Open-BIO

Opening bio-based markets via standards, labelling and procurement

Work package 5

In situ biodegradation

Deliverable:

Environmental safety of biodegradation residuals of polymers

Public

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List of abbreviations, acronyms and used standards

ASTM D5988	Standard Test Method for Determining Aerobic Biodegradation of Plastic Materials in Soil (2012)
DM	Dry Matter
EN 13432	Requirements for packaging recoverable through composting and biodegradation - Test scheme and evaluation criteria for the final acceptance of packaging (2000)
ISO 11268-1	Soil quality — Effects of pollutants on earthworms — Part 1: Determination of acute toxicity to Eisenia fetida/Eisenia andrei
ISO 14238	Soil quality – Biological methods – Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes (2013)
ISO 15685	Soil quality - Determination of potential nitrification and inhibition of nitrification - Rapid test by ammonium oxidation (2012)
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 (2012)
KBBPPS	Knowledge Based Bio-based Products' Pre-Standardization
LDPE	Low Density Polyethylene
OECD 207	Earthworm, acute toxicity test
OECD 208	Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test
OECD 217	Soil microorganisms: Carbon Transformation Test
PBSe	Polybutylene Sebacate
PBSeT	Polybutylene Sebacate-co-butylenterephtalate
PHB	Polyhydroxyalkanoate copolymer
TC	Total Carbon

TOC	Total Organic Carbon
VS	Volatile Solids
WHC	Water Holding Capacity

1 Publishable summary

Open-Bio is a research project funded by the European Commission within FP7 (*7th Framework Programme for Research and Technological Development*). The goal is to investigate how bio-based products can be integrated into the market, using standardisation, labelling and procurement. Work Package 5 of Open-Bio investigates biodegradability test methods for bio-based products in several natural environments: soil, freshwater and marine environment. Besides the research on biodegradability in WP5, also work was performed on environmental safety of biodegradation residuals of polymers (obtained after a biodegradation phase in soil) on request of CEN/TC 249 Plastics/WG7 Thermoplastic films for use in agriculture/TG 1 Biodegradable mulch films. The performed pre-standardisation work was used for the development of prEN 17033 *Plastics - biodegradable mulch films for use in agriculture and horticulture - requirements and test methods*.

In the task group working on Biodegradable mulch film a proposal was done in order to evaluate environmental safety of biodegradation residuals by means of three organism groups:

- Plants
  - OECD 208 *Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test*
- Invertebrates (earthworms)
  - ISO 11268-1 *Soil quality — Effects of pollutants on earthworms — Part 1: Determination of acute toxicity to Eisenia fetida/Eisenia andrei* and/or OECD 207 *Earthworm, acute toxicity test*
- Soil micro-organisms
  - ISO 14238 *Soil quality – Biological methods – Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes*
  - OECD 217 *Soil microorganisms: Carbon Transformation Test*
  - ISO 15685 *Soil quality - Determination of potential nitrification and inhibition of nitrification - Rapid test by ammonium oxidation*

As little experience is available related to the applicability of toxicity test methods for chemicals (= direct toxicity test method) as toxicity test methods for polymer residuals obtained after a biodegradation phase in soil, pre-standardisation research was performed in the Open-Bio project. This pre-standardisation research is summarised in this document. This document contains the results of (1) biodegradation tests in soil on the different polymers, (2) a disintegration test in soil on the different polymers[1] and (3) toxicity tests with plants, invertebrates (earthworms) and micro-organisms on the biodegradation residuals of the polymers.

[1] The disintegration test is performed in order to check if the parameter “disintegration” could give an indication about the biodegradation of the polymer. This would be useful when a producer would use polymers that have all proven to be biodegradable in soil, but when the toxicity tests would not yet be available. In this way, the disintegration test could give an indication about the necessary incubation period in soil before the toxicity tests should be started (as at least a minimum biodegradation per-

When a biodegradable substance is added to soil, the soil characteristics change (at least temporarily). During biodegradation, organic carbon of the sample is converted to carbon dioxide by means of micro-organisms. Not all carbon is immediately converted to carbon dioxide. Part of the carbon is also converted to microbial biomass. In order to produce microbial biomass, the microorganisms also need nitrogen. Therefore, the nitrogen content (ammonium and/or nitrate) and consequently also the electrical conductivity, which is representative for the salt content, of the soil both decrease during the biodegradation phase. Such changes can strongly influence the results of plant toxicity tests (as ammonium and nitrate are fertilisers that influence the plant biomass), earthworm toxicity tests (as earthworms are sensitive for high salt contents) and microbial toxicity tests that monitor the carbon and nitrogen transformation in soil.

In order to discover the weaknesses of the above mentioned test methods towards testing of biodegradation residuals of polymers, several polymers with varying biodegradability (LDPE, cellulose, PHB, PBSe and PBSeT) were added in a 1% concentration to soil. Two types of soil were used: natural soil and standard soil as prescribed by ISO 17556. During the active biodegradation phase and/or in the plateau phase, the obtained soils were used for the toxicity tests.

From **the soil biodegradation test**, it can be concluded that test item LDPE is a not biodegradable polymer, cellulose is a positive reference material and PHB, PBSe and PBSeT are polymers that are biodegradable in soil. PHB, PBSe and PBSeT biodegraded at a similar rate at Novamont laboratory, while PBSeT was characterised by a lower biodegradation rate at OWS laboraratory (which used natural soil to which no nutrients were added).

The performed **plants toxicity tests** when using natural soil (without addition of nutrients) clearly illustrate that the nutrient content of the soil after the biodegradation phase should be carefully monitored before starting the plant toxicity tests. Due to the biodegradation of the test item that was added in a 1% concentration at start of the incubation phase, the nutrient content in the test soil decreases. Consequently, a significantly lower plant yield is measured for the plants in the test soil when compared to the blank soil. This lower plant yield is also observed in the cellulose soil (= positive reference). The lower plant yield in the cellulose soil illustrates that the lower plant yield is not caused by a toxic effect, but by a fertilizing effect. In order to evaluate the toxicity of the test item in a correct way, it is therefore recommended to compared the germination and plant yield also with a positive reference soil. Moreover, it is recommended to measure the nitrogen content before the plant toxicity tests. In case it is observed that the nitrate content in the test soil is indeed lower than the blank soil, the fertilising effect can be solved by adding a fertilizer till a similar nitrogen content is obtained as in the blank soil.

centage is required to be sure that biodegradation residuals are already present). Otherwise, it would always be necessary to require that biodegradation is again evaluated.

When using standard soil (to which a nutrient solution has been added) or natural soil to which nutrients were added, it is observed that the nutrient content in the blank soil can also be too high to allow normal plant germination and plant growth. Several tests (OWS standard soil and Novamont) illustrated that the validity criterion was not reached (< 70% germination in blank soil) due to the high nutrient levels in the blank soil. When the nutrient content in the blank was too high, it is observed that the germination and the plant biomass in the test soils is higher than the blank soil. This can be explained by the fact that the nutrient content in the test soils is lower when compared to the blank soil (and in this case more optimal for the plant germination and growth). It is recommended to avoid the use of standard soil or natural soil to which a lot of nutrients were added. In case standard soil would be used, the concentration of the salt solution as prescribed by ISO 17556 should be significantly reduced to avoid invalid toxicity tests. Moreover, in case of effects on blank soil the use of reference soil (after cellulose degradation) is recommended to interpret the results of the toxicity test correctly.

The performed **earthworm toxicity tests** when using natural soil (without addition of nutrients) does not reveal problems. Earthworm weight is even generally higher when biodegradable polymers are added. However, when using natural soil to which nutrients are added or standard soil as prescribed by ISO 17556, results can become very difficult to interpret. The high nutrient content in the blank soil can result in total mortality of the earthworms and invalid results. When 100% mortality was observed in the nutrient rich blank soil, it was also observed that the survival in the test soils was significantly higher (due to the fact that the nutrient and salt content has decreased due to the biodegradation). It is recommended to avoid the use of standard soil or natural soil to which a lot of nutrients were added. In case standard soil would be used, the concentration of the salt solution as prescribed by ISO 17556 should be significantly reduced to avoid invalid toxicity tests. Moreover, in case of effects on blank soil the use of reference soil (after cellulose degradation) is recommended to interpret the results of the toxicity test correctly.

The **long term nitrification test (ISO 14238)** was evaluated by means of the addition of Luzerne meal and ammonium sulfate. Both nitrogen source gave comparable results. Results in natural soil to which no nutrients were added showed no nitrate formation in the test soils. This was most probably caused by the fact that nitrogen had become limiting and that the microorganisms immediately consumed the ammonium instead of converting it to nitrate. When performing the test on the standard soil series, a nitrate formation was observed which was in most cases even higher than the nitrate formation in the blank soil. From these results, it can be concluded that it is important that nitrogen does not become limiting during the biodegradation phase. Besides requiring that the nitrate formation should be at least 90% when compared to the blank soil or positive reference soil, the pass criteria of this test could be expanded by also requiring that (1) the trend of $N\text{-}NH_4$ decrease should be similar of blank soil (or reference soil) and after 28 days the $N\text{-}NH_4$ content should be less than 10 mg/kg and (2) after 28 days no nitrite should be measurable in the soil.

The **carbon transformation test (OECD 217)** can be used to evaluate the toxicity of biodegradation residuals, but the performed tests showed that it is important to test in parallel also the soil as such (without addition of glucose). The results should be corrected by means of the background activity measured in the series without glucose. It must be noted that the biodegradation test (ISO 17556) is in fact also a kind of carbon transformation test. Therefore, it can be argued that this test might be superfluous. Moreover, in our opinion the carbon transformation test is not very sensitive for the evaluation of toxicity of biodegradation residuals of polymers as glucose is easily biodegradable and only in presence of strong toxicity this test might be useful.

The short term **rapid ammonification test** (ISO 15685) has been performed several times by both laboratories. Most of the results seem rather promising, but still some problems were detected for which no clear explanation was found (e.g. lower nitrite formation in LDPE series and PHB series). The standardization of the test method for the evaluation of the toxicity of biodegradation residuals of polymers after an incubation period in soil, seems not (yet) possible based on the performed research. Additional research is needed in order to demonstrate and confirm the suitability of this test method for the evaluation of toxicity towards soil microorganisms of biodegradation residuals of polymers.

In general to perform ecotoxicity tests during the active biodegradation phase could be a risk due to the fact that a lot of processes are taking place at the same moment. In fact different “strange” results were obtained when performing toxicity tests during the active biodegradation phase. The biodegradation of a material is a transitory phenomenon and it is probably better to determine the effects on the soil at the end of the biodegradation process.

Finally, it can be concluded that the direct toxicity test methods can be suitable to evaluate also the toxicity of biodegradation residuals of polymers. Especially the toxicity test with higher plants and the toxicity test with earthworms are suitable to use as test method for the evaluation of toxicity of biodegradation residuals of polymers. More problems were observed for the toxicity tests with soil micro-organisms (most probably caused by the fact that the soil characteristics change due to the addition of biodegradable substances) and therefore caution is needed when interpreting the results of such toxicity tests. During the research activity some false positive results were obtained for the toxicity tests with soil micro-organisms, this fact makes this kind of test not yet ready for standardization. All performed tests clearly illustrate that it is useful to determine the soil characteristics (pH, nutrients, etc.) at least before the toxicity tests (and even during the incubation period to monitor if nutrients do not become limiting) and it is recommended to positive (cellulose) reference soil as “control” to calculate the eventual effects. The use of only blank soil could underestimate the effects.

Website: www.open-bio.eu

2 Introduction

Work package 5 of Open-Bio investigates biodegradability test methods for bio-based products in several natural environments: soil, freshwater and marine environment. This work is a follow-up of work carried out earlier in Work Package 6 of European project KBBPPS, in which the focus was mainly on biodegradability and environmental safety of lubricants.

Besides the research on biodegradability in WP5, also work was performed on environmental safety of biodegradation residuals of polymers (obtained after a biodegradation phase in soil) on request of CEN/TC 249 Plastics/WG7 Thermoplastic films for use in agriculture/TG 1 Biodegradable mulch films. The performed pre-standardisation work was used for the development of prEN 17033 *Plastics - biodegradable mulch films for use in agriculture and horticulture - requirements and test methods*. Advisory partner BASF, Novamont and OWS participated to the meetings of this task group and informed the task group about the results of the performed research.

BASF made a suggestion in the task group to evaluate environmental safety of biodegradation residuals by means of three organism groups:

- Plants
- Invertebrates (earthworms)
- Soil micro-organisms

As little experience is available related to the applicability of toxicity test methods for chemicals (= direct toxicity test method) as toxicity test methods for polymer residuals obtained after a biodegradation phase in soil, pre-standardisation research was performed by BASF (not shown in this deliverable), Novamont and OWS.

The report contains the results of (1) biodegradation tests in soil on the different polymers, (2) a disintegration test in soil on the different polymers[2] and (3) toxicity tests with plants, invertebrates (earthworms) and micro-organisms on the biodegradation residuals of the polymers.

[2] The disintegration test is performed in order to check if the parameter “disintegration” could give an indication about the biodegradation of the polymer. This would be useful when a producer would use polymers that have all proven to be biodegradable in soil, but when the toxicity tests would not yet be available. In this way, the disintegration test could give an indication about the necessary incubation period in soil before the toxicity tests should be started (as at least a minimum biodegradation percentage is required to be sure that biodegradation residuals are already present). Otherwise, it would always be necessary to require that biodegradation is again evaluated.

3 Materials and methods

3.1 Test materials

The characteristics of the biopolymers are given in Table 1 (provided by Novamont).

Table 1. Characteristics of the test materials

Test material	Note	TOC (%)	TC (%)	H (%)	N (%)
Low Density Polyethylene **LDPE**	Film 30 µm Grade: LUPOLEN 2420K Lyondelbasell	85.03	85.37	14.68	< 0.1
Low Density Polyethylene **LDPE**	Powder Aldrich (Analytical grade)	Not determined			
Cellulose filter paper	(Whatman No. 1)	41.7			
Polyhydroxyalkanoate Copolymer **PHB**	Film 85 µm Grade: Mirel™ P5001 (> 70% PHB copolymer, plasticizer & fillers)	47.82	49.11	6.03	0.52
Polybutylene Sebacate **PBSe**	Film 25 µm Aliphatic polyester	65.26	65.58	7.69	< 0.1
Polybutylene Sebacate-co-butylenterephtalate **PBSeT**	Film 25 µm Aliphatic-Aromatic polyester	65.25	65.81	9.54	< 0.1

3.2 Soil biodegradation test method

The evaluation of the biodegradation was executed in line with the international standard 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* (2012). As the main objective of the biodegradation test was the determination of the moment at which the environmental safety should be started, the test was only executed in duplicate instead of in triplicate as required by ISO 17556. The amount of carbon dioxide evolved was determined in an incubation apparatus as shown in the American standard ASTM D5988 - 12 *Standard Test Method for Determining Aerobic Biodegradation of Plastic Materials in Soil* (this apparatus is allowed by ISO 17556). Vessels with an air-tight seal with a volume of 4 L (OWS) and 3 L (Novamont) were used. The vessels were incubated at 25°C at OWS and at 28°C at Novamont.

3.3 Soil disintegration test method

No international nor European test method is available in order to determine the disintegration of products in soil. Therefore, the disintegration of the materials is evaluated qualitatively by means of slide frames.

3.4 Soil preparation for subsequent toxicity tests

The test materials were milled and added in a specific concentration to the soil inoculum.

3.5 Toxicity test method by means of higher plants

The evaluation of the toxicity of the biodegradation residuals by means of higher plants was performed in line with OECD 208 *Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test* and the principles of Annex E of EN 13432 *Requirements for packaging recoverable through composting and biodegradation - Test scheme and evaluation criteria for the final acceptance of packaging* (2000). Plant toxicity was evaluated by means of barley (Hordeum vulgare) and garden cress (Lepidium sativum). At the end of the test the amount of plants per pot was determined, the fresh weight was measured per pot and after a drying period of 2 days the dry weight of the plants was measured.

3.6 Toxicity test method by means of invertebrates

The evaluation of the toxicity of the biodegradation residuals by means of earthworms was performed in line with ISO 11268-1 *Soil quality — Effects of pollutants on earthworms — Part 1: Determination of acute toxicity to Eisenia fetida/Eisenia andrei* and OECD 207 *Earthworm, acute toxicity test*.

One day before start-up of the test, the worms were conditioned in the artificial soil. At the start of the test, each glass jar was filled with the soil. Subsequently, 10 viable earthworms were put on top of the soil. The weight of the worms was determined at start. After all glass jars were filled, they were closed and put at room temperature (20°C ± 2°C) and with continuous lighting. The test was stopped after 14 days.

3.7 Toxicity test method by means of soil micro-organisms

The evaluation of the toxicity of the biodegradation residuals by means of soil micro-organisms was performed in line with following test methods:

- ISO 14238 *Soil quality – Biological methods – Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes* (2013)
- OECD 217 *Soil microorganisms: Carbon Transformation Test*
- ISO 15685 *Soil quality - Determination of potential nitrification and inhibition of nitrification - Rapid test by ammonium oxidation* (2012)

At start of **the long term nitrification test performed in line with ISO 14238**, 100 mg N was added per kg of dry soil. Nitrogen was added under the form of $(NH_4)_2SO_4$ and under the

form of Lucerne meal. Information with regard to these nitrogen sources is given in Table 2. The C/N ratio of Lucerne meal (14) is somewhat lower when compared to the suggested C/N ratio of 16 in the international standard.

Table 2. Characteristics of N sources

	$(NH_4)_2SO_4$	Lucerne meal
Molecular weight	132.16 g/mol	Not determined
Dry matter (DM, %)	Not determined	90.3
Moisture content (%)	Not determined	9.7
Volatile solids (VS, % on DM)	Not determined	82.8
Ash content (% on DM)	Not determined	17.2
Total N	212 g/kg DS (Calculated)	29 g/kg DS
C/N ratio	Not determined	14

The soils were incubated at 20°C and after 0 days, 7 days, 14 days and 28 days the ammonium-N content, the nitrate-N content and the nitrite-N content was determined. Each week the weight of the reactors was measured and moisture is added if necessary.

The principles of **the carbon transformation test in line with OECD 217** are summarised in Table 3.

Table 3. Characteristics of carbon transformation test

Parameter	Test set-up
Glucose addition	0 days, 7 days, 14 days and 28 days
Replicates	3
Glucose concentration	4000 mg/kg dry weight
Addition method of glucose	Mixing of glucose with 1 g clean quartz sand
Amount soil per OxiTop flask	100 g soil dry weight/flask
Amount glucose per OxiTop flask	400 mg glucose/flask
Temperature incubation	20°C ± 2°C (dark)
Analyses	pH (after start of exposure) Determination of released carbon dioxide or consumed oxygen for 12 consecutive hours and measurements start within 1 to 2 hours after glucose supplement (results can be expressed as mg O_2/kg DW soil/h)
Validation criterion	The variation in the replicates of the control samples should be less than ± 15% of the glucose induced respiration rates at the end of the exposure

The principles of **the rapid test by ammonium oxidation ISO 15685 (2012)** are given in Table 4.

Table 4. Characteristics of rapid test by ammonium oxidation

Parameter	Description
Required analysis before start of test	Dry matter content of soils after biodegradation test
Minimum amount of replicates	3
Amount of soil per replicate	25 g moist soil
Preparation of stock solution A	28 ml KH_2PO_4 solution + 72 ml K_2HPO_4 solution + 100 ml distilled water
Preparation of test medium	10 ml stock solution A + 10-30 ml $NaClO_3$ solution (BASF test: 15 ml) + 0.198 g $(NH_4)_2SO_4$ + add distillated water up to 1000 ml
Preparation of final mixture	25 g moist soil + XX ml test medium to reach a precise total liquid volume of 100 ml (It is consequently necessary to know the amount of water in the 25 g moist soil)
Volume of flasks	250 ml
Incubation temperature	25°C
Incubation conditions	Orbital shaking incubator (175 rpm)
Sampling	After 2 hours and after 6 hours (=end of test)
Analysis at sampling	Take 2 ml of the final mixture Add 2 ml KCl (reason = stop ammonium oxidation) Centrifugation (3000 *g* for 2 min (BASF test: 4000 rpm for 10 minutes)) or filtration Nitrite analysis on supernatant (storage in refrigerater in meantime at 4°C-8°C and analyses need to be executed within 24 hours after sampling)

4 Results

4.1 Soil characteristics

4.1.1 OWS laboratory - Run 1

The characteristics of the natural soil and the standard soil (ISO 17556) are given in Table 5. The inoculum should have a water content between 40% and 60% of the total water holding capacity and a pH between 6.0 and 8.0. The criterion with regard to water content was met for both soils, while the pH criterion was met for the natural soil. The pH of the standard soil (8.3) was slightly higher when compared to the prescribed range.

Table 5. Characteristics of natural soil and standard soil

Characteristics	Natural soil	Standard soil
Dry matter (DM, %)	76.5	87.6
Moisture content (%)	23.5	12.4
Volatile solids (VS, % on DM)	7.9	3.9
Ash content (% on DM)	92.1	96.1
pH	7.9	8.3
EC (µS/cm)	260	1160
WHC_{tot} (%)	58.3	30.2
Moisture content (% on DM)	30.7	14.2
Moisture content (% on DM) on WHC_{tot} (%)	52.7	46.9
Total N (g/kg DM)	5.1	2.0
NO_X^--N (mg/l)	32	156
NH_4^+-N (mg/l)	b.r.	199
C/N ratio	8	10

b.r. = below reporting limit; reporting limit NH_4^+-N = 9.0 mg NH_4^+-N/l

During the incubation period the soils were regularly manually mixed. If necessary moisture was added to the reactors. After 3 weeks the nitrate and ammonium content was measured of all soils prepared for the toxicity tests (except for the PBSeT soil) (Table 6). Nitrate is clearly consumed by the microorganisms during the biodegradation.

Table 6. Overview of measured nitrate and ammonium values (mg/l) after 3 weeks of incubation

Test series	Nitrate (mg/l)	Ammonium (mg/l)
Blank natural soil	63.9	0
Blank standard soil	348.7	20.4
Cellulose filter paper - natural	0.6	0
Cellulose filter paper - standard	63.1	2.9
PHB - natural	0.6	0
PBSe - natural	0.8	0

4.1.2 OWS laboratory - Run 2

Mr. Olivier De Beaurepaire (BASF) suggested to select a natural soil with following properties for the repetition of the toxicity tests with the soil micro-organisms: (1) C/N > 10 (preferably 10-12) and (2) pH: 6.5 - 7.5 (H_2O extraction method). A mixture of two natural soils was selected in order to execute the test. Both soils were collected from the surface layer in two localities in Belgium (forest soil from Moerbeke and sandy soil of a field in Lokeren). The soils were sieved over a 2 mm sieve to remove stones. recognizable roots and plant debris and other impurities and then mixed in a ratio 1:2 forest soil : sandy soil. The characteristics of the natural soil are given in Table 7. The inoculum should have a water content between 40 % and 60 % of the total water holding capacity and a pH between 6.0 and 8.0. Both criteria were reached.

