO and PBSA+5%AO. During the first 3 months a relative fast biodegradation until 60% was obtained after which the biodegradation rate slowed down. A second round of compounds showed slower biodegradation rates in soil. Compound PBSA+5%OI reached only 60% biodegradation after about 400 days, while at that moment compound PBSA+5%OI+10%PBAT showed a degradation level below 50%. After 510 days a biodegradation of 69.7% and 56.8% was measured for PBSA+5%OI and PBSA+5%OI+10%PBAT, respectively (84.1% and 68.5% relative to cellulose). At the current speed it is

still possible to reach 90% relative biodegradation within 2 years for both samples. However, the addition of PBAT decreased the biodegradation rate and might result in insufficient biodegradation.
Film PBSA + 5% oligomer in a thickness of 90 µm showed a rather fast and complete disintegration in soil and when placed at the surface of the soil. After 8 weeks the film incubated in the soil had started to fall apart into small pieces, while the pieces on top of the soil started to degrade (holes). The test material showed complete disintegration after 16 weeks of incubation for both the PBSA + 5% oligomer film (90 µm) on top of the soil as the material incubated in soil. This disintegration rate might be too fast as a mulching film must often remain intact for several months. The introduction of PBAT in the compound reduced the disintegration rate, especially when situated at the surface of the soil. After 16 weeks all pieces of Film PBSA + 5% oligomer + 10% PBAT in the soil had also completely disintegrated, however, the pieces on top of the soil were still rather intact. After 52 weeks of incubation still large pieces of test item were retrieved on top of the soil. The PBAT content decreased the disintegration rate on the soil surface considerably. By tuning with the amount of PBAT suitable disintegration rates in line with the harvest periods might be obtained. However, it must be demonstrated that the applied PBAT also biodegrades completely in soil.
Apart from these films also the disintegration in soil of trilayer films consisting of PBSA, PBAT and optionally oligomer was evaluated. These films, produced by AIMPLAS in a thickness around 45 µm – 49 µm, proceeded well. After 16 weeks Trilayer film A/B/C; A and B = PBSA +10%PBAT, C= PBSA+5%oligomer + 10% PBAT (45 µm) showed complete disintegration, while the other 2 films (Trilayer film A/B/C; A=B=C= PBSA + 10% PBAT (49 µm) and Trilayer film A/B/C; A and B = PBSA +10%PBAT; C= PBSA+5%oligomer(45 µm)) were completely disappeared after 22 weeks.

Furthermore plant pots were developed within AGRIMAX project. The first range of pots produced in small scale at AIMPLAS from PBS and potato fiber showed promising biodegradation and disintegration characteristics under industrial composting conditions. However, the biodegradation in soil was clearly insufficient. These results formed the basis of the final plant pot demonstrators, produced by FEMTO. The biodegradation and disintegration of these plant pots was evaluated in soil, under industrial composting conditions and in dry thermophilic anaerobic digestion plants. The composition and behaviour of these plant pots under the different condition are show in are *Table 48*. In total 4 different compounds were developed of which plant pots with a body thickness of 1.5 mm and bottom thickness around 1.7-1.9 mm were produced: Cellulose diacetate + 30% ATEC+ 10% Bran, PBS FZ71 PB + 10% PF, PLA + 11% Bran and PHBV + 15% Bran. It was investigated if these plant pots were suitable for organic recycling such as industrial composting and anaerobic digestion.
The European norm EN 13432 *Requirements for packaging recoverable through composting and biodegradation - Test scheme and evaluation criteria for the final acceptance of packaging* (2000) defines 4 requirements for industrial compostability, namely material characteristics, biodegradation, disintegration and effect on compost quality including plant toxicity. No problems with regard to material characteristics and compost quality were expected for the developed grades and therefore focus was on biodegradation and disintegration. The grades of PBS, PLA and PHBV were already certified according to EN 13432 as industrially compostable and therefore biodegradation has already been demonstrated. Moreover, as Bran is wheat bran of Barilla, a natural product without chemical modifications, also this constituent fulfills the biodegradation of EN 13432. Only Cellulose diacetate + 30% ATEC + 10% Bran needed to be tested for biodegradation under controlled composting conditions and this test was passed successfully. From the tests it can be concluded that plant pots (thickness body: 1.5 mm and bottom: 1.7-1.9 mm) produced from PLA + 11% Bran and PHBV + 15% Bran do fulfil the requirements on biodegradation and disintegration defined by EN 13432. However, for the Cellulose diacetate + 30% ATEC+ 10% Bran and PBS FZ71 PB + 10% PF the thickness must be reduced

to obtain sufficient disintegration. Especially for PBS FZ71 PB + 10% PF a considerable reduction in thickness would be needed, which might negatively impact the mechanical properties.