Table 7. Characteristics of natural soil

Characteristics	Natural soil
Dry matter (DM, %)	81.7
Moisture content (%)	18.3
Volatile solids (VS, % on DM)	6.2
Ash content (% on DM)	93.8
pH	7.4
EC (µS/cm)	174
WHC_{tot} (%)	57.4
Moisture content (% on DM)	23.5
Moisture content (% on DM) on WHC_{tot} (%)	40.9
Total N (g/kg DM)	2.1
NO_X^--N (mg/l)	32.7
NH_4^+-N (mg/l)	b.r.
C/N ratio	15

b.r. = below reporting limit
reporting limit NH_4^+-N = 9.0 mg NH_4^+-N/l

4.1.3 OWS laboratory - Run 3

Comparable as in run 2, the incubation will took place in a natural soil with following characteristics (as suggested by Mr. Olivier De Beaurepaire (BASF)):

- C/N > 10 (preferably 10-12)
- pH: 6.5 - 7.5 (H_2O extraction method)

The natural soil consisted of a mixture of two natural soils. which were collected from the surface layer in two localities in Belgium (forest soil from Moerbeke with a pH of 8.0 and sandy soil of a field in Lokeren with a pH of 6.9). The soils were sieved over a 2 mm sieve to remove stones, recognizable roots and plant debris, and other impurities and then mixed in a ratio 33% forest soil from Moerbeke and 67% sandy soil of a field in Lokeren. The characteristics of the natural soil are given in Table 8. The inoculum should have a water content between 40 % and 60 % of the total water holding capacity and a pH between 6.0 and 8.0. Both criteria were reached.

Table 8. Characteristics of natural soil

Characteristics	Natural soil
Dry matter (DM, %)	86.7
Moisture content (%)	13.3
Volatile solids (VS, % on DM)	4.2
Ash content (% on DM)	95.8
pH	7.4
EC (µS/cm)	404
WHC_{tot} (%)	38.7
Moisture content (% on DM)	15.3
Moisture content (% on DM) on WHC_{tot} (%)	40
Total N (g/kg DM)	2.2
NO_X^--N (mg/l)	150
NH_4^+-N (mg/l)	53
C/N ratio	10

After 28 days the pH of the control soil, the cellulose filter paper soil and the PBSe soil were determined (Table 9). The pH of the control soil and the cellulose filter paper soil had clearly increased during the incubation period.

Table 9. pH of soils after incubation period of 28 days

Soil	pH
Control soil	7.8
Cellulose filter paper soil	7.9
PBSe soil	7.5

At the end of the incubation period the electrical conductivity (EC), the pH, the ammonium content and the nitrate content of the different soils were determined (Table 10). It can clearly be observed that the nitrate content in the cellulose filter paper soil and the PBSe soil had clearly decreased during the incubation period.

Table 10. Characteristics of the soil after the incubation period

Soil	EC (µS/cm)	pH	NH_4^+-N (mg/l)	NO_x^--N (mg/l)
Control soil	442	7.4	< 10	216
Cellulose filter paper soil	202	7.8	< 10	80
PBSe soil	125	8.0	< 10	24

4.1.4 OWS laboratory - Run 4

The soil characteristics are given in Table 11. The inoculum should have a water content between 40 % and 60 % of the total water holding capacity and a pH between 6.0 and 8.0. Both criteria were reached.

Table 11. Characteristics of natural soil

Characteristics	Natural soil
Dry matter (DM, %)	80.1
Moisture content (%)	19.9
Volatile solids (VS, % on DM)	5.4
Ash content (% on DM)	94.6
pH	7.7
EC (µS/cm)	344
WHC_{tot} (%)	49.8
Moisture content (% on DM)	24.8
Moisture content (% on DM) on WHC_{tot} (%)	49.9
Total N (g/kg DM)	3.8
C/N ratio	7

4.1.5 Novamont laboratory - Run 1

The characteristics of the natural soil and the standard soil are shown in Table 12.

Table 12. Characteristics of natural soil and standard soil

Characteristics	Natural soil	Standard soil
Dry matter (DM, %)	82.61	82.24
Moisture content (%)	17.39	17.76
Volatile solids (VS, % on DM)	5.31	2.75
Ash content (% on DM)	94.69	97.25
pH	7.08	7.32
Total N (g/kg DS)	3.36	0.67
C/N ratio	7.9	20.5

4.2 Biodegradation

4.2.1 OWS laboratory

Biodegradability of all samples was evaluated in natural soil, while reference material cellulose filter paper was also evaluated in standard soil. An overview of the test set-up of the biodegradation test is given in Table 13 (inoculum = natural soil) and Table 14 (inoculum = standard soil as defined by ISO 17556). A higher amount of test material was added to the reactors containing standard soil (3 g per 500 g or 0.6% on wet weight basis) when compared to natural soil (1 g per 500 g or 0.2% on wet weight basis).

Table 13. Test set-up of biodegradation test in natural soil

Reactor number	Test series	Natural soil (g)	Test item (g)
0	Technical control	-	-
00	Technical control	-	-
1	Control	500.1	-
6	Control	500.0	-
2	Cellulose filter paper	500.1	0.9985
7	Cellulose filter paper	499.9	1.0018
3	PHB	499.9	1.0034
8	PHB	499.9	1.0027
4	PBSe	500.0	0.9958
9	PBSe	500.1	1.0064
5	PBSeT	500.1	1.0008
10	PBSeT	499.8	1.0037

Table 14. Test set-up of biodegradation test in standard soil (as defined by ISO 17556)

Reactor number	Test series	Standard soil (ISO 17556) (g)	Test item (g)
0	Technical control	-	-
00	Technical control	-	-
11	Control	500.0	-
13	Control	500.0	-
12	Cellulose filter paper	499.9	2.9983
14	Cellulose filter paper	500.0	2.9995

A summary of the biodegradation percentages and the standard deviation is given in Table 15. The evolution of the biodegradation of the different samples in natural soil is given in Figure 1, while the evolution of the biodegradation in standard soil is given in Figure 2. From this test it can be concluded that the absolute biodegradation percentage of cellulose filter paper in natural soil is significantly higher when compared to the absolute biodegradation percentage in standard soil (after 318 days: 92.5% in natural soil ↔ 77.5% in standard soil).

The biodegradation test in natural soil was stopped after 120 days for samples PHB copolymer and PBSe. The test was further extended for the positive reference material (Cellulose filter paper) and PBSeT.

Table 15. Biodegradation percentage (average and standard deviation)

Test series	Biodegradation	
	Average (%)	Standard deviation (%)
After 120 days		
Cellulose filter paper – natural soil	86.2	1.4
Cellulose filter paper – standard soil	69.9	1.3
PHB – natural soil	90.2	2.3
PBSe – natural soil	91.7	1.0
PBSeT – natural soil	37.2	1.3
After 318 days		
Cellulose filter paper – natural soil	92.5	7.9
Cellulose filter paper – standard soil	77.5	1.2
PHB – natural soil		
PBSe – natural soil		
PBSeT – natural soil	76.7	3.5

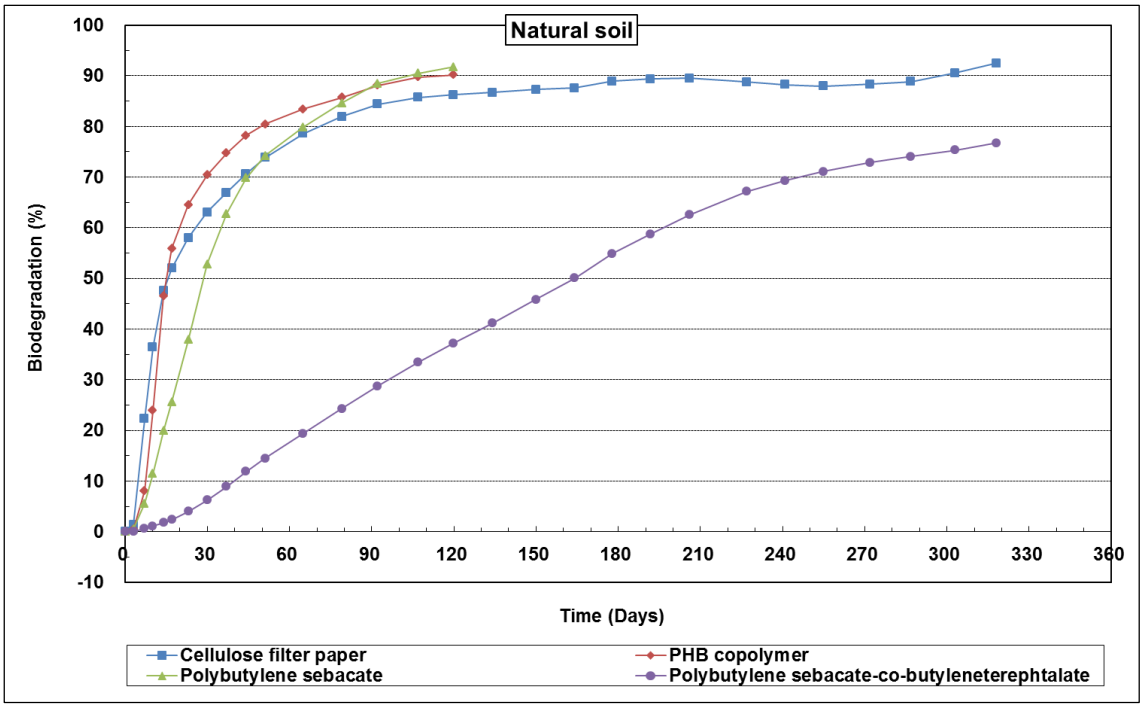

Figure 1. Biodegradation in natural soil

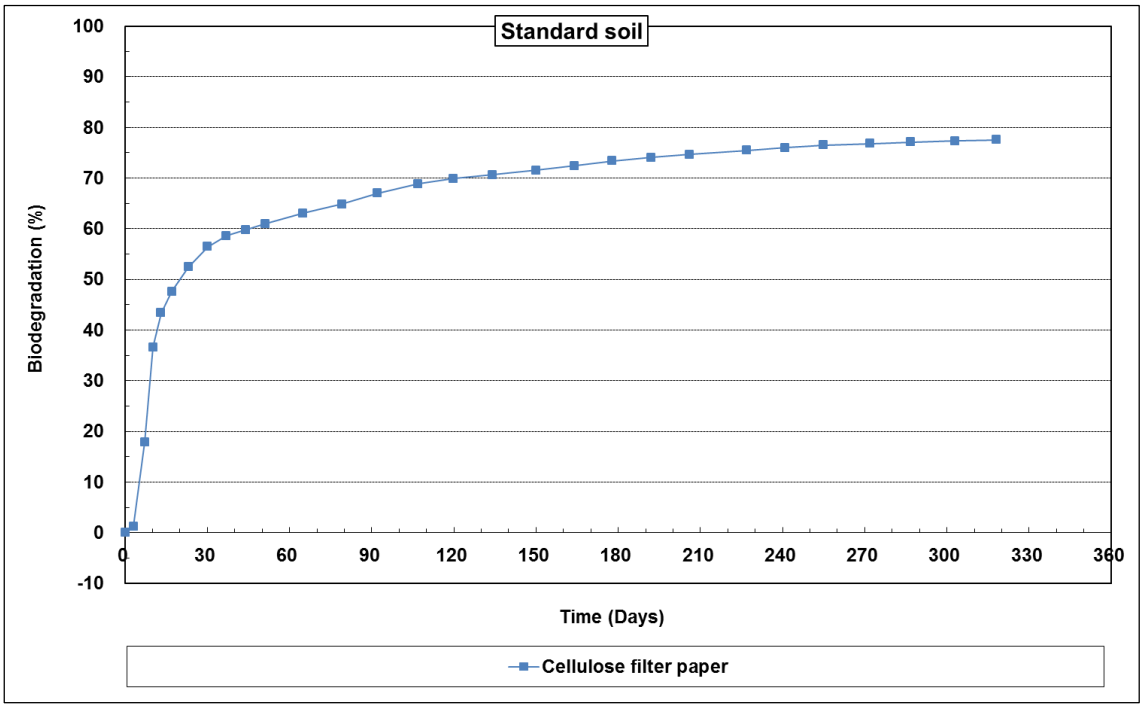

Figure 2. Biodegradation in standard soil

4.2.2 Novamont laboratory

The set-up of the biodegradation test in natural soil and in standard soil is shown in Table 16 and Table 17.

Table 16. Test set-up of biodegradation test in natural soil

Reactor number	Test series	Natural soil (g)	Test item (mg)
1	Control	201.77	-
2	Control	201.7	-
3	PBSe	200.6	998.64
4	PBSe	201.04	1007.28
5	PBSeT	201.34	1001.7
6	PBSeT	201.54	1006.98
7	PHB	201.58	998.14
8	PHB	200.34	1001.26
9	Cellulose Filter Paper	200.6	999.95
10	Cellulose Filter Paper	201.31	995.55

Table 17. Test set-up of biodegradation test in standard soil

Reactor number	Test series	Natural soil (g)	Test item (mg)
1	Control	201.08	-
2	Control	201.22	-
3	PBSe	200.71	1000.62
4	PBSe	201.28	1008.05
5	PBSeT	201.4	997.8
6	PBSeT	201.42	1008.14
7	PHB	201.94	1009.29
8	PHB	201.77	1001.25
9	Cellulose Filter Paper	200.57	999.59
10	Cellulose Filter Paper	200.91	1000.2

In Figure 3, Figure 4 and Table 18 the results of biodegradation tests in natural and standard soil are shown. A comparable biodegradation was observed for the different samples.

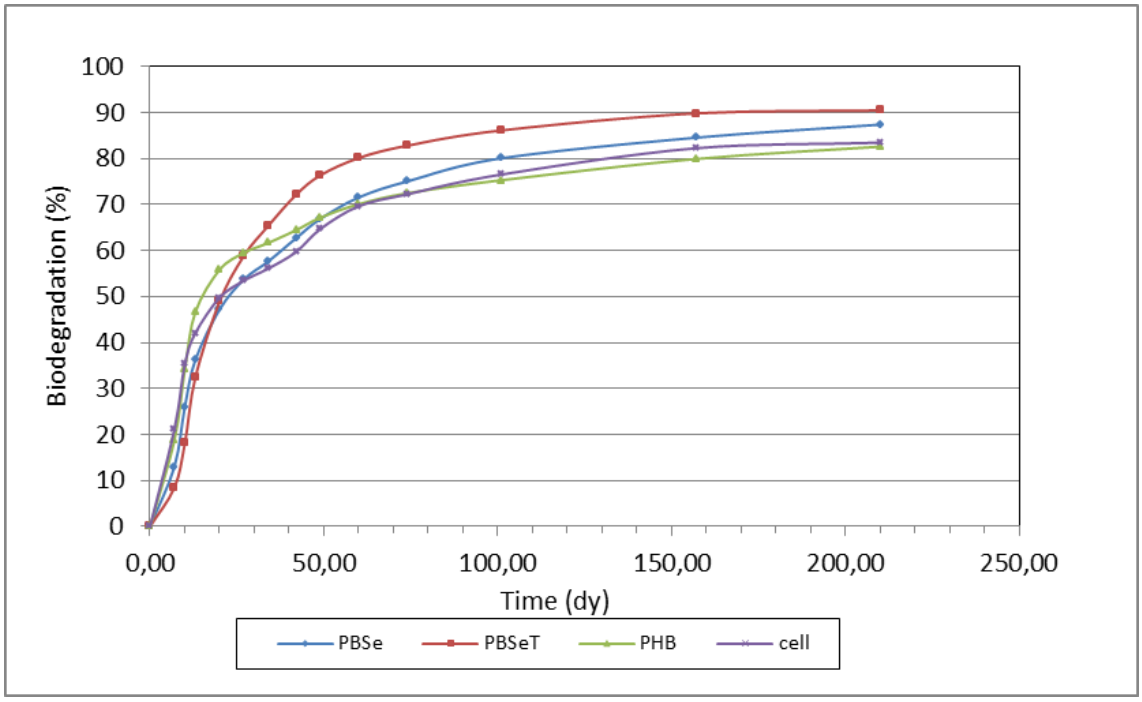

Figure 3. Biodegradation in natural soil

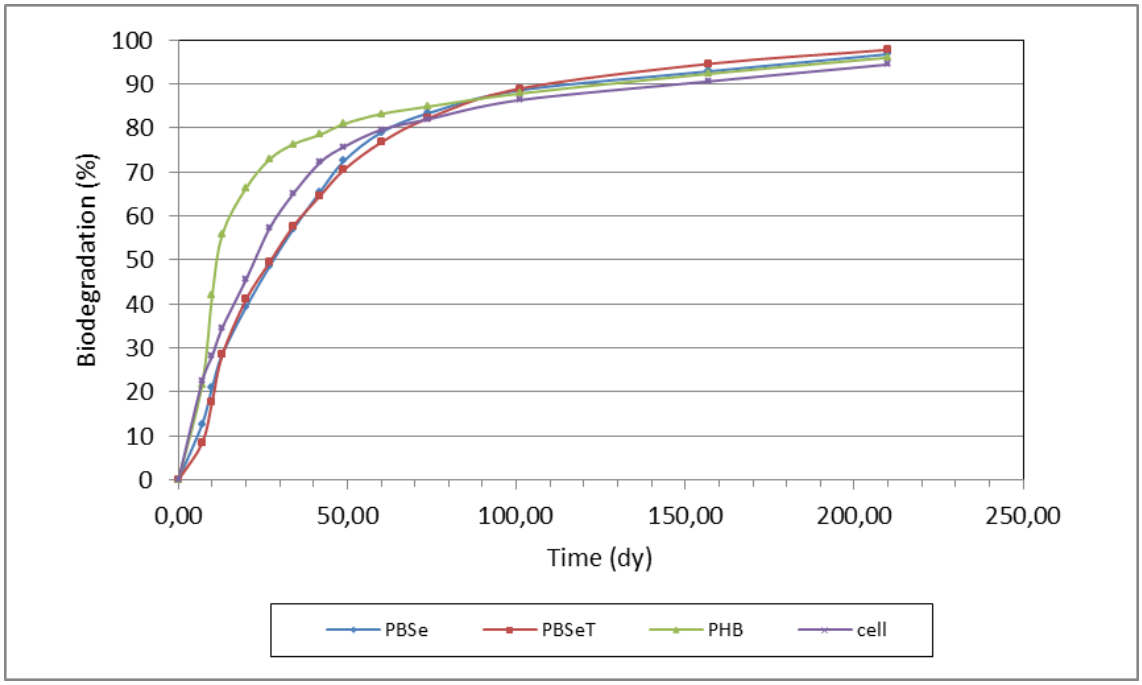

Figure 4. Biodegradation in standard soil

Table 18. Biodegradation: results after 210 days (end of test)

Test series	Biodegradation (210 days)	
	Average (%)	Standard deviation
Cellulose filter paper – natural soil	83.46	5.5
PHB copolymer – natural soil	82.60	18.4
Polybutylene sebacate – natural soil	87.39	12.5
Polybutylene sebacate-co-butylene terephthalate – natural soil	90.47	0.6
Cellulose filter paper – standard soil	94.47	0.4
PHB copolymer – standard soil	96.02	0.8
Polybutylene sebacate – standard soil	96.80	1.6
Polybutylene sebacate-co-butylene terephthalate – standard soil	97.80	1.4

4.3 Disintegration

The test materials were put in slide frames and added to the soil inoculum. An overview of the test set-up is given in Table 19 (inoculum = natural soil) and Table 20 (inoculum = standard soil as prescribed by ISO 17556). 2.5 kg natural soil was added per box with a volume of approximately 5 L, while 3.0 kg standard soil was added per box. More standard soil was added as the density of standard soil is higher when compared to natural soil. The reactors were incubated at 25°C.

Table 19. Test set-up of disintegration test in natural soil

Reactor number	Test series	Natural soil (kg)	Test item (g)
1	Cellulose filter paper	2.5	13 slide frames
2	PHB copolymer	2.5	13 slide frames
3	PBSe	2.5	13 slide frames
4	PBSeT	2.5	13 slide frames

Table 20. Test set-up of disintegration test in standard soil (as defined by ISO 17556)

Reactor number	Test series	Standard soil (ISO 17556) (kg)	Test item (g)
5	Cellulose filter paper	3.0	13 slide frames

A visual presentation of the evolution of the disintegration of test materials Cellulose filter paper (Whatman No. 1), PHB, PBSe and PBSeT in slide frames during 12 weeks is given in Figure 5 up to Figure 9. A summary of the visual observations is given in Table 21 and Table 22. The disintegration percentages given in this table refer to the disintegration percentage of the slide frame shown in the overview of the evolution of the disintegration.

A few observations when comparing results of biodegradation tests with disintegration tests:

- Disintegration of Cellulose filter paper in standard soil (ISO 17556 mixture) proceeded somewhat faster when compared to disintegration in natural soil in spite of the fact that the reverse is observed for the biodegradation.
- Disintegration of PHB copolymer has started after 2 weeks, but less fast when compared to the positive reference material Cellulose filter paper. This is contradictory with the results of the biodegradation test. After 14 days approximately the same biodegradation percentage was observed for Cellulose filter paper and PHB copolymer. This is most probably caused by the fact that Cellulose filter paper absorbs very easily water, while this is not the case for the PHB copolymer.
- Disintegration of PBSe has slowly started after 2 weeks, while disintegration of PBSeT has not yet started. This is in line with results of the biodegradation test.

⇨ Based on these results, it can be stated that there exists certainly a link between the results of the biodegradation and the disintegration tests.

Table 21. Overview of visual observation during disintegration test (part 1)

	Soil	2 weeks	4 weeks	6 weeks
Filter paper	N	Medium holes Light brown colour Fragile 55% disintegration	Large holes Brown colour Very fragile 78% disintegration	Tiny pieces at borders Brown colour Very fragile 91% disintegration
Filter paper	S	Large holes Light brown colour Fragile 65% disintegration	Only small border Light brown colour Very fragile 88% disintegration	Tiny pieces at borders Light brown colour Very fragile 91% disintegration
PHB	N	Small holes White/yellow colour Fragile 9% disintegration	Medium holes Light brown colour Fragile 33% disintegration	Large holes Light brown colour Very fragile 69% disintegration
PBSe	N	Tiny holes Transparent <5% disintegration	Small – medium holes Transparency decreases Fragile 21% disintegration	Large holes Fragile 73% disintegration
PBSeT	N	Intact Transparent 0% disintegration	Intact Transparent 0% disintegration	Tiny tears and holes Transparent 0% disintegration

Table 22. Overview of visual observation during disintegration test (part 2)

	Soil	8 weeks	12 weeks
Filter paper	N	100% disintegration	-
Filter paper	S	100% disintegration	-
PHB	N	Only border of test material Light brown colour Very fragile 86% disintegration	Only small test item pieces Light brown colour Very fragile 90% disintegration
PBSe	N	Only border of test material Fragile 93% disintegration	Only small test item pieces Light brown colour 94% disintegration
PBSeT	N	Small tears and holes Light brown discoloration ±2-5% disintegration	Holes in test material Light brown discoloration 19% disintegration

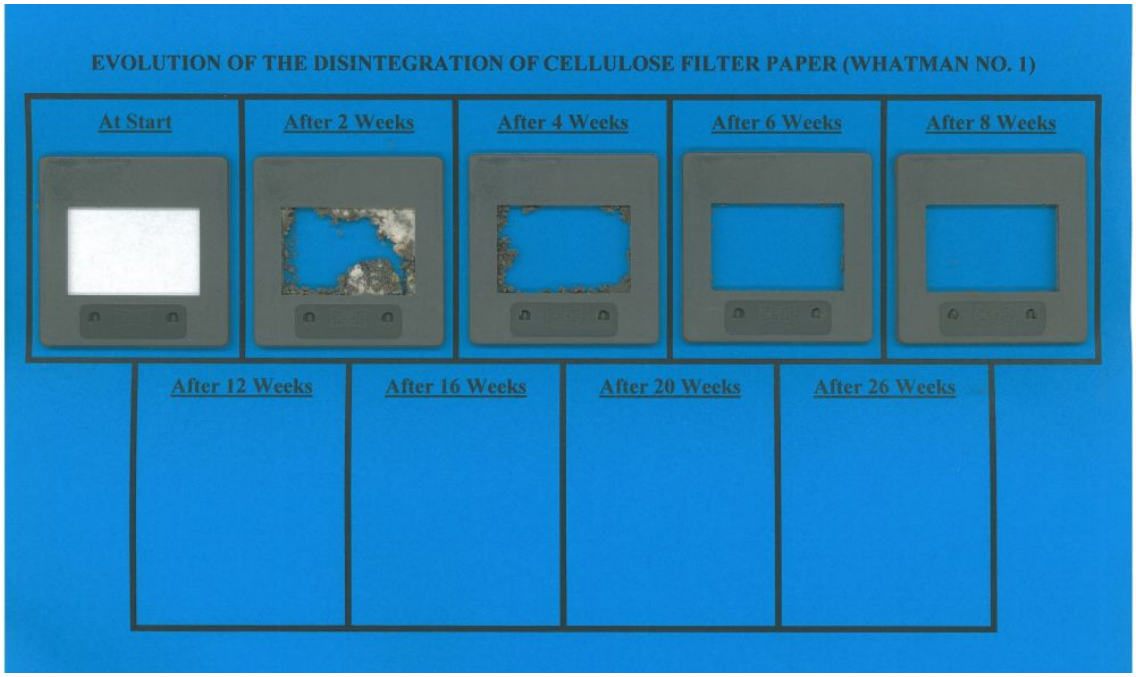

Figure 5. Visual presentation of the evolution of the disintegration of Cellulose filter paper in natural soil

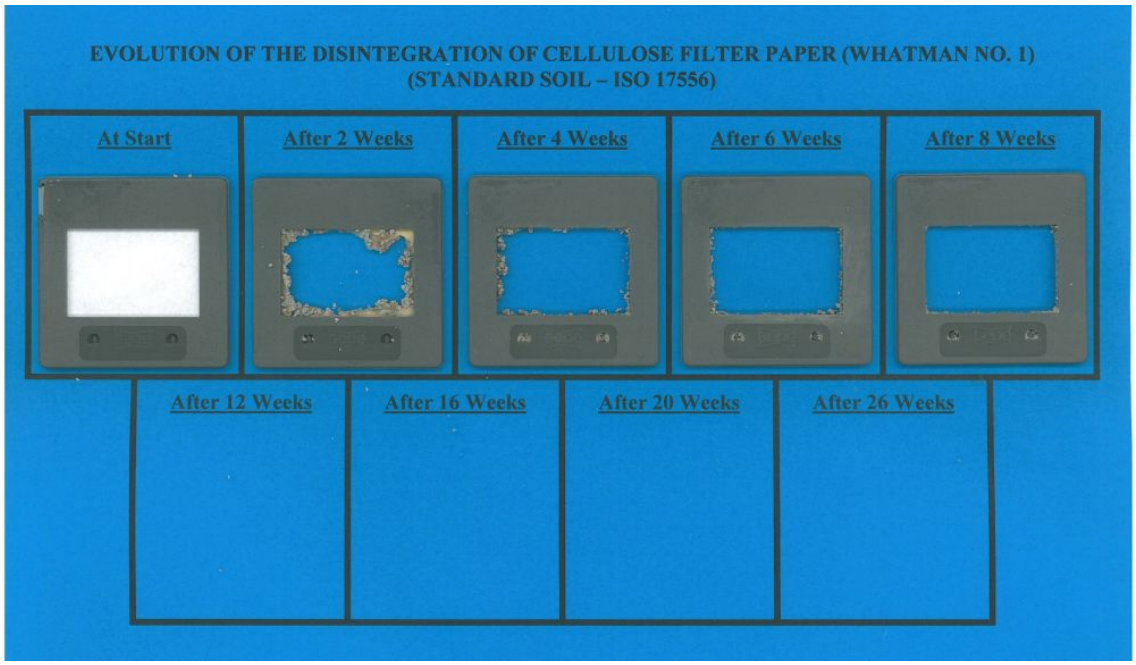

Figure 6. Visual presentation of the evolution of the disintegration of Cellulose filter paper in standard soil

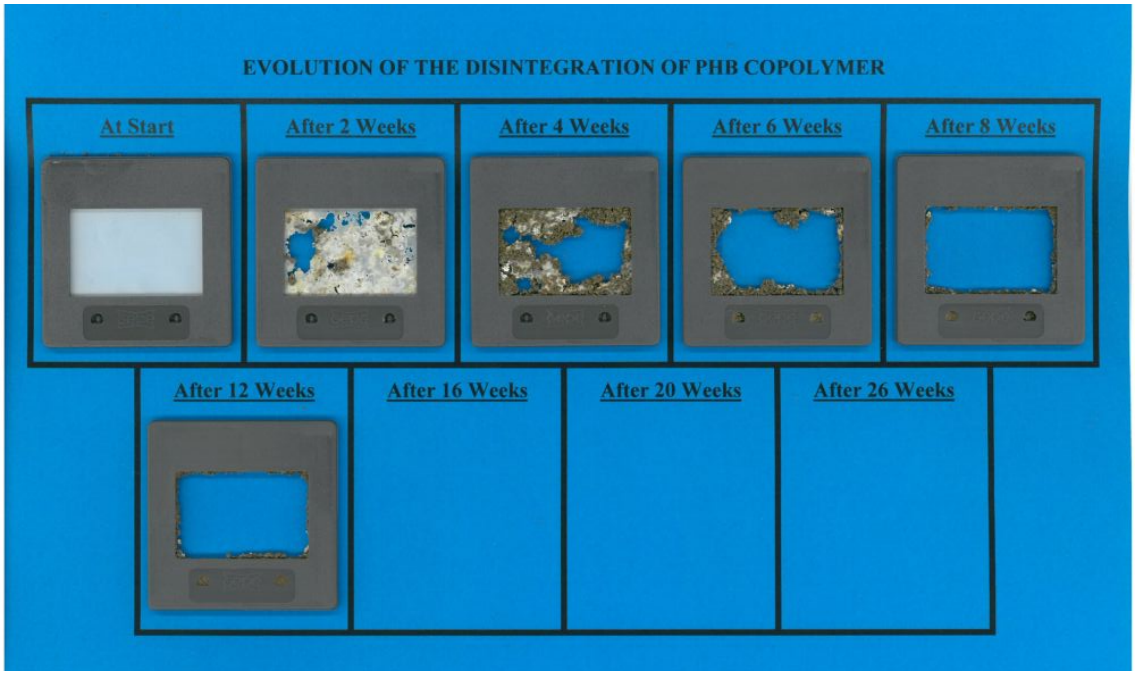

Figure 7. Visual presentation of the evolution of the disintegration of PHB in natural soil

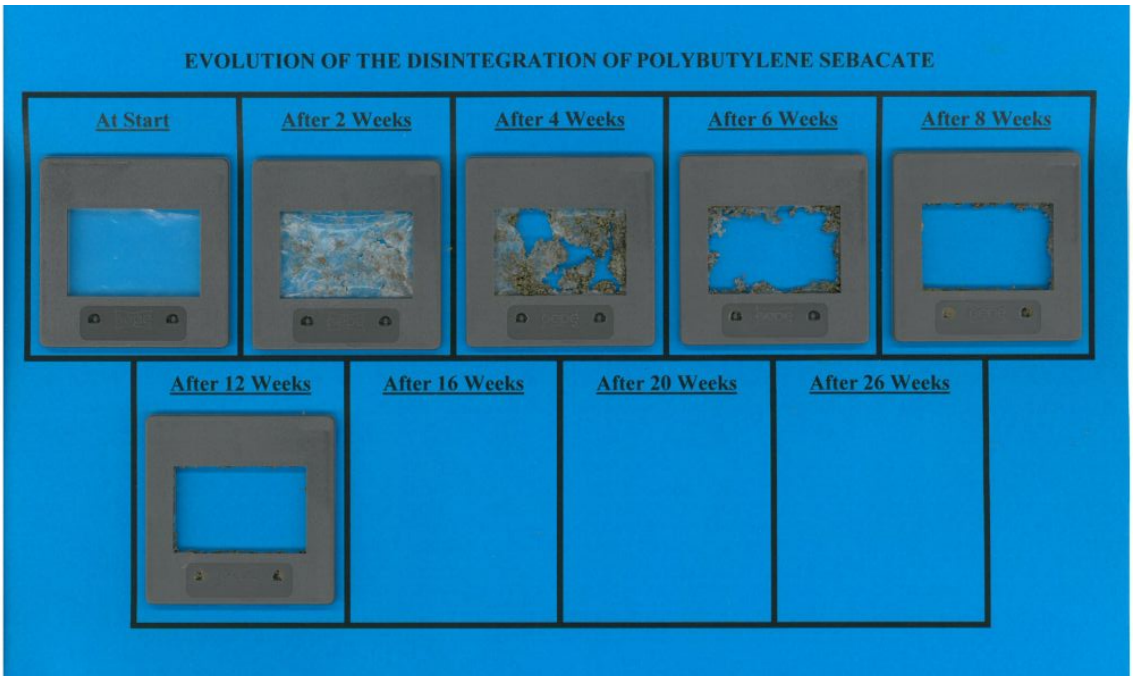

Figure 8. Visual presentation of the evolution of the disintegration of PBSe in natural soil

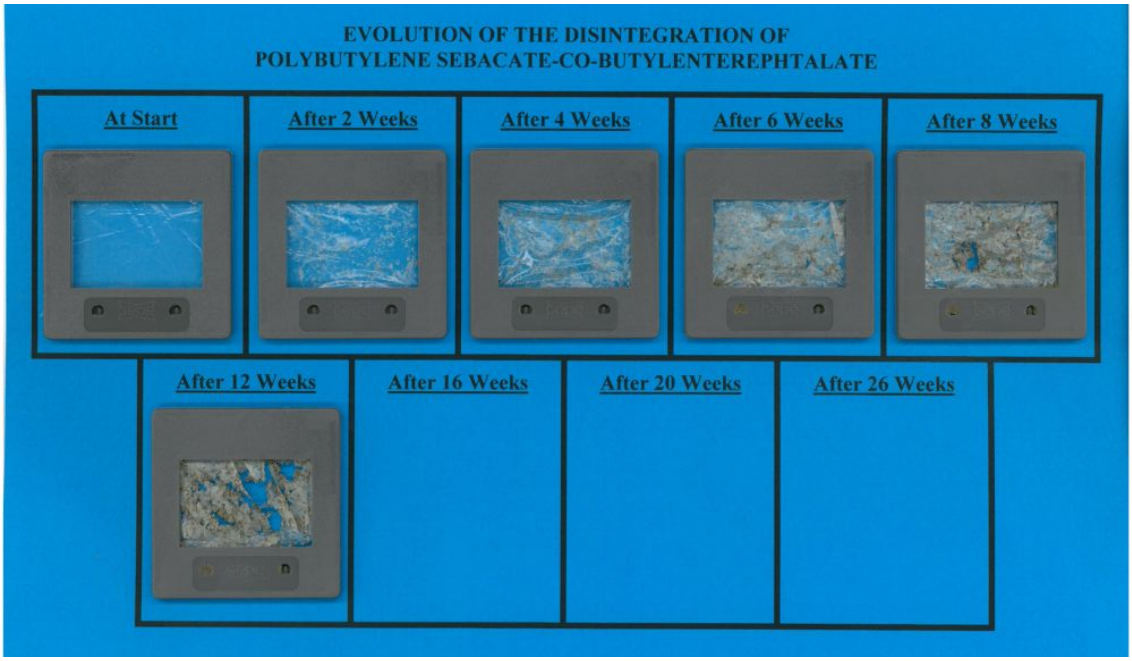

Figure 9. Visual presentation of the evolution of the disintegration of PBSeT in natural soil

4.4 Soil preparation for subsequent plant toxicity tests

4.4.1 OWS laboratory - Run 1

The test materials were milled and added to the soil inoculum in a 1.0% concentration on wet weight basis. The mixtures were thoroughly mixed. An overview of the test set-up is given in Table 23 (inoculum = natural soil) and Table 24 (inoculum = standard soil as prescribed by ISO 17556). The reactors were incubated at 25°C. Only control soil, cellulose filter paper soil and PBSe soil are used for plant toxicity tests and earthworm toxicity tests. Therefore, a higher quantity of these soils was prepared when compared to the other soils.

Table 23. Test set-up of soil preparation for subsequent ecotoxicity tests in natural soil

Reactor number	Test series	Natural soil (g)	Test item (g)
1	Control	5180.03	-
2	Cellulose filter paper	4220.08	42.23
3	PHB	480.09	4.81
4	PBSe	3740.07	37.42
5	PBSeT	480.03	4.80

Table 24. Test set-up of soil preparation for subsequent ecotoxicity tests in standard soil (as defined by ISO 17556)

Reactor number	Test series	Standard soil (ISO 17556) (g)	Test item (g)
6	Control	4220.12	-
7	Cellulose filter paper	4220.00	42.18

4.4.2 OWS laboratory - Run 2

Based on the results of run 1, it was decided to repeat the soil production and to perform another type of toxicity tests with soil micro-organisms (after discussions with CEN/TC 249/WG 7/TG1). The test was executed with Cellulose filter paper. The test material was milled (< 0.5 mm) and added to the soil inoculum in a 1.0% concentration on wet weight basis. The mixtures were thoroughly mixed.

An overview of the test set-up is given in Table 25.

Table 25. Test set-up of soil preparation for subsequent ecotoxicity tests in natural soil.

Reactor number	Test series	Natural soil (g wet)	Natural soil (g dry)	Test item (g)
1A	Control	3250.2	2655.4	-
1B	Control	3250.0	2655.3	-
2A	Cellulose filter paper (milled)	3249.9	2655.2	32.49
2B	Cellulose filter paper (milled)	3249.8	2655.1	32.53

The reactors were incubated at (25 ± 2)°C for 28 days. During the incubation period the soils were mixed and, if necessary, distilled water was added in order to restore the initial weight.

4.4.3 OWS laboratory - Run 3

Based on the results of run 2, the soil production was repeated again to perform another type of toxicity tests with soil micro-organisms (after discussions with CEN/TC 249/WG 7/TG1). An overview of the test set-up is given in Table 26.

Table 26. Test set-up of soil preparation for subsequent ecotoxicity tests in natural soil.

Reactor number	Test series	Natural soil (g wet)	Test item (g)
1	Control soil	4720	-
2	Control soil	4720	-
3	Control soil	4720	-
4	Cellulose filter paper soil	4200	36.33
5	Cellulose filter paper soil	4200	36.33
6	Cellulose filter paper soil	4200	36.33
7	LDPE soil	1400	12.11
8	PBSe soil	4200	36.33
9	PBSe soil	4200	36.33
10	PBSeT soil	4200	36.33
11	PBSeT soil	4030	34.86

All test materials were milled (< 1 mm) and added to the soil inoculum in a 1.0% concentration on dry weight basis. The reactors were incubated at (25 ± 2)°C. During the incubation period the soils were mixed and, if necessary, distilled water was added in order to restore the initial weight.

4.4.4 OWS laboratory - Run 4

The test was executed with Cellulose, PHB, PBSe, LDPE (source: Open-BIO) and LDPE (source: Aldrich - analytical grade). All test materials were milled (< 1 mm) and added to the soil inoculum in a 1.0% concentration on dry weight basis. The soil incubation was started on Feb-05-2016. The reactors were incubated at (25 ± 2)°C. During the incubation period the soils were mixed twice per week and, if necessary, distilled water was added in order to restore the initial weight.

An overview of the test set-up is given in Table 27.

Table 27. Test set-up of soil preparation for subsequent ecotoxicity tests in natural soil

Reactor number	Test series	Natural soil (g wet)	Test item (g)
1	Control soil	200.00	-
2	Cellulose filter paper soil	200.05	1.60
3	PHB soil	200.04	1.60
4	PBSe soil	200.03	1.60
5	LDPE soil (source: Open-Bio)	200.02	1.60
6	LDPE soil (source: Aldrich)	200.05	1.60

4.4.5 Novamont laboratory - Run 1

Plastic boxes with 3 kg of soil and 1% test material were prepared (Table 28). These soils have been used for ecotoxicity tests at different moments: during the active biodegradation phase (biodegradation percentage between 30-50%) and at the plateau phase.

Table 28. Test set-up of soil preparation for subsequent ecotoxicity tests in natural soil and in standard soil

Reactor number	Test series	Natural soil (kg)	Standard soil (kg)
1	Blank	3.0	-
2	PBSe 1%	3.0	-
3	PBSeT 1%	3.0	-
4	PHB 1%	3.0	-
5	Filter Paper 1%	3.0	-
6	Blank	-	3.0
7	PBSe 1%	-	3.0
8	PBSeT 1%	-	3.0
9	PHB 1%	-	3.0
10	Filter Paper 1%	-	3.0

4.5 Toxicity by means of higher plants

4.5.1 OWS laboratory - Plant toxicity tests with soil of run 1

The evaluation of the environmental safety by means of plants of Cellulose filter paper (in both inocula) and PBSe was started after an incubation period of 36 days. After an incubation period of 36 days a biodegradation percentage of approximately 67% and 59% was obtained for Cellulose filter paper in natural soil and standard soil, respectively, while a biodegradation percentage of approximately 63% was obtained for Polybutylene sebacate in natural soil (see Figure 1 and Figure 2). Initially it was suggested in CEN/TC 249/WG 7/TG 1 to start the evaluation of the environmental safety after 30%-40% biodegradation was reached. Consequently biodegradation is already at a higher percentage when compared to the initial proposed biodegradation level.

An overview of the start-up is given in Table 29. The weights are expressed per replicate. Three replicates were evaluated per test series.

Table 29. Test set-up plant toxicity test

Test series	Soil (g wet weight per pot)
Control soil (Natural soil)	200
Cellulose filter paper soil (Natural soil)	200
Polybutylene sebacate (Natural soil)	200
Control soil (Standard soil)	200
Cellulose filter paper soil (Standard soil)	200

The barley test was stopped after 9 days, while the cress test was stopped after 14 days.

The results of **the toxicity tests with barley** are given in Table 30 and Table 31 and in Figure 10 up to Figure 13. Both in the natural soil series and the standard soil series no significant difference was observed with regard to the germination between the blank soil and the test soil. In the natural soil series the plant yield in the blank soil was significantly higher when compared to the cellulose filter paper soil and the PBSe soil, while the reverse was observed in the standard soil (plant yield in cellulose filter paper soil was significantly higher when compared to blank standard soil). It must be noticed that the cellulose filter paper soil and the PBSe soil based on natural soil were characterized by a comparable plant yield. No significant difference was observed between these series.

Table 30. Germination rate and plant yield of barley plants

Test series	Germination rate (%)	
	AVG	STD
Blank soil (natural soil)	100.0	0.0
Cellulose filter paper soil (natural soil)	96.0	0.0
PBSe soil (natural soil)	100.0	0.0
Blank soil (standard soil)	100.0	0.0
Cellulose filter paper soil (standard soil)	99.3	1.2
Test series	**Fresh Weight Yield (g)**	
	AVG	STD
Blank soil (natural soil)	7.93	0.18
Cellulose filter paper soil (natural soil)	6.23	0.04
PBSe soil (natural soil)	6.43	0.33
Blank soil (standard soil)	5.35	0.54
Cellulose filter paper soil (standard soil)	7.49	0.72
Test series	**Dry Weight Yield (g)**	
	AVG	STD
Blank soil (natural soil)	0.83	0.04
Cellulose filter paper soil (natural soil)	0.67	0.01
PBSe soil (natural soil)	0.69	0.03
Blank soil (standard soil)	0.60	0.05
Cellulose filter paper soil (standard soil)	0.77	0.06

Table 31. Relative germination and plant yield (as % of blank soil) (N = Natural soil; S = Standard soil)

Test series	Germination	Wet weight plant yield	Dry weight plant yield
Cellulose filter paper soil (N)	96	79	81
PBSe soil (N)	100	81	83
Cellulose filter paper soil (S)	99	140	128