Organic recycling of biodegrable plastics by anaerobic digestion is a new concept and a standard specifications with clear requirements does not yet exist. However, based on the obtained results it can be concluded that the PHBV + 15% Bran is convertible in dry thermophilic digestion plants. The Cellulose diacetate + 30% ATEC + 10% Bran pot showed a somewhat too slow disintegration rate to be completely convertible in these biogas plants, but the material is compatible with these systems. Moreover, when a subsequent post-composting is performed, as often is part of these plants, then it is expected that also these pots are completely degradable. The biodegradation of PLA + 11% Bran is rather slow to be fully convertible, but it will also partly degrade in these conditions. The PBS FZ71 PB + 10% PF plant pot is not suitable for organic recycling by anaerobic digestion.

Finally also the disintegration in soil of these plant pots were evaluated. The plant pots composed of PBS FZ71 PB + 10% PF and PHBV + 15% Bran showed complete disintegration in soil within 38 weeks, while the other plant pots were still rather intact after 52 weeks. PHBV is expected to be fully biodegradation in soil within a reasonable duration, but for PBS FZ71 PB and PLA this is most likely not the case. The biodegradation in soil of Cellulose diacetate + 30% ATEC + 10% Bran was not evaluated in this project. Therefore, only PHBV + 15% Bran plant pot can remain in soil.

Table 48: Biodegradation and disintegration of FEMTO plant pots (body thickness: 1.5 mm and bottom thickness: 1.7-1.9 mm) in different environments

Composition	Biodegradation (90%, absolute or relative)		Disintegration (90%)		
	Industrial composting (58°C; 180d)	Anaerobic digestion (52°C)	Industrial composting (12w)	Soil (25°C)	Anaerobic digestion (52°C)
Cellulose diacetate + 30% ATEC + 10% Bran	✓	✓	X	X	V
PBS FZ71 PB + 10% PF	✓	X	X	✓	X
PLA + 11% Bran	✓	X	✓	X	V
PHBV + 15% Bran	✓	✓	✓	✓	✓

Bran = wheat bran of Barilla; PF = potato fibers

Mogu plant pot (thickness: ± 15.6 mm (edge), ± 18.8 mm (wall), ± 28.0 mm (bottom); 13.9 mm (bottom middle)) showed complete disintegration under industrial composting conditions. As the material is produced from natural lignocellulosic materials by a natural process no additional biodegradation testing is needed according to EN 13432. This material has also the potential for organic recycling by industrial composting.

As a general conclusion in can be stated that agricultural films were developed that can degrade on the field, while several plant pots were produced that can be treated by composting and even by anaerobic digestion (PHBV + 15% Bran).

6.8 References

ASTM D5511 *Standard Test Method for Determining Anaerobic Biodegradation of Plastic Materials Under High-Solids Anaerobic-Digestion Conditions* (2018)

EN 13432 *Requirements for packaging recoverable through composting and biodegradation - Test scheme and evaluation criteria for the final acceptance of packaging* (2000)

EN 17033 *Plastics - Biodegradable mulch films for use in agriculture and horticulture - Requirements and test methods* (2018)

ISO 14855-1 *Determination of the ultimate aerobic biodegradability of plastic materials under controlled composting conditions – Method by analysis of evolved carbon dioxide* (2012)

ISO 15985 *Plastics – Determination of the ultimate anaerobic biodegradation and disintegration under high-solids anaerobic-digestion conditions - Method by analysis of released biogas* (2014).

ISO 16929 *Plastics – Determination of the Degree of Disintegration of Plastics Materials under Defined Composting Conditions in a Pilot-Scale Test* (2019)

ISO 17556 *Plastics - Determination of the ultimate aerobic biodegradability in soil by measuring the oxygen demand in a respirometer or the amount of carbon dioxide evolved* (2019)