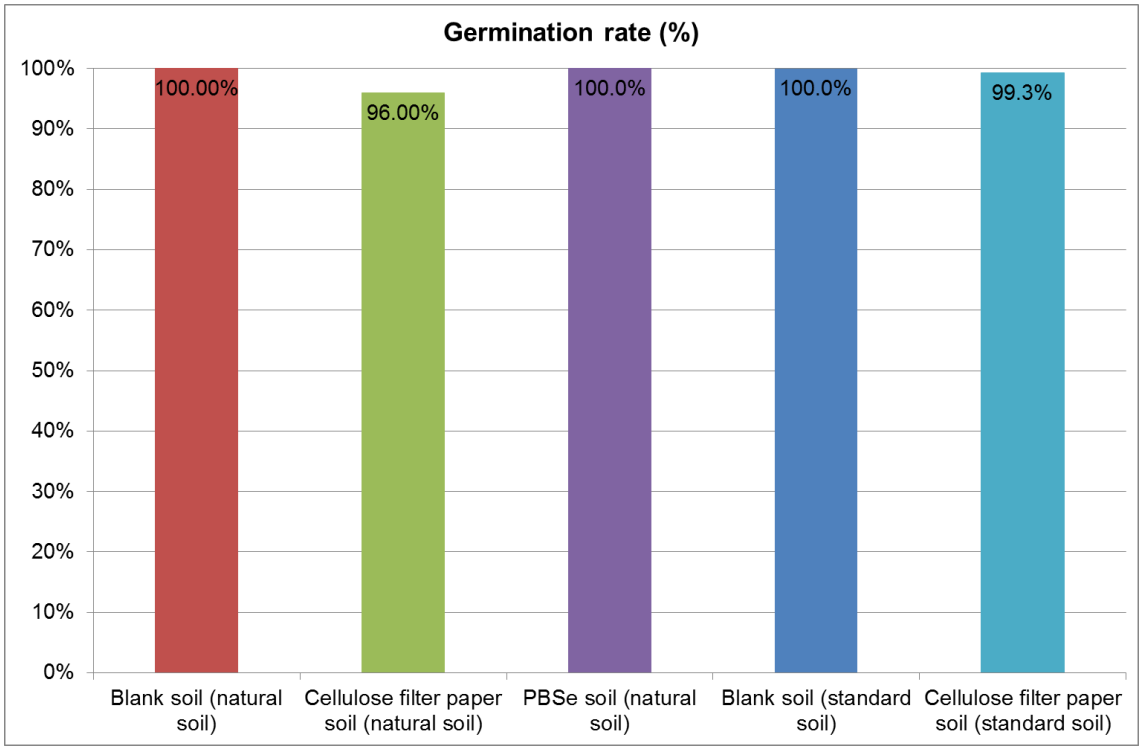

Figure 10. Germination rate of barley plants

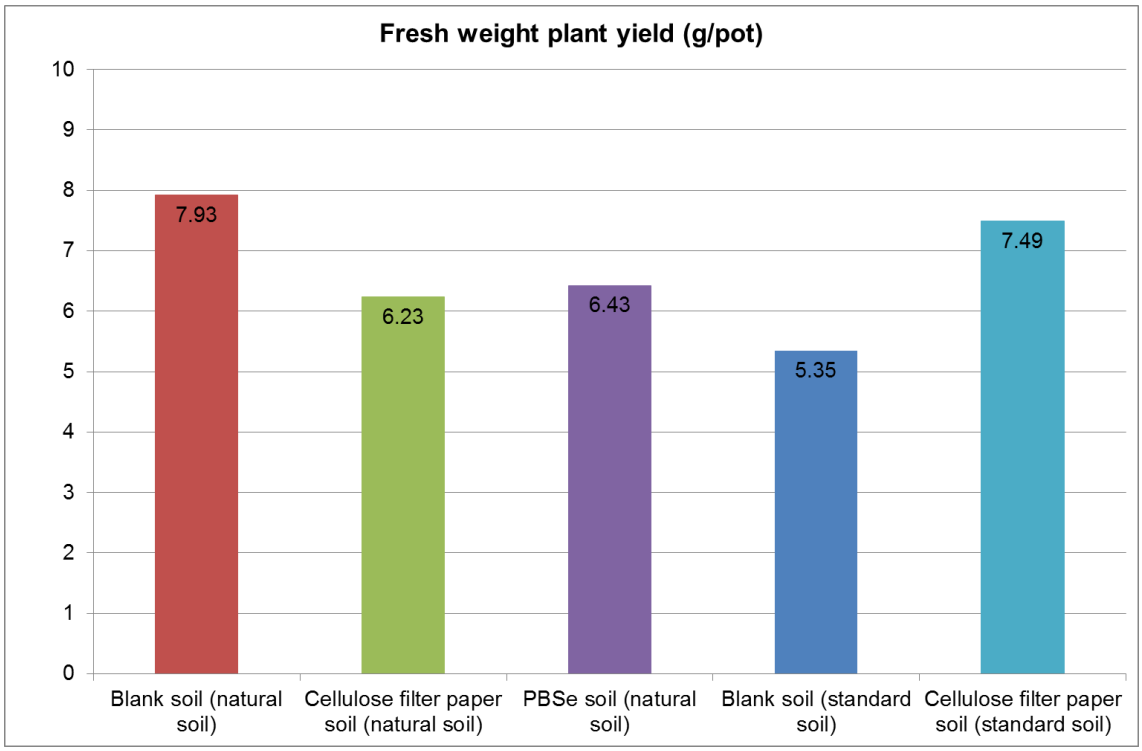

Figure 11. Fresh weight plant yield of barley plants

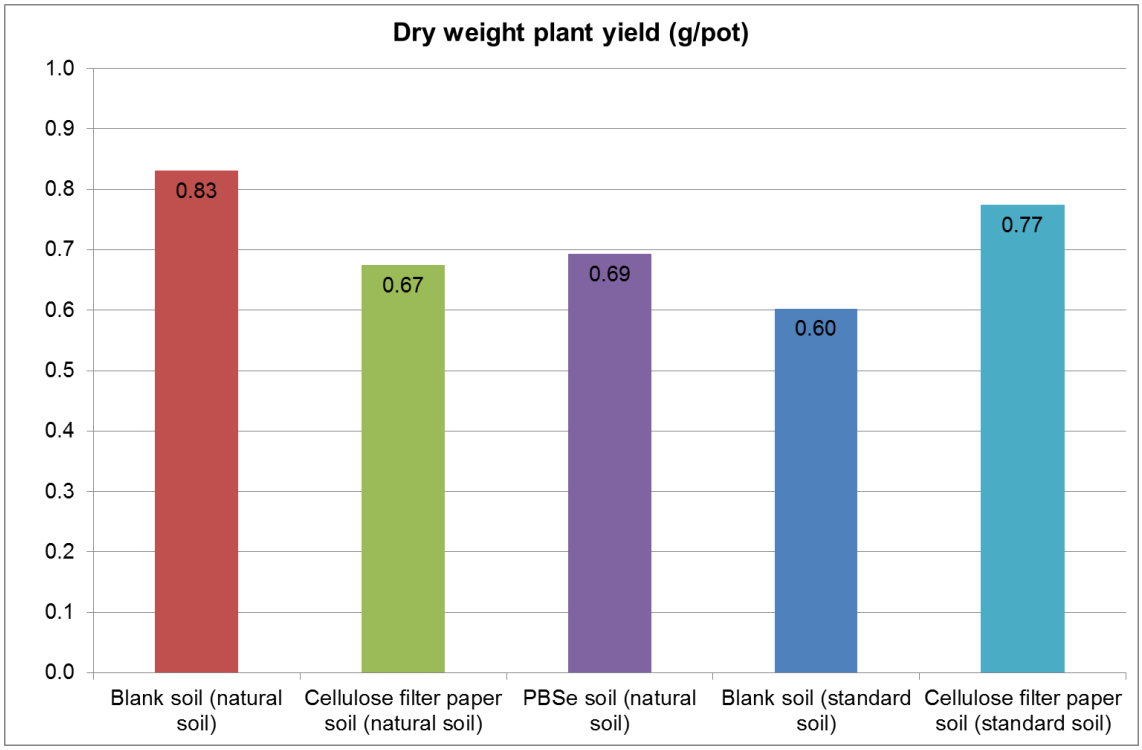

Figure 12. Dry weight plant yield of barley plants

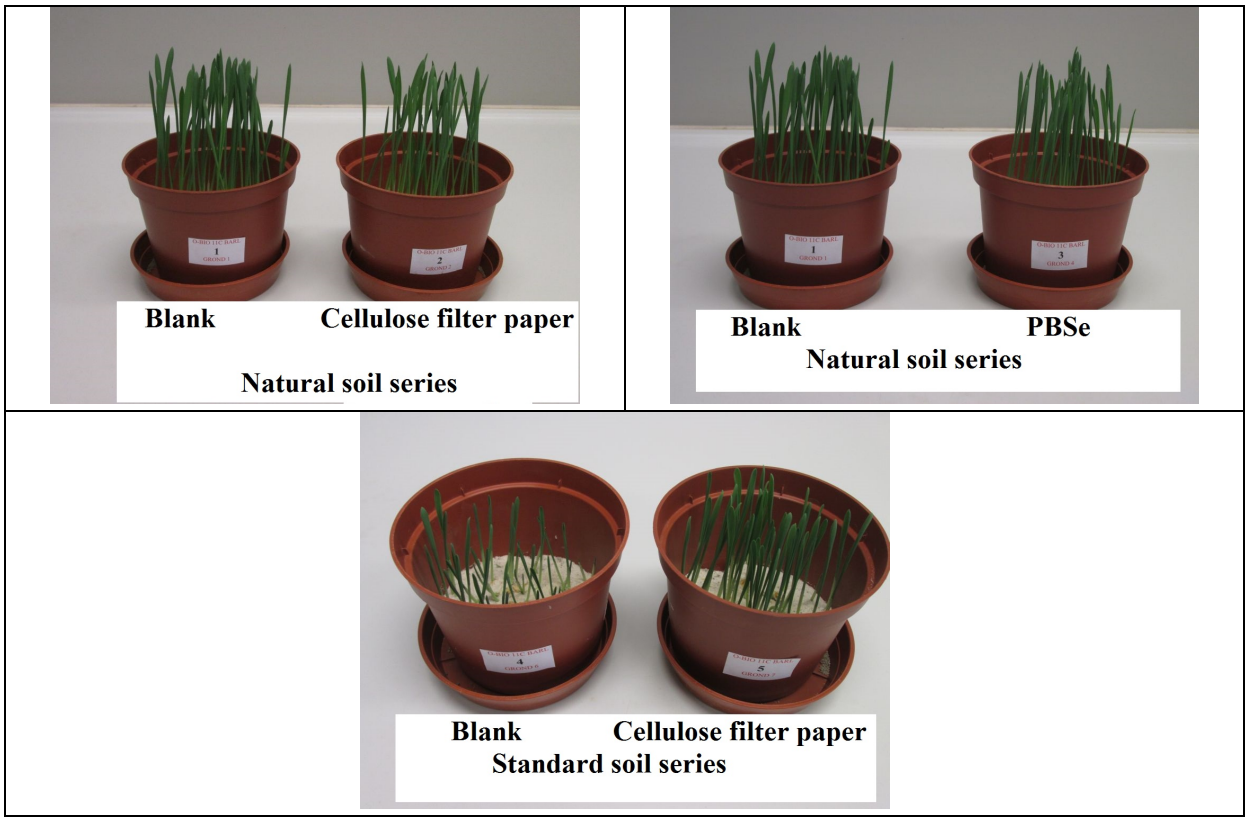

Figure 13. Visual presentation of barley plants

The results of **the toxicity tests with cress** are given in Table 32 and Table 33 and in Figure 14 up to Figure 17.

In the natural soil series, no significant difference was observed with regard to the germination between the blank soil and the test soil, while the germination in the standard soil was significantly lower when compared to the cellulose filter paper series in standard soil.

In the natural soil series the plant yield in the blank soil was significantly higher when compared to the cellulose filter paper soil and the PBSe soil, while the reverse was observed in the standard soil (plant yield in cellulose filter paper soil was significantly higher when compared to blank standard soil). It must be noticed that the cellulose filter paper soil and the PBSe soil based on natural soil were characterized by a comparable plant yield. No significant difference was observed between these series.

Table 32. Germination rate and plant yield of cress plants

Test series	**Germination rate (%)**	
	AVG	**STD**
Blank soil (natural soil)	99.0	1.0
Cellulose filter paper soil (natural soil)	97.7	2.3
PBSe soil (natural soil)	96.7	2.1
Blank soil (standard soil)	45.0	10.5
Cellulose filter paper soil (standard soil)	95.7	1.5
Test series	**Fresh Weight Yield (g)**	
	AVG	**STD**
Blank soil (natural soil)	3.55	0.61
Cellulose filter paper soil (natural soil)	2.31	0.25
PBSe soil (natural soil)	2.26	0.32
Blank soil (standard soil)	1.14	0.33
Cellulose filter paper soil (standard soil)	3.29	0.22
Test series	**Dry Weight Yield (g)**	
	AVG	**STD**
Blank soil (natural soil)	0.30	0.01
Cellulose filter paper soil (natural soil)	0.21	0.02
PBSe soil (natural soil)	0.21	0.01
Blank soil (standard soil)	0.12	0.05
Cellulose filter paper soil (standard soil)	0.25	0.01

Table 33. Relative germination rate and plant yield (as % of blank soil) (N = Natural soil; S = Standard soil)

Test series	Germination	Wet weight plant yield	Dry weight plant yield
Cellulose filter paper soil (N)	99	65	72
PBSe soil (N)	98	64	72
Cellulose filter paper soil (S)	213	288	216

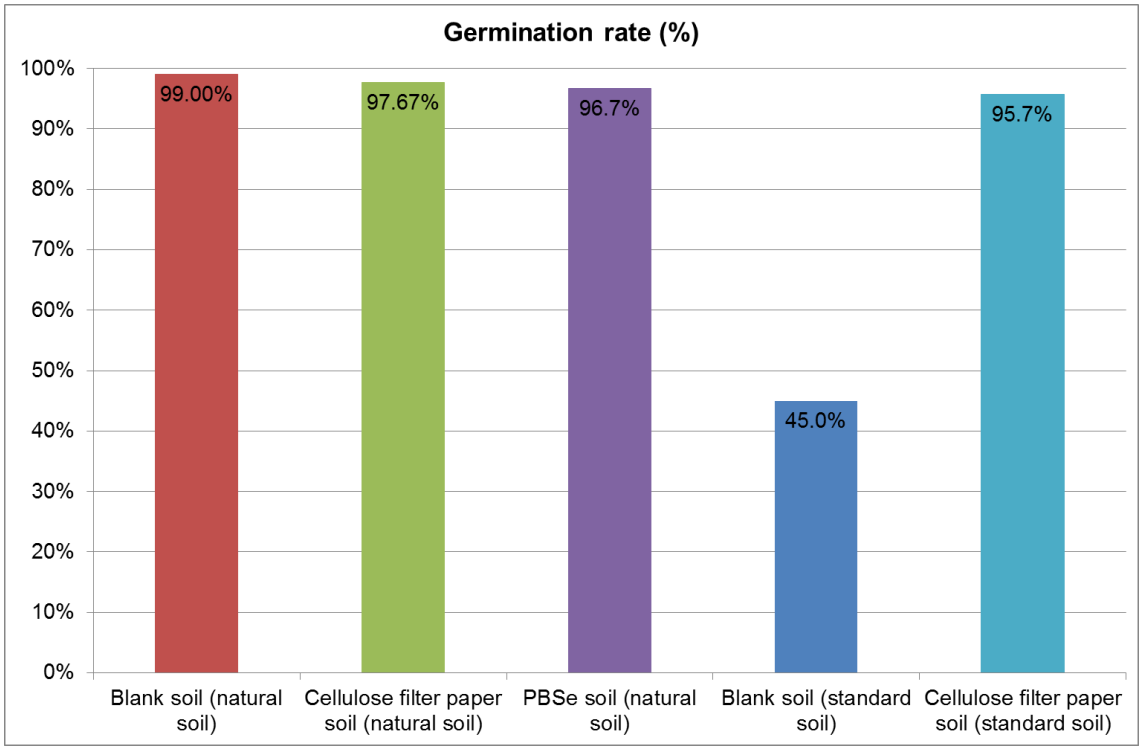

Figure 14. Germination rate of cress plants

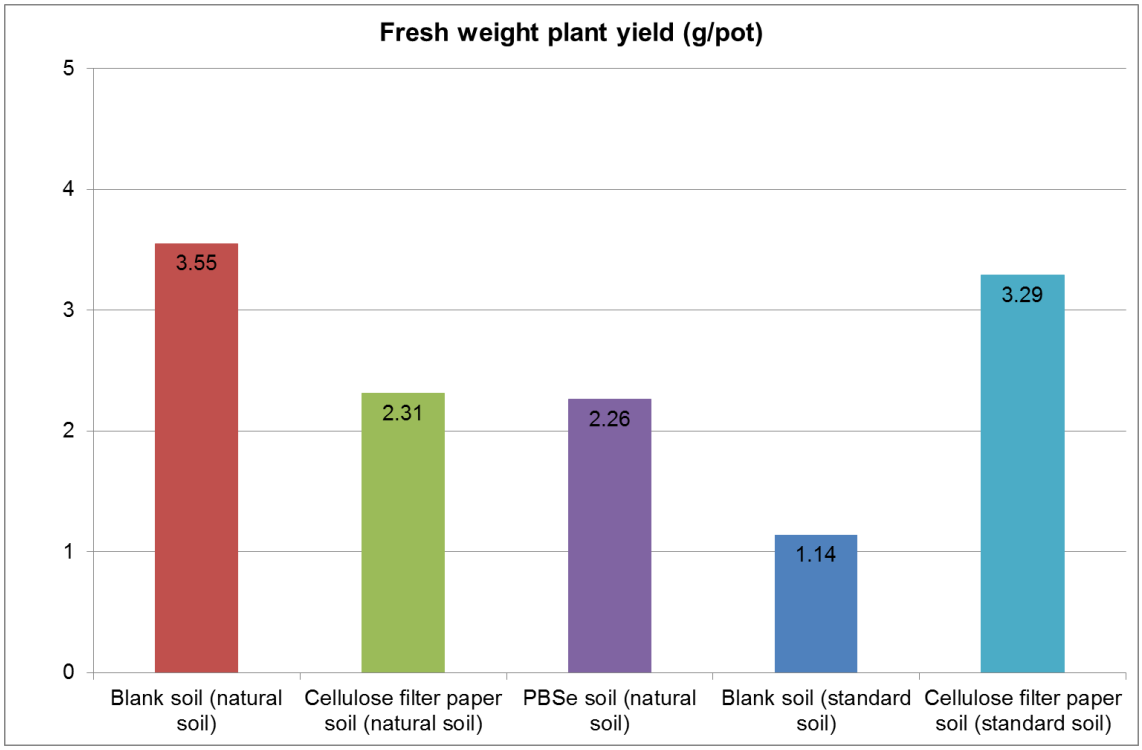

Figure 15. Fresh weight plant yield of cress plants

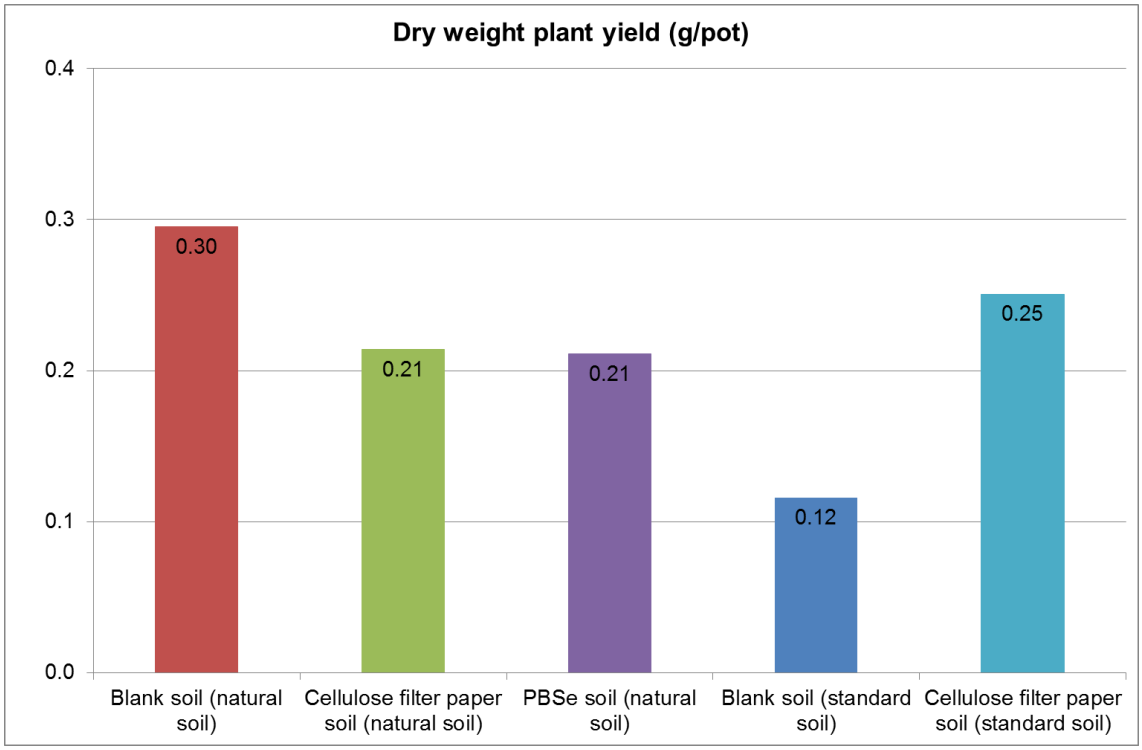

Figure 16. Dry weight plant yield of cress plants

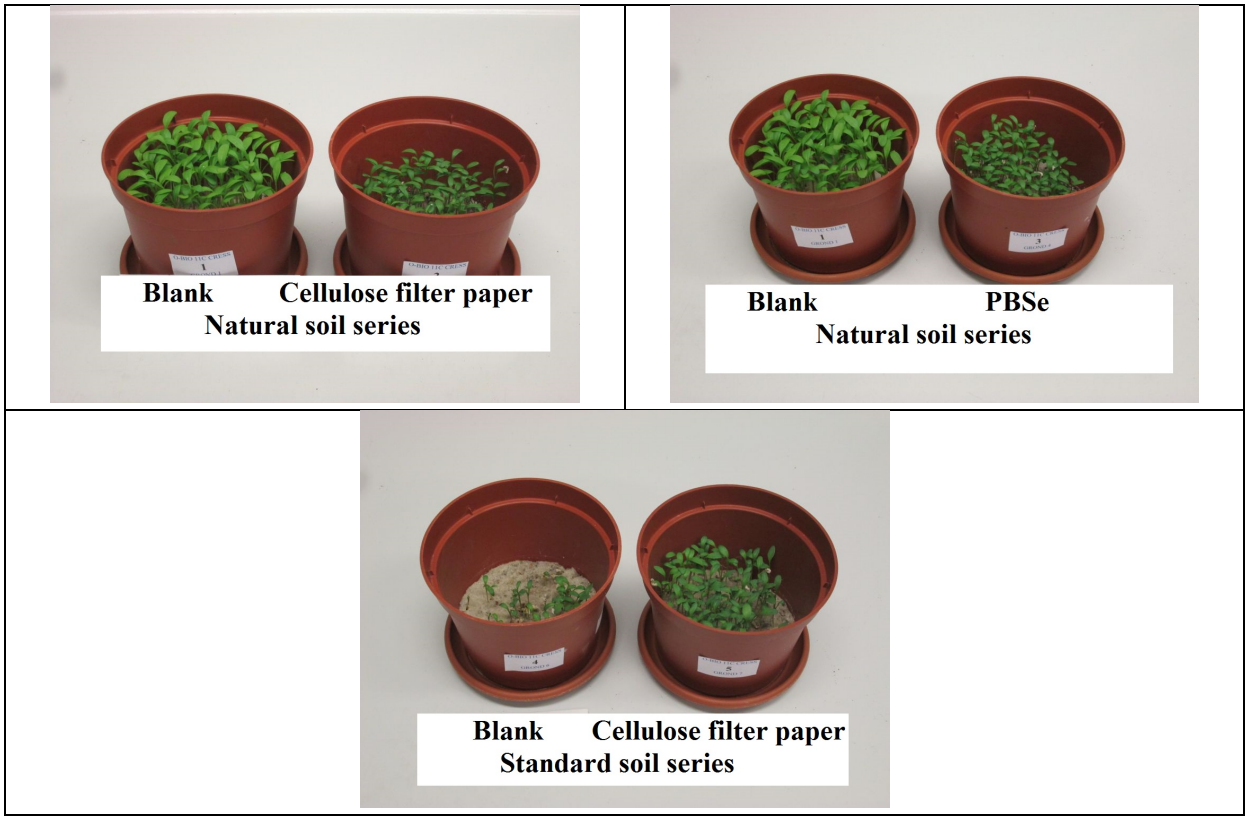

Figure 17. Visual presentation of cress plants

Disadvantage natural soil: plant yield in the blank natural soil is significantly higher when compared to the test soil. This is most probably caused by the fact that nutrients are consumed by the micro-organisms during the biodegradation of the sample. The solution for this problem could be that the test soil is compared with the cellulose filter paper soil as reference. Alternatively the fertilizing effect could be eliminated by adding a nutrient solution.

Disadvantage standard soil: germination rate in blank standard soil is very low (for cress plants) and therefore test is not valid. This will probably also be the case for other plant species (which are sensitive for high nutrient values). Moreover, the plant yield in the blank standard soil is considerably lower when compared to the test series. This is probably caused by the fact that the nutrient content in the blank standard soil is still too high (due to the fact that no nutrients are consumed during the degradation).

4.5.2 OWS laboratory - Plant toxicity tests with soil of run 3

Due to the difference in nitrate content between control soil and test soils (see Chapter 3.1.4), it is possible that negative results are observed in the test soils, which are not caused by a toxic effect of the residuals of the test items, but by a fertilizing effect due to the difference in nitrate content. Therefore the cellulose filter paper soil and the PBSe soil were both enriched with nitrate under the form of HNO_3. For the barley test, the soils were enriched till a similar level as in the control soil, while for the cress test the soils were enriched till a lower level (175 mg/l) as cress plants are normally sensitive for the addition of nitrate under the form of nitric acid.

An overview of the start-up is given in Table 34. The weights are expressed per replicate. Three replicates were evaluated per test series.

Table 34. Test set-up plant toxicity test

Test series	Soil (g wet weight per pot)
Control soil	200.0
Cellulose filter paper soil	200.0
Cellulose filter paper soil + nitrate addition	200.0
PBSe soil	200.0
PBSe soil + nitrate addition	200.0

The barley test was stopped after 10 days, while the cress test was stopped after 14 days.

The results of the toxicity tests with barley are given in Table 35 and in Figure 18 up to Figure 20.

The germination rate and the plant biomass (on fresh weight basis and on dry weight basis) of the cellulose filter paper soil (with nitrate addition) and the PBSe soil (with nitrate addition) were both higher than 90% when compared to the control soil. This was also the case for the germination rate of the cellulose filter soil (without nitrate addition) and for the PBSe soil (without nitrate addition) and for the plant biomass (on fresh weight basis) of the cellulose filter paper soil (without nitrate addition). However, for the plant biomass (on fresh weight basis) of the PBSe soil without nitrate addition and for the plant biomass (on dry weight basis) of the cellulose filter paper soil without nitrate addition and the PBSe soil without nitrate addition, less than 90% was reached when compared to the control soil.

From the NO_x^- -N analyses at start of the ecotoxicity test, it was seen that the nitrification process in the cellulose filter paper soil and the PBSe soil was not as far proceeded as in the control soil. This could have a fertilizing effect and favour plant growth in the control soil. This fertilizing effect can be confirmed by the results shown below. It can be concluded that, due to the fertilizing effect in the control soil, the 90% pass level was not reached for the plant biomass (on fresh weight basis and/or on dry weight basis). By adding an extra test series, in

which the cellulose filter paper soil and the PBSe soil were enriched with nitrate till a similar level as the control soil, the fertilizing effect is eliminated.

Table 35. Germination rate and plant yield of barley plants

Test series	Germination rate (%)	
	AVG	STD
Control soil	95.3	1.2
Cellulose filter paper soil	93.3	3.1
Cellulose filter paper soil + NO_3^--N	96.0	4.0
PBSe soil	99.3	1.2
PBSe soil + NO_3^--N	94.7	6.1
Test series	**Fresh Weight Yield (g)**	
	AVG	**STD**
Control soil	7.87	0.08
Cellulose filter paper soil	7.22	0.14
Cellulose filter paper soil + NO_3^--N	7.71	0.18
PBSe soil	6.44	0.14
PBSe soil + NO_3^--N	7.55	0.85
Test series	**Dry Weight Yield (g)**	
	AVG	**STD**
Control soil	0.81	0.02
Cellulose filter paper soil	0.65	0.01
Cellulose filter paper soil + NO_3^--N	0.76	0.02
PBSe soil	0.60	0.01
PBSe soil + NO_3^--N	0.72	0.09

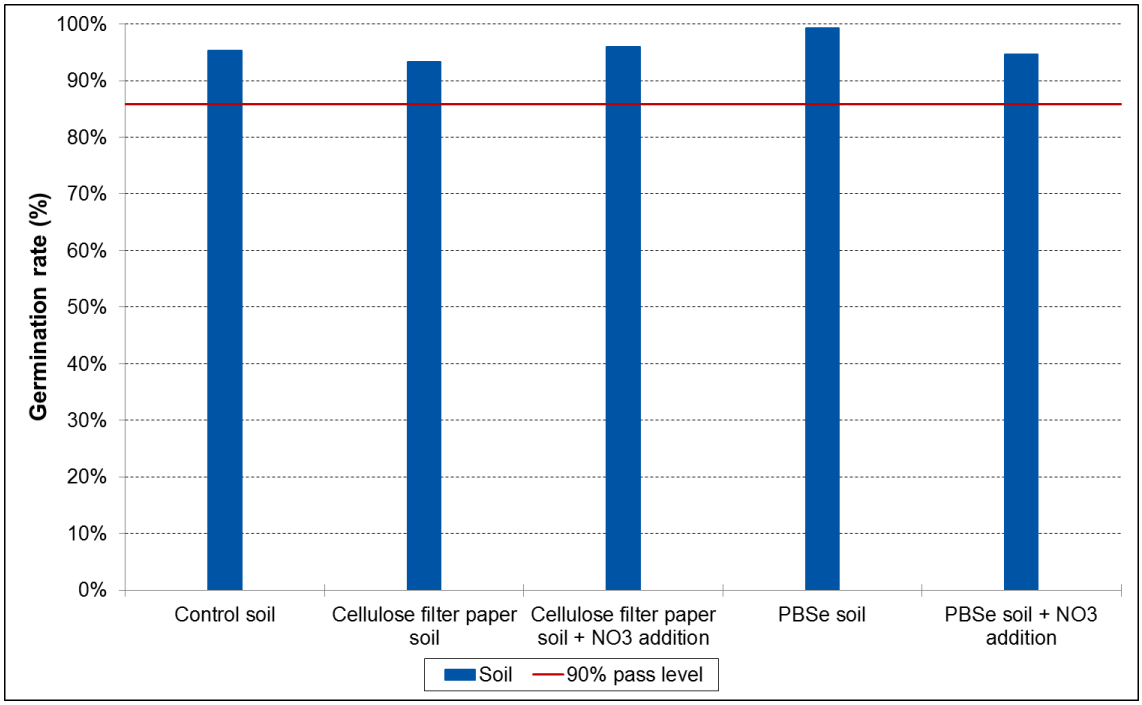

Figure 18. Germination rate of barley plants

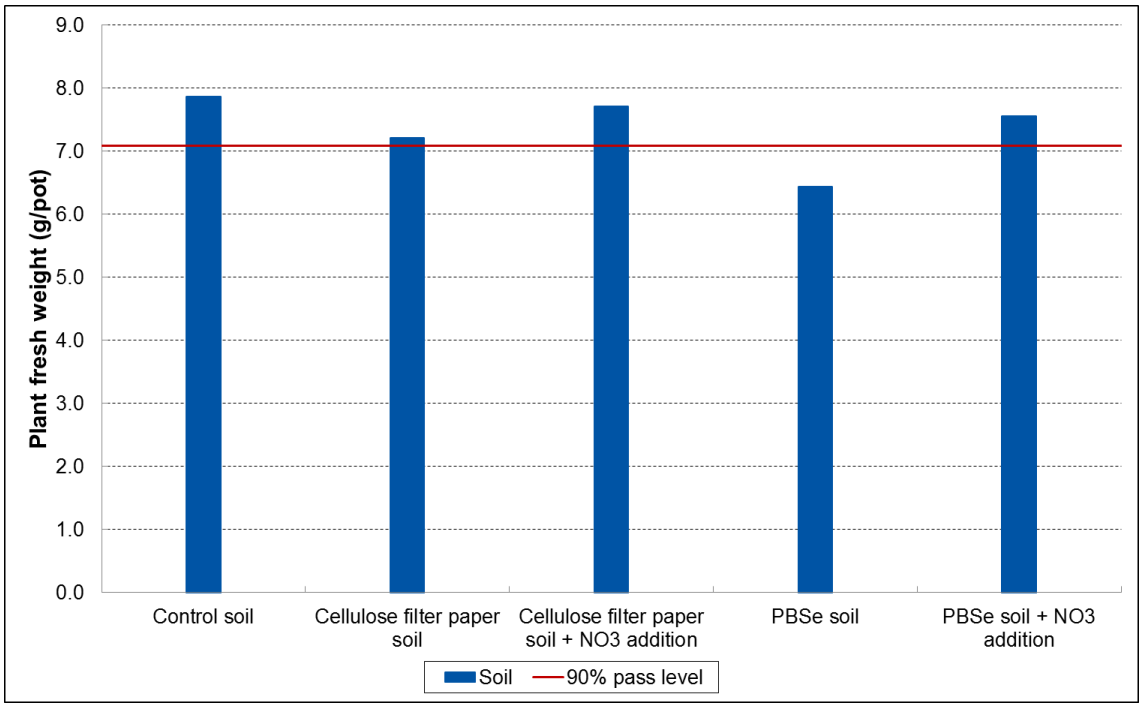

Figure 19. Fresh weight plant yield of barley plants

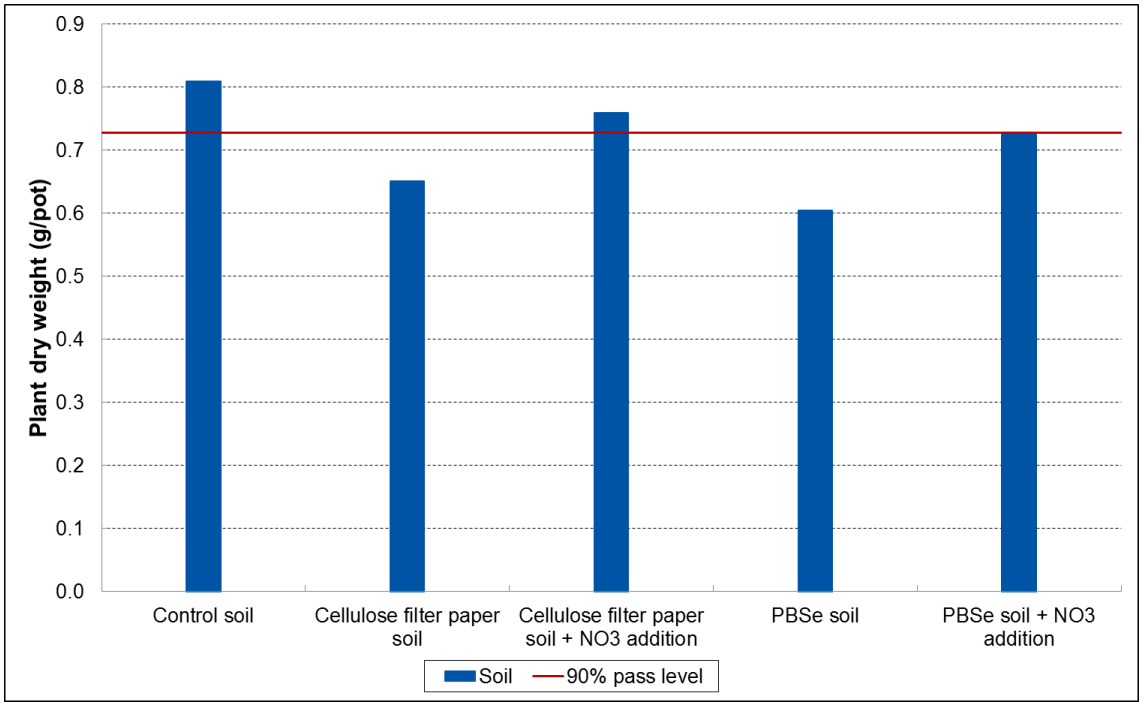

Figure 20. Dry weight plant yield of barley plants

The results of the toxicity tests with cress are given in Table 36 and in Figure 21 up to Figure 23.

The germination rate and the plant biomass (on fresh weight basis and on dry weight basis) of the cellulose filter paper soil (with and without nitrate addition) and the PBSe soil (with nitrate addition) were both higher than 90% when compared to the control soil. This was also the case for the germination rate of the PBSe soil (without nitrate addition). However, for the plant biomass of the PBSe soil (without nitrate addition), less than 90% was reached when compared to the control soil.

From the NO_x^- -N analyses at start of the ecotoxicity test, it was seen that the nitrification process in the cellulose filter paper soil and the PBSe soil was not as far proceeded as in the control soil. This could have a fertilizing effect and favour plant growth in the control soil. This fertilizing effect can be confirmed for the PBSe soil by the results shown below. It can be concluded that, due to the fertilizing effect in the control soil, the 90% pass level was not reached for the plant biomass (on fresh weight basis and on dry weight basis). By adding an extra test series, in which the PBSe soil were enriched with nitrate till approximately 175 mg/l, the fertilizing effect is eliminated.

Table 36. Germination rate and plant yield of cress plants

Test series	Germination rate (%)	
	AVG	STD
Control soil	98.7	0.6
Cellulose filter paper soil	99.0	0.0
Cellulose filter paper soil + NO_3^--N	98.0	2.0
PBSe soil	95.7	1.5
PBSe soil + NO_3^--N	97.3	1.5
Test series	**Fresh Weight Yield (g)**	
	AVG	**STD**
Control soil	5.62	0.06
Cellulose filter paper soil	6.13	0.21
Cellulose filter paper soil + NO_3^--N	5.70	0.10
PBSe soil	5.00	0.33
PBSe soil + NO_3^--N	5.72	0.18
Test series	**Dry Weight Yield (g)**	
	AVG	**STD**
Control soil	0.37	0.01
Cellulose filter paper soil	0.38	0.02
Cellulose filter paper soil + NO_3^--N	0.36	0.00
PBSe soil	0.33	0.01
PBSe soil + NO_3^--N	0.35	0.02

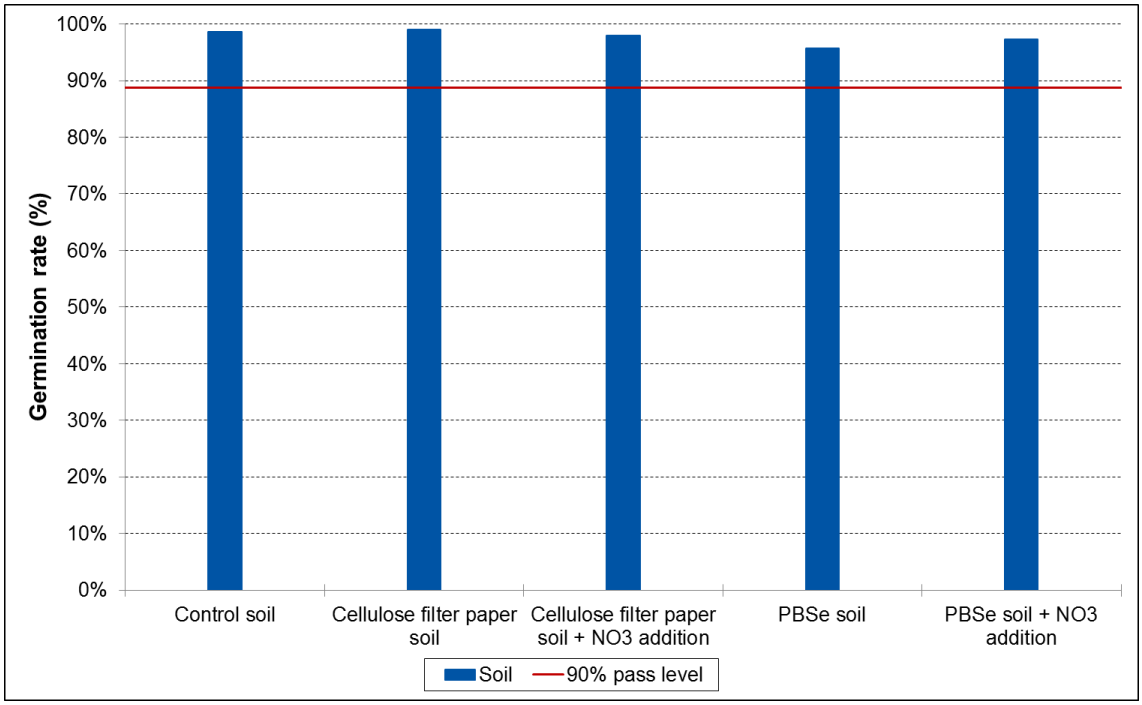

Figure 21. Germination rate of cress plants

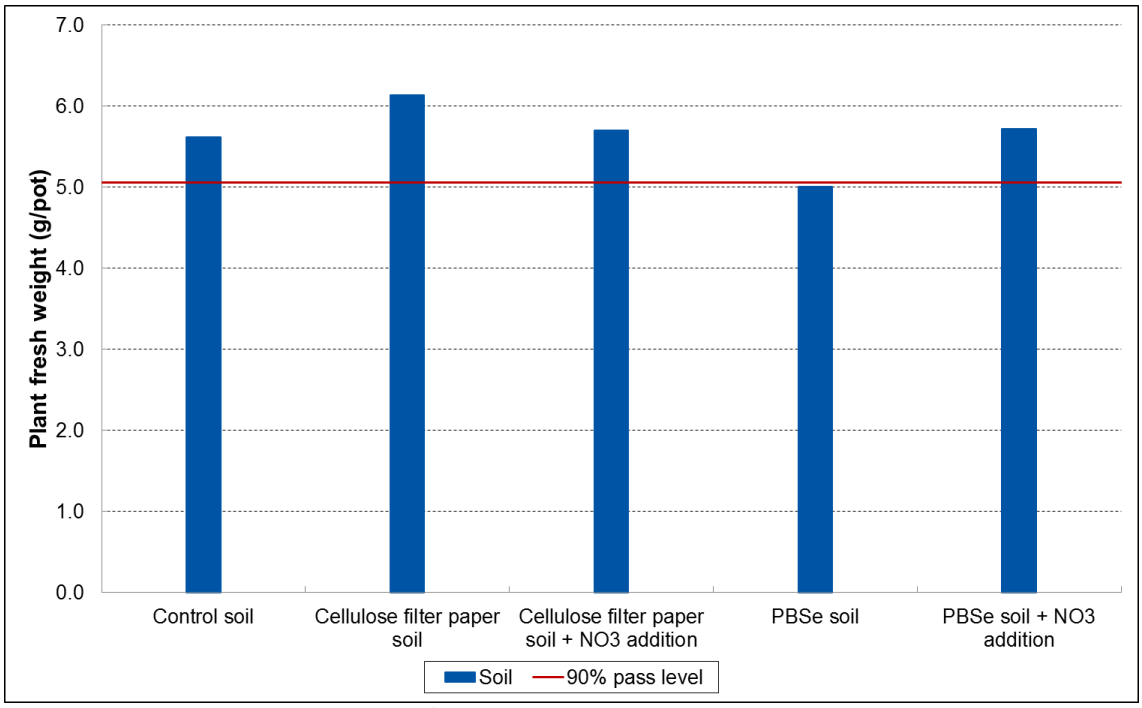

Figure 22. Fresh weight plant yield of cress plants

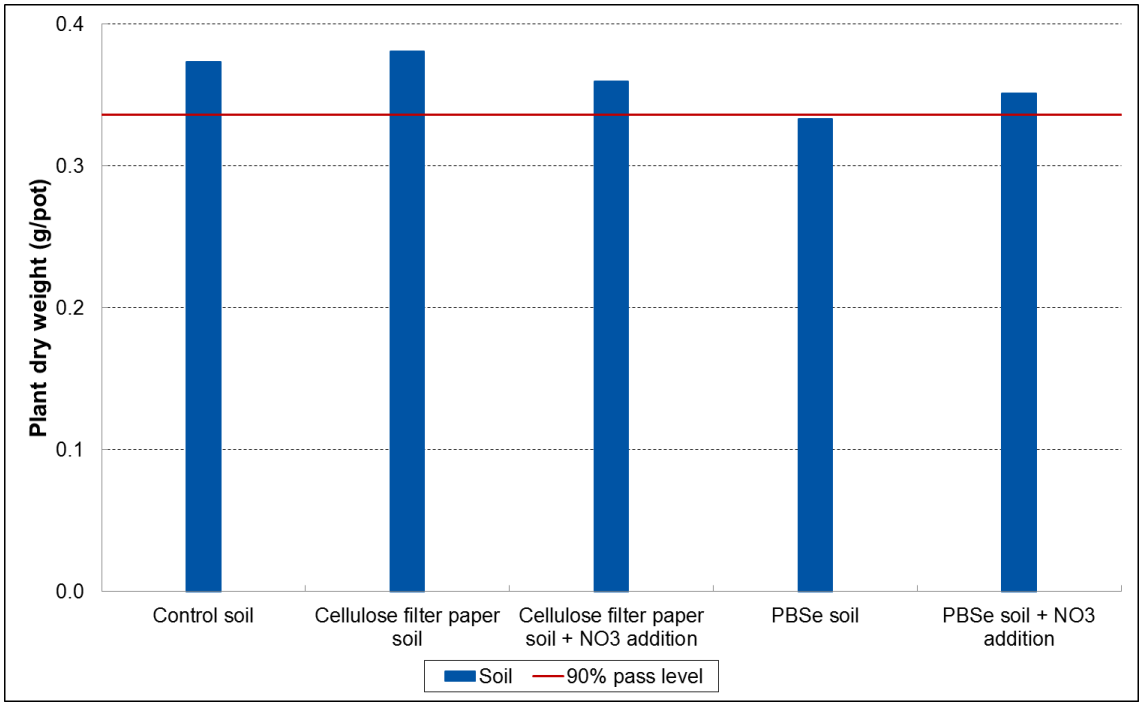

Figure 23. Dry weight plant yield of cress plants

4.5.3 Novamont laboratory - Plant toxicity tests during active biodegradation phase

Plant toxicity tests were performed on the natural soil samples.

Blank soil, PHB soil and cellulose filter paper soil were mixed with a substrate made of peat and sand. The soil samples were tested in a concentration of 25% and 50%. The test was started after an incubation period of 14 days in soil. After 14 days PHB had reached a biodegradation percentage of approximately 48% and cellulose filter paper a biodegradation percentage of about 43%.

Blank soil, PBSe soil and PBSeT soil were tested without dilution. The test was started after an incubation period of 25 days in soil. After 25 days a biodegradation percentage of about 52% was reached for PBSe, while a biodegradation percentage of about 56% was reached for PBSeT.

In the first CEN/TC 249/WG 7/TG 1 meeting it was suggested to start the evaluation of the environmental safety when at least 30%-40% biodegradation was reached. Consequently biodegradation is already at a higher percentage when compared to the proposed biodegradation level.

In each pot 150 seeds of radish are laid on the surface of soil. The test duration was 15 days.

The results of the tests are shown in Table 37 and Table 38 and in Figure 24 and Figure 25. No toxic effects were observed, when testing the soil directly (100%) or diluted (25 and 50%). The germination and the plants biomass are similar and in general statistical differences were not recorded. Only the soil without dilution showed an increase of germination % and plant biomass; however the difference is rather low and it should be tested again to confirm this tendency.

Table 37. Experimental data

Sample	Soil (%)	Substrate (g)	Soil (g)	Germination (number)	Wet Weight (g)	Dry Weight (g)
Control	-	350	-	138	12.9175	0.7329
Control	-	350	-	120	9.1864	0.6154
Control	-	350	-	123	8.6614	0.6019
Blank	25	262.5	87.5	125	10.3989	0.8791
Blank	25	262.5	87.5	109	8.7598	0.7503
Blank	25	262.5	87.5	106	9.3518	0.7308
Blank	50	175	175	126	9.4867	0.9132
Blank	50	175	175	119	7.9757	0.8728
Blank	50	175	175	130	10.8331	0.8221
PHB	25	262.5	87.5	119	12.0733	0.8385
PHB	25	262.5	87.5	121	10.7456	0.6134
PHB	25	262.5	87.5	132	11.622	0.7289
PHB	50	175	175	118	11.9926	0.8092
PHB	50	175	175	106	8.6748	0.6587
PHB	50	175	175	136	12.8933	0.8134
Cellulose	25	262.5	87.5	118	12.2416	0.7761
Cellulose	25	262.5	87.5	109	8.9991	0.5436
Cellulose	25	262.5	87.5	137	12.1277	0.8494
Cellulose	50	175	175	118	9.3009	0.6530
Cellulose	50	175	175	108	4.5462	0.8184
Cellulose	50	175	175	136	11.0765	0.8816
PBSe	100	0	350	142	15.3323	0.9048
PBSe	100	0	350	148	13.8185	0.8330
PBSe	100	0	350	143	11.5035	0.8240
PBSeT	100	0	350	132	13.6825	0.8242
PBSeT	100	0	350	137	5.5219	0.9174
PBSeT	100	0	350	131	10.2128	0.9538
Blank	100	0	350	124	11.3835	0.8034
Blank	100	0	350	115	7.6745	0.7790

Table 38. Final results

	Germination Rate %		Fresh Weight Yield (g)		Dry Weight Yield (g)	
	AVG	STD	AVG	STD	AVG	STD
Blank 25%	75.56	6.81	9.50	0.83	0.79	0.08
Blank 50%	83.33	3.71	9.43	1.43	0.87	0.05
PHB 25%	82.67	4.67	11.48	0.68	0.73	0.11
PHB 50%	80.00	10.07	11.19	2.22	0.76	0.09
Cell 25%	80.89	9.53	11.12	1.84	0.72	0.16
Cell 50%	80.44	9.46	8.31	3.38	0.78	0.12
Blank 100%	79.67	4.24	9.53	2.62	0.79	0.02
PBSe 100%	96.22	2.14	13.55	1.93	0.85	0.04
PBSeT 100%	88.89	2.14	9.81	4.10	0.90	0.07

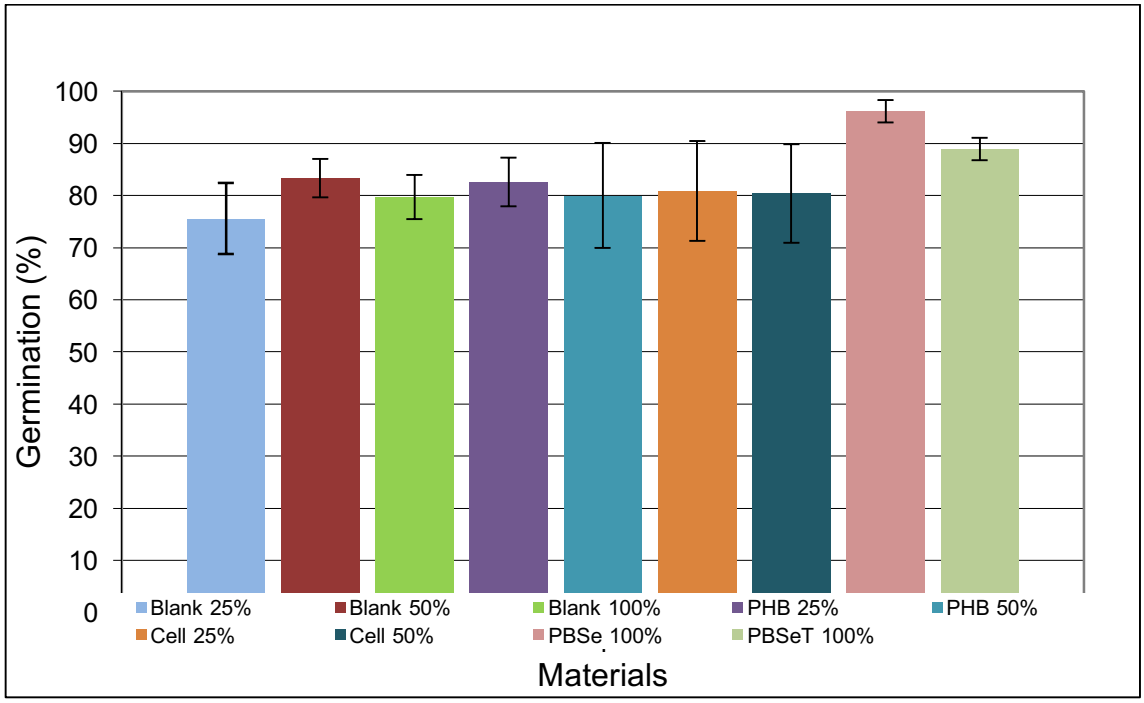

Figure 24. Germination %

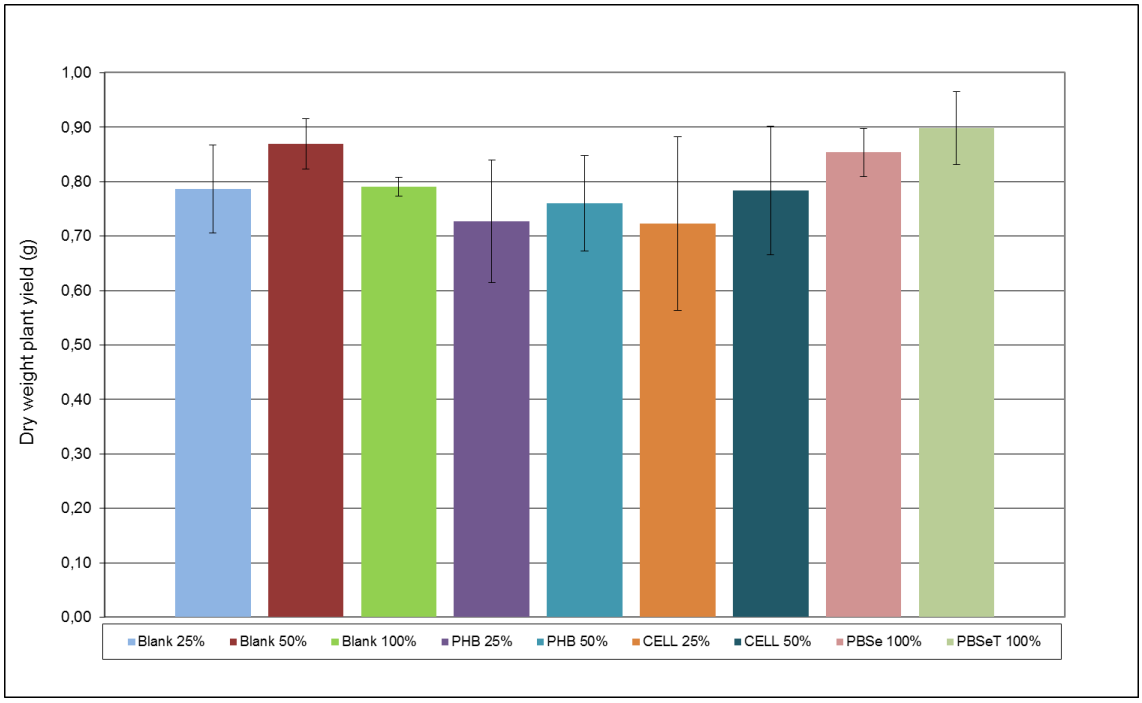

Figure 25. Dry weight plant yield (g)

4.5.4 Novamont laboratory - Plant toxicity tests at plateau phase

At the end of biodegradation test after 210 days (= plateau phase), the natural soil series and the standard soil series were tested (without any dilution). In each pot 350 g of "substrate" (control: peat+sand) or test soil (natural and standard) was added and 150 radish seeds were laid on the surface. Test duration was 15 days. Table 39 up to Table 42 and Figure 26 up to Figure 29 summarize the results.

No toxic effects were observed when testing the natural soil series and the standard soil series. The germination and the plants biomass of the soils after biodegradation of test materials are always higher than the "Blank" soils. Using natural soil a germination between 64-83% was obtained, while using the standard soil the germination was lower (57-75%). OECD 208 prescribes that a test can only be considered valid if the seedling emergence is at least 70%. Taken into account this criterion, it can be concluded that both tests (using natural soil and standard soil) are not valid. The low germination rate in the blank soil series (64% in the natural soil series and 57% in the standard soil series) is most probably caused by the high level of nutrients and salts (= high electrical conductivity) in the blank soil. The electrical conductivity in the test soils is lower as part of the nutrients are consumed during the biodegradation process. This effect was not observed when performing the tests during the active biodegradation phase. No explanation was found for this difference. On the contrary the dry weight was higher using the standard soil (0.74-0.91g) when compared to natural soil (0.66-0.89g).

Table 39. Experimental data (natural soil)

Sample	Soil (%)	Substrate (g)	Soil (g)	Germination (number)	Wet Weight (g)	Dry Weight (g)
Control	-	350	-	88	7.88	0.42
Control	-	350	-	80	7.29	0.40
Control	-	350	-	101	11.44	0.57
Blank	100	-	350	88	14.55	0.63
Blank	100	-	350	107	18.91	0.77
Blank	100	-	350	93	13.03	0.58
PBSe	100	-	350	100	15.56	0.64
PBSe	100	-	350	113	20.36	0.77
PBSe	100	-	350	105	18.62	0.73
PBSeT	100	-	350	112	19.42	0.78
PBSeT	100	-	350	114	19.09	0.77
PBSeT	100	-	350	119	22.79	0.86
PHB	100	-	350	124	23.93	0.91
PHB	100	-	350	118	21.42	0.83
PHB	100	-	350	131	20.05	0.82
CELLULOSE	100	-	350	112	21.22	0.82
CELLULOSE	100	-	350	125	22.27	0.89
CELLULOSE	100	-	350	129	25.34	0.96

Table 40. Final results (natural soil)

	Germination Rate %		Fresh Weight Yield (g)		Dry Weight Yield (g)	
	AVG	STD	AVG	STD	AVG	STD
Blank	64.00	6.57	15.50	3.05	0.66	0.01
PBSe	70.67	4.37	18.18	2.42	0.71	0.07
PBSeT	76.67	2.40	20.43	2.05	0.80	0.05
PHB	82.89	4.34	21.80	1.97	0.85	0.05
Cell	81.33	5.93	22.94	2.14	0.89	0.07

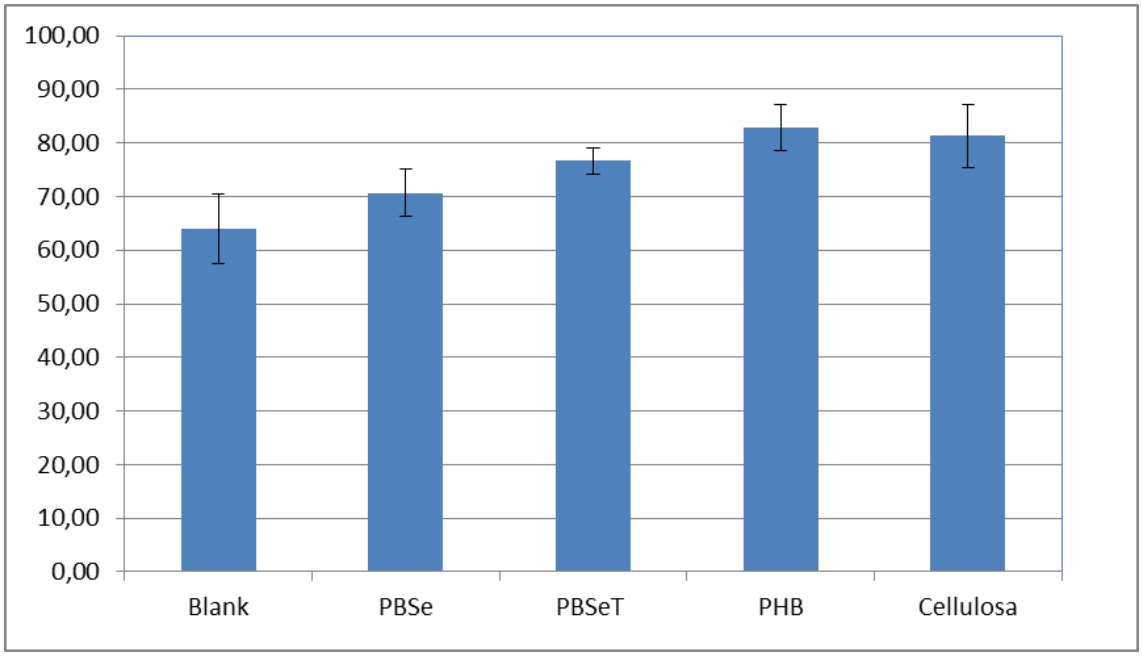

Figure 26. Germination % (Natural soil)

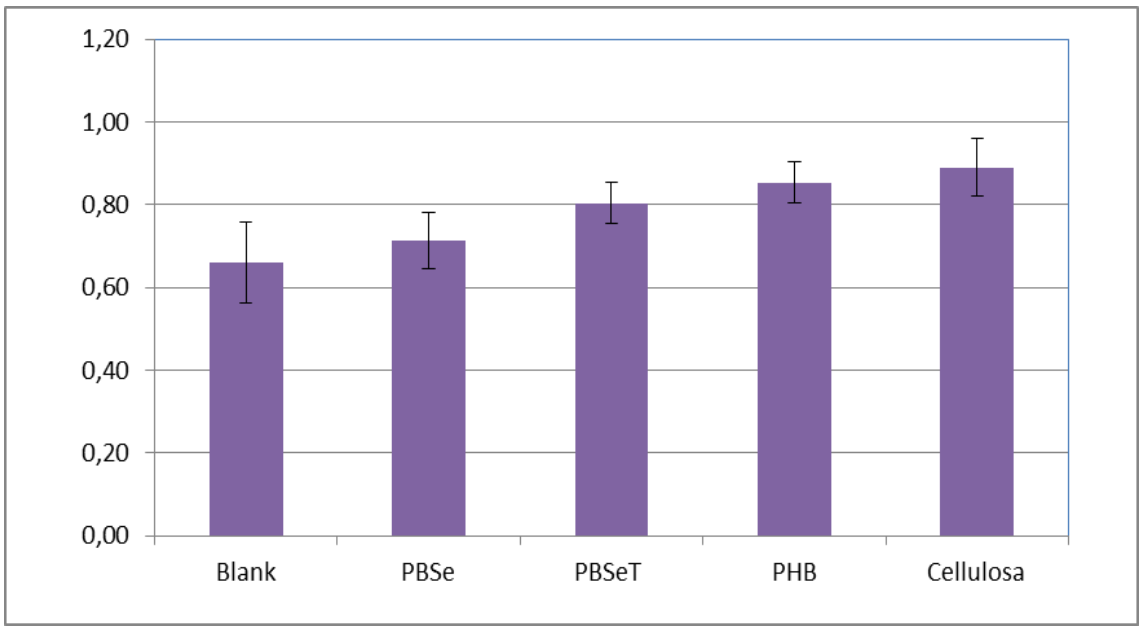

Figure 27. Dry weights (Natural soil)

Table 41. Experimental data (Standard soil)

Sample	Soil (%)	Substrate (g)	Soil (g)	Germination (number)	Wet Weight (g)	Dry Weight (g)
Control	-	350	-	77	8.99	0.50
Control	-	350	-	68	8.31	0.46
Control	-	350	-	71	7.66	0.48
Blank	100	-	350	85	17.15	0.79
Blank	100	-	350	92	16.82	0.77
Blank	100	-	350	80	13.53	0.65
PBSe	100	-	350	100	16.08	0.63
PBSe	100	-	350	117	23.43	0.97
PBSe	100	-	350	111	18.82	0.82
PBSeT	100	-	350	101	18.04	0.75
PBSeT	100	-	350	107	20.01	0.77
PBSeT	100	-	350	85	16.26	0.73
PHB	100	-	350	108	21.09	0.89
PHB	100	-	350	117	23.31	0.92
PHB	100	-	350	112	22.9	0.92
Cellulose	100	-	350	95	17.81	0.76
Cellulose	100	-	350	99	19.81	0.82
Cellulose	100	-	350	98	18.13	0.73

Table 42. Final results (Standard soil)

	Germination Rate %		Fresh Weight Yield (g)		Dry Weight Yield (g)	
	AVG	STD	AVG	STD	AVG	STD
Blank	57.11	4.02	15.83	2	0.74	0.08
PBSe	72.89	5.75	19.44	3.71	0.81	0.17
PBSeT	65.11	7.58	18.10	1.88	0.75	0.02
PHB	74.89	3.01	22.43	1.18	0.91	0.02
Cellulose	64.89	1.39	18.58	1.07	0.77	0.05

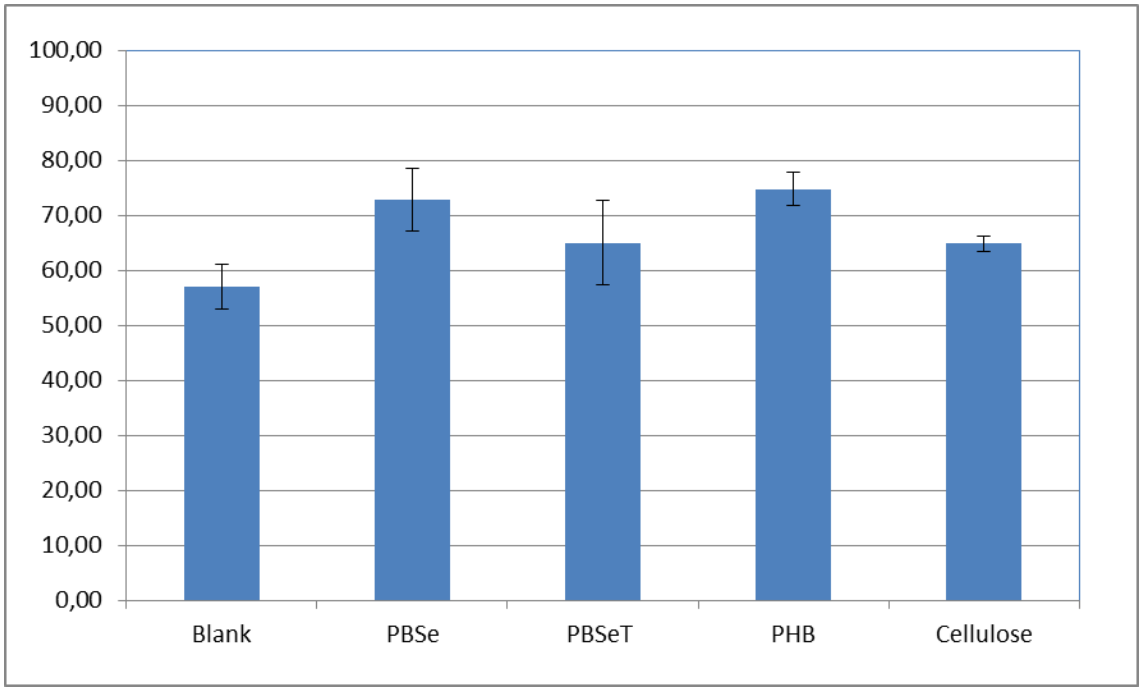

Figure 28. Germination % (Standard soil)

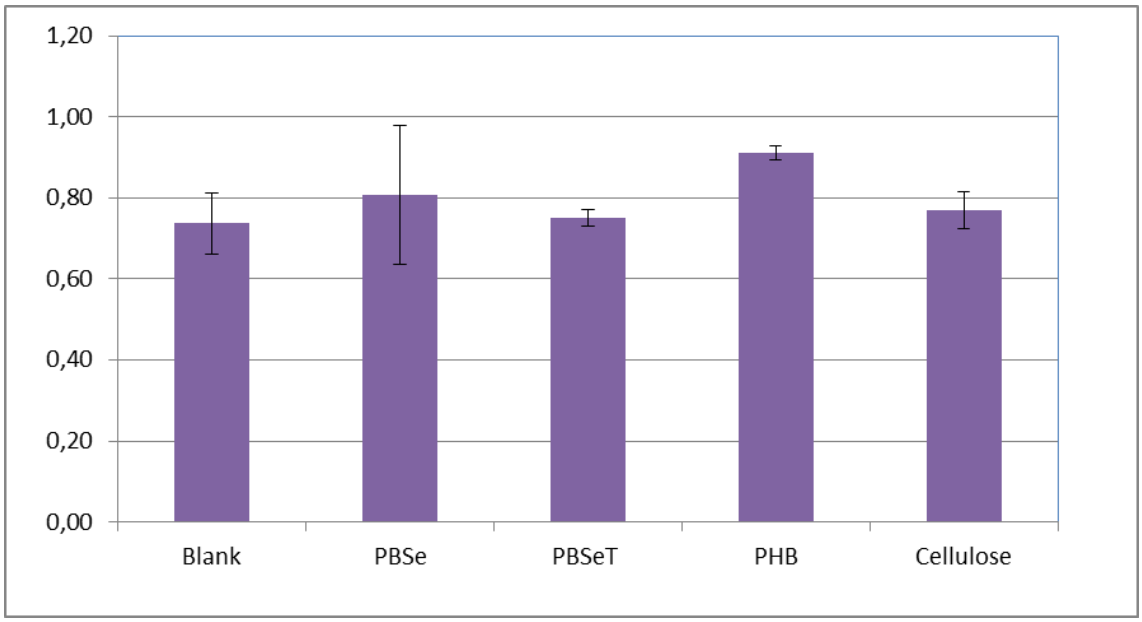

Figure 29. Dry weights (Standard soil)

4.6 Toxicity by means of earthworms

4.6.1 OWS laboratory - Earthworm toxicity tests with soil of run 1

The evaluation of the environmental safety by means of earthworms of Cellulose filter paper (in both inocula) and PBSe was started after an incubation period of 43 days. After an incubation period of 43 days a biodegradation percentage of approximately 71% and 60% was obtained for Cellulose filter paper in natural soil and standard soil. Respectively, while a biodegradation percentage of approximately 70% was obtained for PBSe in natural soil (see Figure 1 and Figure 2). Initially it was suggested in CEN/TC 249/WG 7/TG 1 to start the evaluation of the environmental safety after 30%-40% biodegradation was reached. Consequently biodegradation is already at a higher percentage when compared to the proposed biodegradation level.

An overview of the start-up is given in Table 43. The weights are expressed per replicate. Three replicates were evaluated per test series.

Table 43. Test set-up earthworm test

Test series	Soil (g wet weight per pot)
Control soil (Natural soil)	600
Cellulose filter paper soil (Natural soil)	600
Polybutylene sebacate soil (Natural soil)	600
Control soil (Standard soil)	600
Cellulose filter paper soil (Standard soil)	600

Table 44 shows the average percentage of survival and mortality at the end of the test. Also the live weight per worm and the average preservation of the weight at start of the surviving worms are given. The survival percentages and the mean weight percentages (as a percentage of weight at start) are also shown in Figure 30 and Figure 31. Figure 32 shows the retrieved earthworms at the end of the test.

No survival was measured in the control soil (= standard soil) after 14 days, while complete survival was measured in the control soil (= natural soil), the cellulose filter paper soil (= natural soil), the PBSe soil (= natural soil) and the cellulose filter paper soil (= standard soil). The results in the control soil (= standard soil) were in contrast with the expectations. It is unusual that complete survival is observed in the standard soil to which cellulose filter paper was added in a 1.0 % concentration at start of the incubation period, while no survival was measured in the control soil (= standard soil) as such. For the control soil (= natural soil) and the natural soil series to which Cellulose filter paper and PBSe was added an increase of the mean weight was observed after 14 days, while the weight of the earthworms in the Cellulose filter paper series in standard soil was significantly reduced up to 70 % of the initial weight.

Table 44. Average and standard deviation of percentage survival, mortality and live weight yield for each test series

Test series	Survival (%)		Mortality (%)	Live weight yield (g per worm)		(% of start)	
	AVG	STD	AVG	AVG	STD	AVG	STD
Blank soil (natural soil)	100	0	0	0.33	0.01	105	4
Cellulose filter paper soil (natural soil)	100	0	0	0.36	0.00	117	1
PBSe soil (natural soil)	100	0	0	0.37	0.02	118	3
Blank soil (standard soil)	0	0	100	0.00	0.00	0	0
Cellulose filter paper soil (standard soil)	100	0	0	0.22	0.03	70	3

With AVG = average and STD = standard deviation.

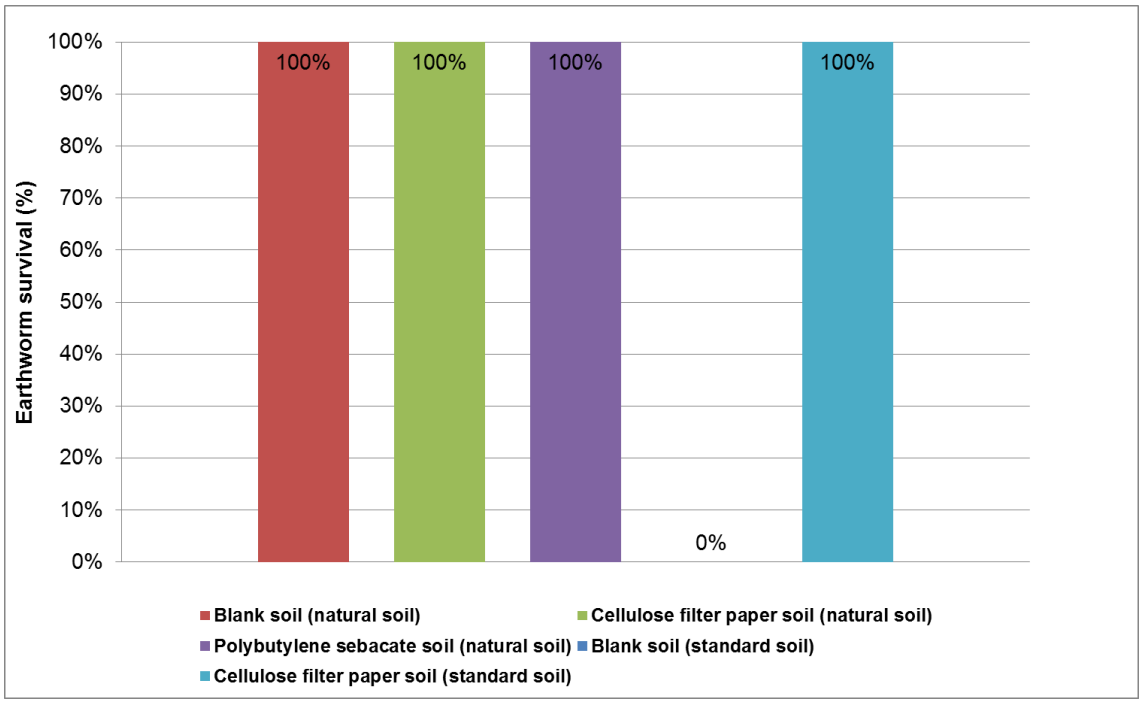

Figure 30. Average survival of earthworms

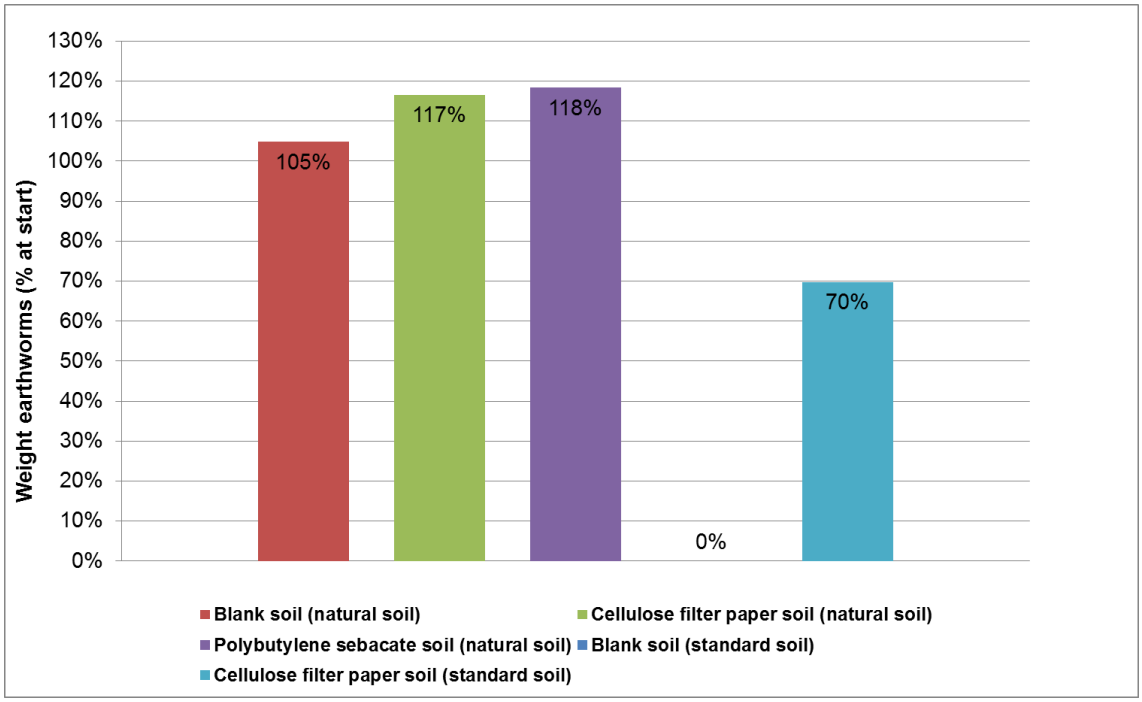

Figure 31. Average weight of earthworms (as % of weight at start)

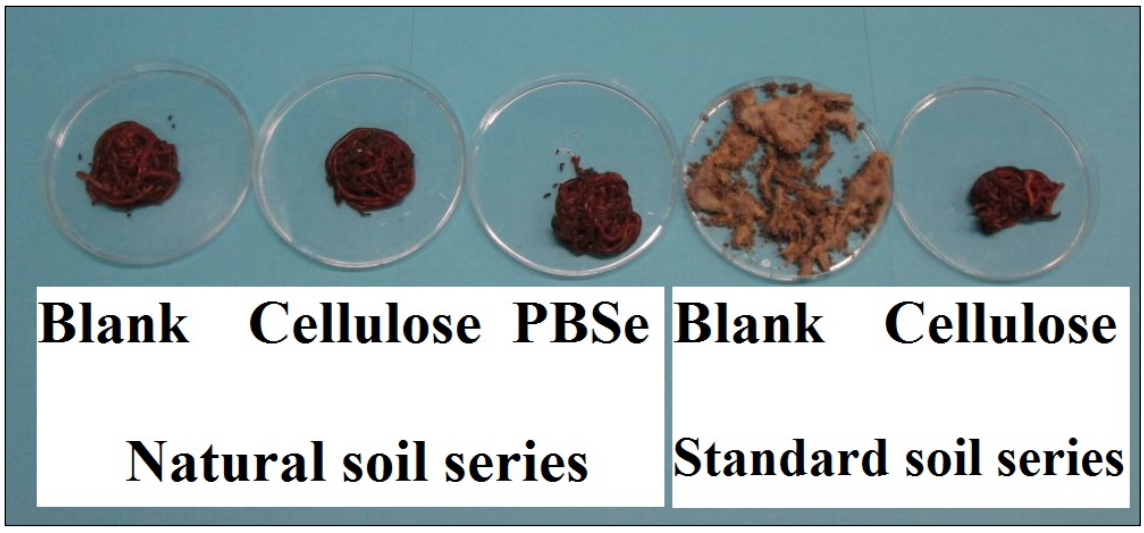

Figure 32. Earthworms at the end of the earthworm toxicity test

It can be concluded that the standard soil as defined by ISO 17556 is not suitable in order to produce soil for subsequent earthworm toxicity tests. This is probably caused by the too high nutrient (= salt) content in the standard soil. In the standard soil to which test material was added, the nutrients were already partly consumed by the micro-organisms during the biodegradation process.

4.6.2 Novamont laboratory - Earthworm toxicity tests during active biodegradation phase

The evaluation of the environmental safety by means of earthworms of the natural soil series was started after 17 days of incubation in soil, when a biodegradation percentage of 51% was obtained for PHB, 46% for Cellulose filter paper, 42% for PBSe and 40% for PBSeT. The test using the standard soil series was started after 18 days of incubation in soil, when a biodegradation percentage of 61% was obtained for PHB and 40% for Cellulose filter paper. In the first CEN/TC 249/WG 7/TG 1 meeting it was suggested to start the evaluation of the environmental safety when 30%-40% biodegradation was reached. Consequently biodegradation is already at a higher percentage when compared to the proposed biodegradation level.

An amount of 375 g soil was put in plastic pots with 5 earthworms, each with a weigh between 300 and 600 mg. The duration of the tests was 14 days. The results are summarized in Table 45 and Table 46.

In the natural soil series 100% mortality was observed in the blank soil after a biodegradation period of 17 days. When the test was repeated after an incubation period of 24 days only 40% mortality was observed. No mortality was observed in the soil without addition of compost and salts (N). Also in the soils with test material, no mortality was observed. The survival of the earthworms is clearly influenced by the amount of nutrients. In the test soils part of the nutrients are used by microorganism during the biodegradation process. This is not the case in the “blank” soil. The higher nutrient content in the blank soil influences the survival of earthworms negatively. In the standard soil series no mortality was observed.

Table 45. Earthworm test in natural soil

Test Series	Survival 7 days (%)	Survival 14 days (%)	Initial Weight (g)	Final Weight (g)	Weight variation (%)
Blank (17 days)	0	0	2.906	0	-100.00
Blank (17 days)	0	0	2.814	0	-100.00
Blank (17 days)	20	0	2.845	0	-100.00
PHB	100	100	2.640	2.887	9.36
PHB	100	100	2.859	2.912	1.85
PHB	100	100	2.721	2.678	-1.58
Paper Filter	100	100	2.676	2.586	-3.36
Paper Filter	100	100	2.762	2.721	-1.48
Paper Filter	100	100	2.444	2.291	-6.26
PBSe	100	100	2.617	2.823	7.87
PBSe	100	60	2.947	1.5558	-47.21
PBSe	100	100	2.823	3.15463	11.75
PBSeT	100	100	2.918	2.77079	-5.04
PBSeT	100	100	2.781	2.66686	-4.10
PBSeT	100	100	2.946	3.07039	4.22
Blank (24 days)	40	40	2.708	0.9242	-65.87
Blank (24 days)	60	60	3.007	1.4664	-51.23
Blank (24 days)	80	80	2.253	1.3257	-41.16
Soil *	100	100	2.70100	2.54683	-5.71
Soil *	100	100	2.59400	2.42581	-6.48
Soil *	100	100	2.72500	2.50103	-8.22

* Without salts and compost

Table 46. Earthworm test in standard soil

Test Series	Survival 7 days (%)	Survival 14 days (%)	Initial Weight (g)	Final Weight (g)	Weight variation (%)
Blank	100	100	2.484	2.2825	-8.11
Blank	100	100	2.436	2.2648	-7.03
Blank	100	100	2.504	2.4257	-3.13
PHB	100	100	2.431	3.0740	26.45
PHB	100	100	2.586	3.1458	21.65
PHB	100	100	2.695	3.0778	14.20
Paper Filter	100	100	2.937	3.6781	25.23
Paper Filter	100	100	2.619	3.2073	22.46
Paper Filter	100	100	2.841	3.3750	18.80

4.6.3 Novamont laboratory - Earthworm toxicity test at plateau phase

The evaluation of the environmental safety by means of earthworms of the natural and standard soil series was carried out after 210 days of incubation in soil (= end of biodegradation test).

An amount of 375 g soil is put in plastic pots with 5 earthworms, each with a weigh between 300 and 600 mg. The results are summarized in Table 47 up to Table 49.

At the plateau biodegradation phase no toxic effects were observed the natural soil series and the standard soil series. Complete survival was observed. Especially in the standard soil a weight loss was observed in the blank soil, while in the series to which test item was added, the weight of the earthworms increased. The weight increase was especially observed in the natural soil series (between 24 and 37%) but also in standard soil series (between 17 and 24%).

Table 47. Earthworm test in natural soil after 210 days

Test Series	Survival 7 days (%)	Survival 14 days (%)	Initial Weight (g)	Final Weight (g)	Weight variation (%)
Blank	100	100	2.169	2.335	7.68
Blank	100	100	1.991	2.0573	3.34
Blank	100	100	2.131	2.395	12.40
PBSe	100	100	1.878	2.5885	37.85
PBSe	100	100	2.169	2.7288	25.79
PBSe	100	100	2.043	2.8902	41.45
PBSeT	100	100	2.459	3.313	34.72
PBSeT	100	100	2.366	3.0722	29.86
PBSeT	100	100	1.969	2.9051	47.56
PHB	100	100	2.364	3.1808	34.56
PHB	100	100	2.121	2.7312	28.80
PHB	100	100	2.163	2.7102	25.32
Cellulose	100	100	2.400	3.007	25.29
Cellulose	100	100	2.159	2.7023	25.16
Cellulose	100	100	2.477	3.0227	22.04

Table 48. Earthworm test in standard soil after 210 days

Test Series	Survival 7 days (%)	Survival 14 days (%)	Initial Weight (g)	Final Weight (g)	Weight variation (%)
Blank	100	100	2.964	2.4973	-15.75
Blank	100	100	2.884	2.4418	-15.33
Blank	100	100	2.673	2.1509	-19.53
PBSe	100	100	2.724	3.5140	29.00
PBSe	100	100	2.526	3.1442	24.47
PBSe	100	100	2.737	3.2576	19.02
PBSeT	100	100	3.070	3.233	5.31
PBSeT	100	100	2.850	3.0065	5.49
PBSeT	100	100	2.248	3.1427	39.80
PHB	100	100	2.645	3.0925	16.92
PHB	100	100	3.068	3.5592	16.01
PHB	100	100	2.225	2.4531	10.25
Cellulose	100	100	2.339	2.6688	14.10
Cellulose	100	100	2.351	2.7322	16.21
Cellulose	100	100	2.486	3.267	31.42

Table 49. Averages

Test Series	Survival 7 days (%)	Survival 14 days (%)	Weight variation (%) Average	Weight variation SD
Natural soil				
Blank	100	100	7.81	4.53
PBSe	100	100	35.03	8.20
PBSeT	100	100	37.38	9.14
PHB	100	100	29.56	4.67
Cellulose	100	100	24.16	1.84
Standard soil				
Blank	100	100	-16.87	2.31
PBSe	100	100	24.16	5
PBSeT	100	100	16.87	19.86
PHB	100	100	14.39	3.61
Cellulose	100	100	20.58	9.45

4.7 Toxicity by means of soil organisms

4.7.1 Long term nitrification test (ISO 14238)

4.7.1.1 OWS laboratory - Long term nitrification test with soil of run 1

After an incubation period of 2 weeks already more than 40% biodegradation was observed for Cellulose filter paper (in both inocula) and for PHB copolymer (see Figure 1 and Figure 2). In the first CEN/TC 249/WG 7/TG 1 meeting it was suggested to start the evaluation of the environmental safety after 30%-40% biodegradation was reached. Consequently, after 2 weeks the suggested biodegradation level is already slightly exceeded.

The evaluation of the environmental safety of Cellulose filter paper (in both inocula) and PHB copolymer was started as soon as practically possible after the minimum biodegradation level was reached (= after an incubation period of 3 weeks and 3 days).

An overview of the start-up is given in Table 50 and Table 51. The weights are expressed per replicate. Three replicates were evaluated per test series.

Table 50. Test set-up (per replicate) using $(NH_4)_2SO_4$ (N = Natural soil; S = Standard soil)

Test series	Soil (g wet weight)	Soil (g dry weight)	$(NH_4)_2SO_4$ (mg dry weight)
Control soil (N)	60	45	21
Cellulose filter paper soil (N)	60	45	21
PHB copolymer (N)	60	45	21
Control soil (S)	60	53	25
Cellulose filter paper soil (S)	60	51	24

Table 51. Test set-up (per replicate) using Luzerne meal (N = Natural soil; S = Standard soil)

Test series	Soil (g wet weight)	Soil (g dry weight)	Luzerne meal (mg dry weight)
Control soil (N)	60	45	174
Cellulose filter paper soil (N)	60	45	172
PHB copolymer (Natural soil)	60	45	173
Control soil (S)	60	53	201
Cellulose filter paper soil (S)	60	51	194

An overview of the measured ammonium-N during the test is given in Figure 33 ($(NH_4)_2SO_4$) and Figure 34 (luzerne), while the measured nitrate-N during the test is given in Figure 35 ($(NH_4)_2SO_4$) and Figure 36 (luzerne). The nitrate formation rate is shown in Figure 37. The measured nitrite levels were below detection limit during the test and therefore these values are not shown. The ammonium levels measured during the test were low. No significant evolution in the ammonium content was measured during the test. The nitrate content of the natural blank soil, the standard blank soil and the cellulose filter paper soil (standard soil) increased during the test, while this was not the case of the cellulose filter paper soil (natural soil) and the PHB copolymer soil (natural soil).

It can be concluded that the blank soil (natural soil or standard soil) can significantly influence the results of the test.

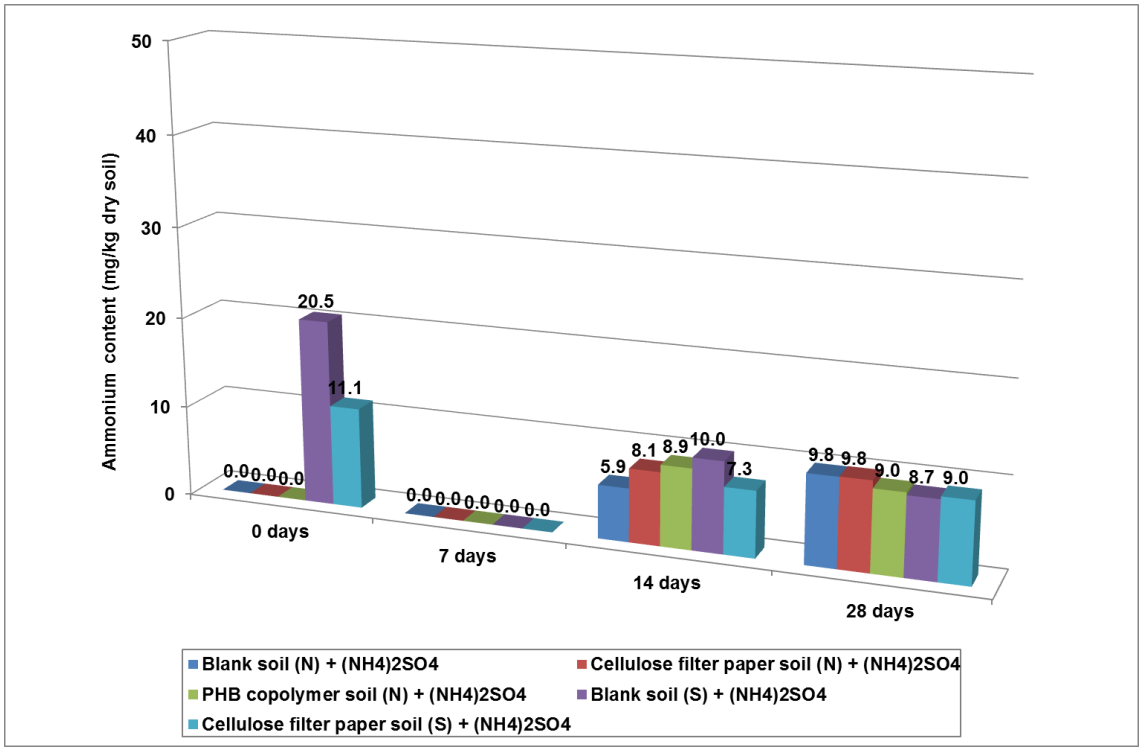

Figure 33. Ammonium content during test ($(NH_4)_2SO_4$)

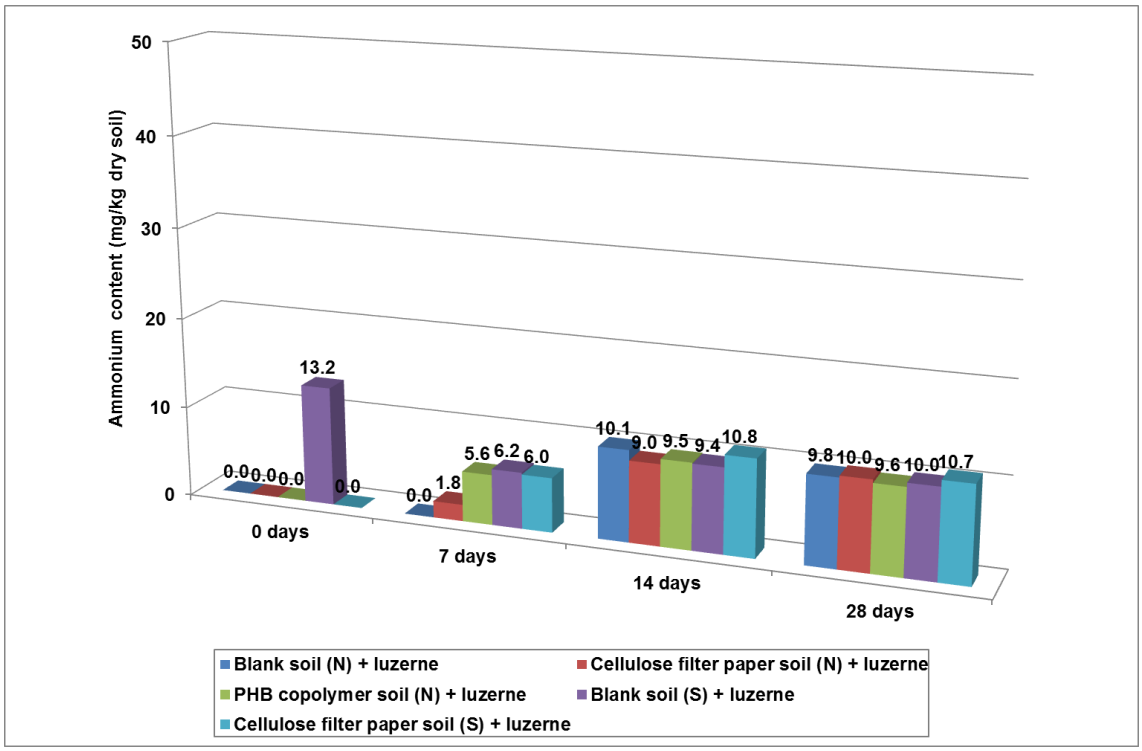

Figure 34. Ammonium content during the test (luzerne)

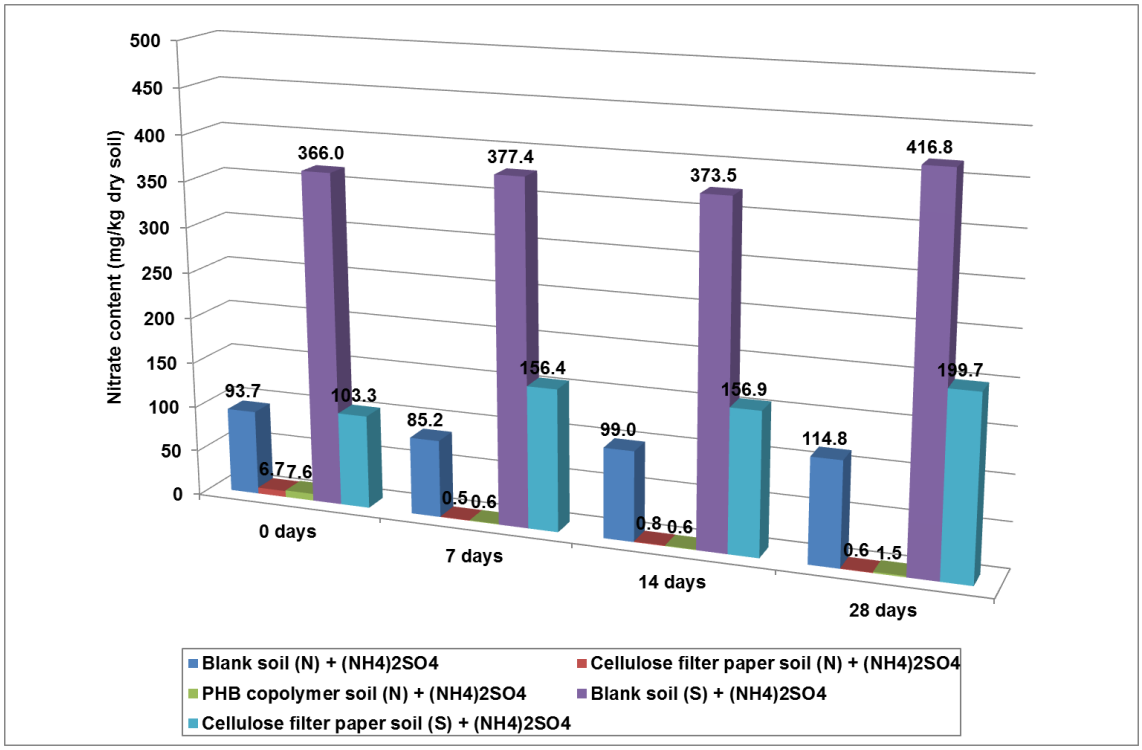

Figure 35. Nitrate content during the test ($(NH_4)_2SO_4$)

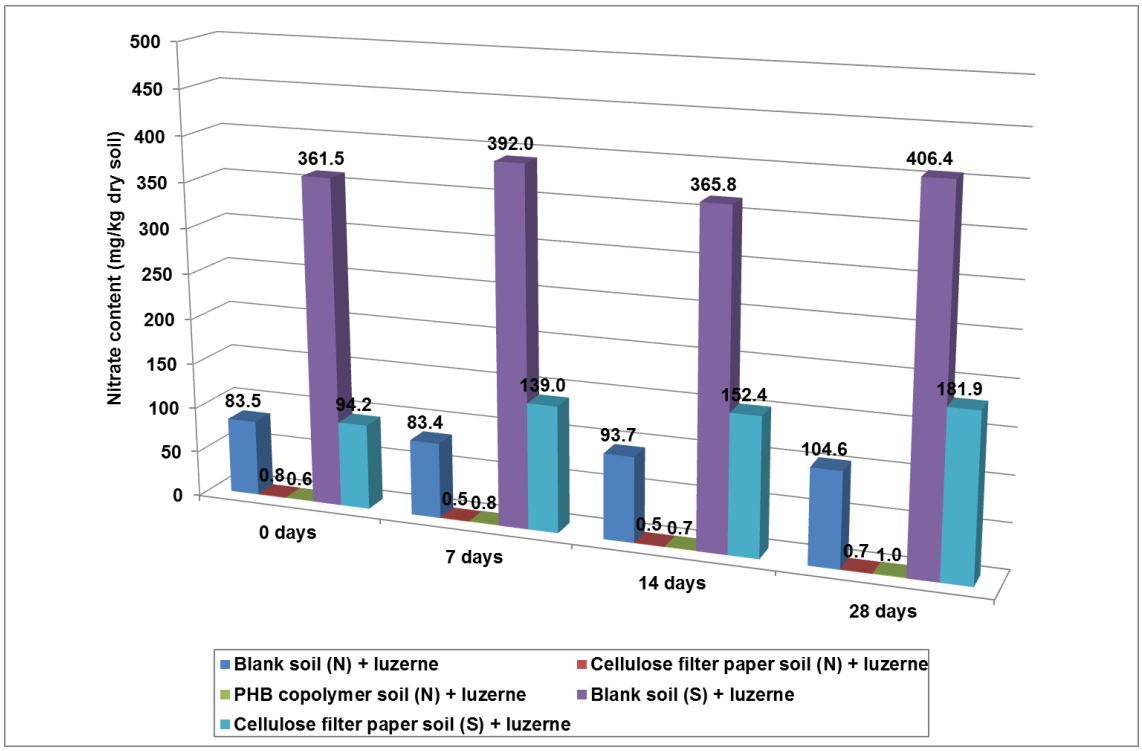

Figure 36. Nitrate content during the test (luzerne)

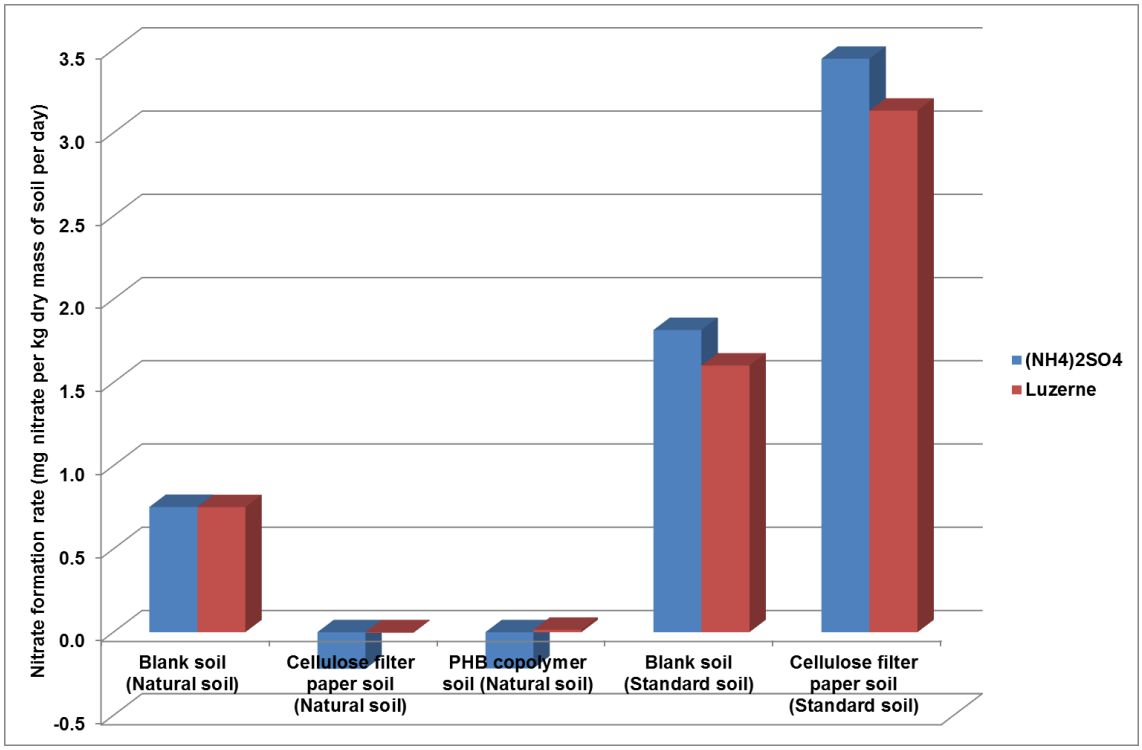

Figure 37. Nitrate formation rate

For sample Cellulose filter paper, it can be concluded that the nitrate formation rate is significantly lower when compared to the blank soil when using natural soil, while the reverse would be concluded when using standard soil as prescribed by ISO 17556. The difference with regard to the nutrient level in the natural soil versus the standard soil will probably be the reason for this observation. Due to the fact that no nutrient solution was added to the natural soil at start of the test, the nitrogen (added under the form of luzerne meal of $(NH_4)_2SO_4$)) will be completely utilised by the micro-organisms in the natural soil as the nitrogen sources were depleted due to the biodegradation of the sample. The higher nitrate transformation rate in the cellulose filter soil using standard soil is probably caused by the fact that the microbial biomass is higher.

4.7.1.2 Novamont laboratory - Long term nitrification test during active biodegradation phase

The test on Cellulose filter paper soil, PHB copolymer soil and blank soil was started after an incubation period of 19 days, while PBSeT soil was started after 27 days of incubation. Characteristics of the natural soil series before start of the test are shown in Table 52.

Table 52. Chemical characteristics of natural soil series before start-up

Test Soil	Time of incubation (dy)	Biod. %	Water Content %	pH	N-NH_4 mg/kg	N-NO_3 mg/kg	N-NO_2 Mg/kg
Blank	19	-	17.81	7.40	0.00	358.50	0.00
PHB	19	55.8	17.84	7.89	3.50	149.85	0.35
Cellulose	19	49.8	18.68	7.69	3.35	214.58	1.12
PBSeT	27	58.9	17.90	7.28	0.00	249.93	0.12

At start of the test 100 mg N was added per kg of dry soil. Nitrogen was added under the form of $(NH_4)_2SO_4$. The test set-up is shown in Table 53.

Table 53. Test set-up (per replicate) using $(NH_4)_2SO_4$

Test series	Soil (g wet weight)	Soil (g dry weight)	$(NH_4)_2SO_4$ (mg dry weight)
Blank	300	246	118
PHB	300	246	117.6
Cellulose	300	246	117.8
PBSeT	300	246	117.8

The soils were incubated at 23°C and after 0 days (one day after the ammonium addition), 7 days, 14 days and 28 days, 15 g of soil (dry weight) was treated with 75 ml KCl 1M. The ammonium-N content, the nitrate-N content and the nitrite-N content were determined. Each week the weight of the reactors was measured and water was added if necessary in order to restore the initial moisture content. The results are shown in Table 54 up to Table 56 and in Figure 38 and Figure 39.

The ammonium content had increased immediately after the addition of $(NH_4)_2SO_4$ in all soil series. The amount of N-NH_4 measured (about 100 mg/kg) is in agreement with the expected results. After 7 days again low ammonium values were measured. Blank soil and the test soils showed the same trend. The presence of the biodegradation residuals does not influence the ammonium transformation.

The nitrate content of the blank soil at start of the test was already higher when compared the test soils. The nitrate formation rate (calculated by means of the values between time = 0 days and time = 28 days) in blank soil was lower when compared to the test soils. It is possible to conclude that during the biodegradation in soil of PHB, PBSeT and cellulose and es-

pecially in the more active phase when the biodegradation level is around 40/50%, there are no effects on the microorganisms involved in the nitrification reactions.

Table 54. Ammonium-N content during test

	Ammonium NH_4^+ mg/kg soil			
Time (day)	**Blank**	**PHB**	**PBSeT**	**Cell**
Before to start the test	0	3.05	0	3.35
0	101.25	105	99	104.5
7	3.05	3.25	2.95	5.7
14	2.75	2.8	3.15	4.3
28	3.2	3.35	3.55	3.15

Table 55. Nitrate-N content during test

	Nitrate NO_3^- mg/kg soil			
Time (day)	**Blank**	**PHB**	**PBSeT**	**Cell**
Before to start the test	358.5	149.855	249.93	214.58
0	499	159.01	217.62	195.87
7	519.475	205.125	333.69	290.215
14	518.715	231.725	302.99	299.8
28	444.5	264.87	296.04	292.675
Nitrate-N formation rate (mg nitrate-N/kg soil/day)	- 1.9	3.8	2.8	3.5

Table 56. Nitrite-N content during test

	Nitrite NO_2^- mg/kg soil			
Time (day)	**Blank**	**PHB**	**PBSeT**	**Cell**
Before to start the test	0.00	0.35	0.12	1.12
0	0.35	0.71	1.59	0.59
7	0.00	2.36	0.18	2.83
14	0.00	0.12	0.47	1.24
28	0.00	1.18	2.06	0.00

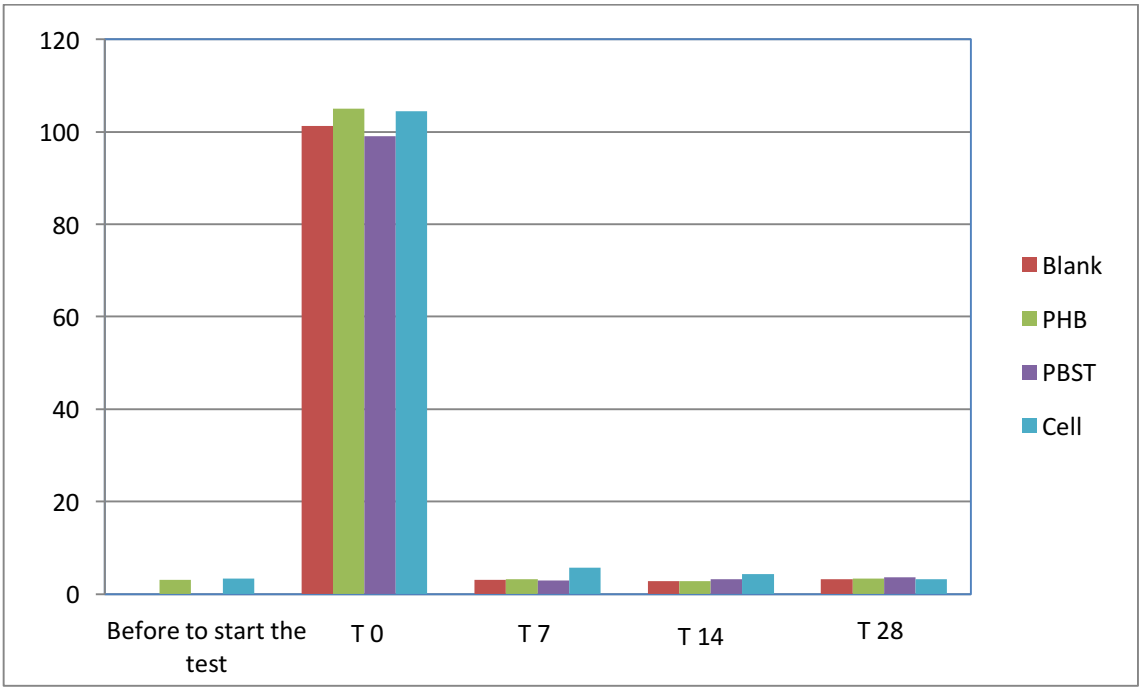

Figure 38. Ammonium-N content (mg/kg soil)

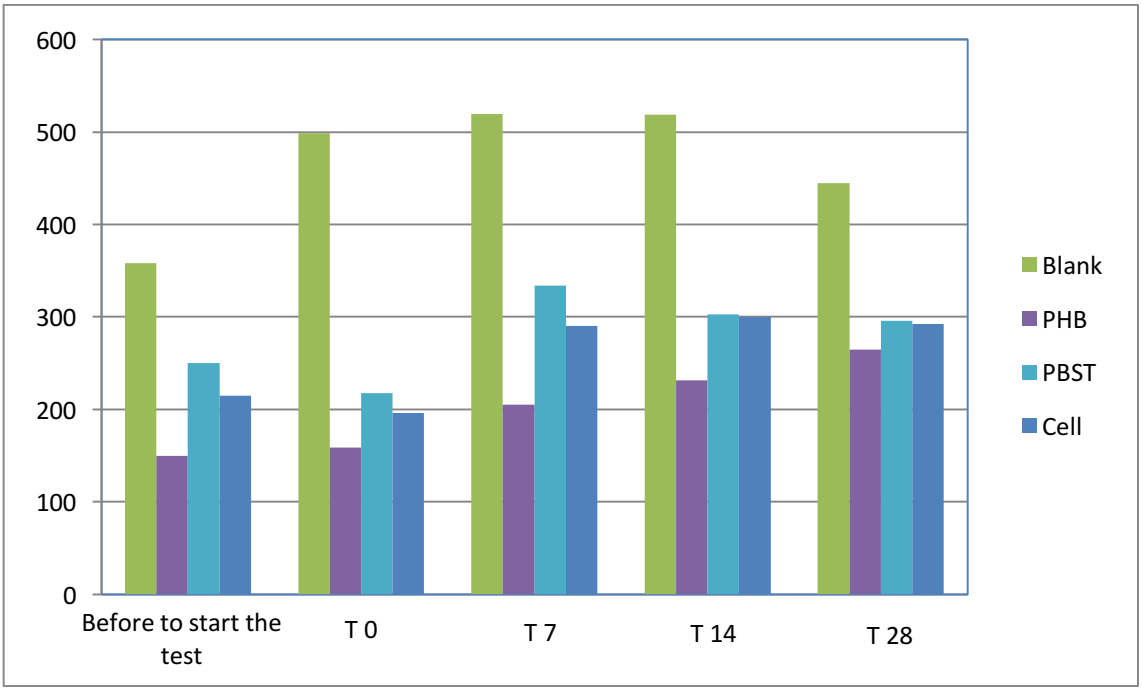

Figure 39. Nitrate-N content (mg/kg soil)

4.7.1.3 Novamont laboratory - Long term nitrification test at plateau phase

The evaluation of the environmental safety of Cellulose filter paper, PHB copolymer and blank soil was carried out after 210 days of incubation in soil at the end of biodegradation test. Characteristics of the natural soil series and the standard soil series before start of the test are shown in Table 57.

Table 57. Chemical characteristics of natural and standard soil series before start-up

Test Soil	Time of incubation (day)	Biod. %	Water Content %	N-NH_4 mg/kg	N-NO_3 mg/kg	N-NO_2 Mg/kg
Natural soil series						
Blank	210	-	17.80	3.35	482.45	0
PBSe	210	87.39	19.04	3.40	127.54	0
PHB	210	82.60	18.88	3.45	164.88	0
Cellulose	210	83.46	19.40	3.40	330.12	0
PBSeT	210	90.47	18.42	3.50	261.91	0.058
Standard soil series						
Blank	210	-	12.57	4.45	322.12	0.009
PBSe	210	96.80	13.80	4.20	-1.26	0
PHB	210	96.02	14.95	9.35	64.49	0
Cellulose	210	94.47	13.78	5.65	101.96	0
PBSeT	210	97.80	12.99	3.60	47.18	0

At start of the test 100 mg N was added per kg of dry soil. Nitrogen was added under the form of $(NH_4)_2SO_4$. The test set-up is shown in Table 58.

Table 58. Test set-up (per replicate) using $(NH_4)_2SO_4$

Test series	Soil (g wet weight)	Soil (g dry weight)	$(NH_4)_2SO_4$ (mg dry weight)
Natural soil series			
Blank Natural	183.7	150.97	71.82
PBSe Natural	183.5	148.56	70.32
PHB Natural	183.3	148.68	70.17
Cellulose Natural	182.9	147.39	69.49
PBSeT Natural	183.5	149.70	71.07
Standard soil series			
Blank Standard	185.8	162.44	78.64
PBSe Standard	185.2	159.63	75.29
PHB Standard	184.8	157.16	75.57
Cellulose Standard	185.2	159.70	75.97
PBSeT Standard	185.8	161.63	77.40

The soils were incubated at 23°C and after 0 days (one day after the ammonium addition), 7 days, 14 days and 28 days, 15 g of soil (dry weight) was treated with 75 ml KCl 1M. The ammonium-N content, the nitrate-N content and the nitrite-N content were determined. Each week the weight of the reactors was measured and water was added if necessary in order to restore the initial moisture. The results are shown in Table 59 up to Table 61 and in Figure 40 up to Figure 45.

The ammonium content increased just after the addition of $(NH_4)_2SO_4$ in all soil samples. The amount of $N\text{-}NH_4$ measured (about 100 mg/kg) different between the different series. Especially the cellulose soils (both natural and standard) and the PBSe and PBSeT natural soils were characterized by a lower $N\text{-}NH_4$ content (60 - 70 mg/kg) as if a portion of nitrogen was already consumed by the microorganism after 1 day. After 7 days the ammonium content decrease in similar way in all soil samples (natural and standard). Blank soil and the test soils showed the same trend. The presence of biodegradation residuals does not influence the ammonium transformation. After 28 days all soils showed a $N\text{-}NH_4$ content < 10 mg/kg. Following could be proposed in the test methodology: the trend of $N\text{-}NH_4$ decrease should be similar to the blank soil and after 28 days the $N\text{-}NH_4$ content should be less than 10 mg/kg.

Also at the plateau phase the nitrate content of the blank soil is higher than in the test soils. The nitrate content of blank soil and of the test soils increased after the addition of ammonium sulphate. In the natural soil series, the nitrate formation rate was higher in the test soils when compared to the blank soil, while the opposite was observed for the standard soil series. In the standard soil series the nitrate formation rate in the different test soils was rather comparable.

The nitrite content was measurable just after the addition of ammonium sulphate and in some cases after 7 days (blank in standard and natural soil and PBSe and cellulose only in standard soil). After 14 and 28 days the nitrite content was not measurable. Following could be proposed in the test methodology: after 28 days no nitrite should be measurable in the soil.

It is possible to conclude that also in the plateau phase, after complete biodegradation in soil of PHB, PBSeT, PHB and Cellulose no toxic effects are observed on the micro-organisms involved in the nitrification reactions. The trends of $N\text{-}NH_4$, $N\text{-}NO_2$ and $N\text{-}NO_3$ are similar in blank soil and in test soils. As already observed in the tests during the active biodegradation phase, in general a higher concentration of N-Nitrate is measured in the blank.

Table 59. Ammonium-N content during test

Natural Soil	Ammonium NH_4^+ mg/kg soil				
Time (day)	Blank	PHB	PBSeT	Cell	PBSe
Before to start the test	3.35	3.45	3.50	3.40	3.40
0	92.50	92.50	72.50	69.50	72
7	2.95	3.40	3.45	3.55	3.40
14	5.85	9.10	7.30	6.15	5.90
28	7.05	7.65	8.10	8.30	ND
Standard Soil	**Ammonium NH_4^+ mg/kg soil**				
Time (day)	Blank	PHB	PBSeT	Cell	PBSe
Before to start the test	4.45	9.35	3.60	5.65	4.20
0	96	91	91	63	107
7	9.45	3.60	3.55	4.80	3.60
14	4.05	3.30	3.60	8.55	3.30
28	3.6	3.80	4.30	3.90	3.80

Table 60. Nitrate-N content during test

Natural Soil	Nitrate NO_3^- mg/kg soil				
Time (day)	Blank	PHB	PBSeT	Cell	PBSe
Before to start the test	482.45	164.88	261.91	330.13	127.54
0	520.83	213.59	245.50	261.52	169.15
7	597.44	140.46	379.48	267.47	314.62
14	546.92	306.87	387.49	425.99	361.39
28	531.55	258.55	350.93	409.84	239.04
Nitrate-N formation rate (mg nitrate-N/kg soil/day)	0.4	1.6	3.8	5.3	2.5
Standard Soil	**Nitrate NO_3^- mg/kg soil**				
Time (day)	Blank	PHB	PBSeT	Cell	PBSe
Before to start the test	322.12	64.50	47.18	101.96	-1.26
0	301.70	73.28	66.82	96.92	7.00
7	360.49	155.71	159.33	175.35	48.08
14	389.82	177.16	166.05	189.43	82.45
28	430.65	165.14	165.79	202.09	92.01
Nitrate-N formation rate (mg nitrate-N/kg soil/day)	4.6	3.3	3.5	3.8	3.0

Table 61. Nitrite-N content during test

Natural Soil	Nitrite NO_2^- mg/kg soil				
Time (day)	**Blank**	**PHB**	**PBSeT**	**Cell**	**PBSe**
Before to start the test	0	0	0.058	0	0
0	8.73	7.08	4.50	3.48	6.08
7	23.56	0	0	0	0
14	0	0	0	0	0
28	0	0	0	0.078	0
Standard Soil	**Nitrite NO_2^- mg/kg soil**				
Time (day)	**Blank**	**PHB**	**PBSeT**	**Cell**	**PBSe**
Before to start the test	0.009	0	0	0	0
0	5.86	1.94	3.02	3.18	3.44
7	41.36	0	0	5.84	28.99
14	0	0	0	0	0
28	0	0	0.018	0	0

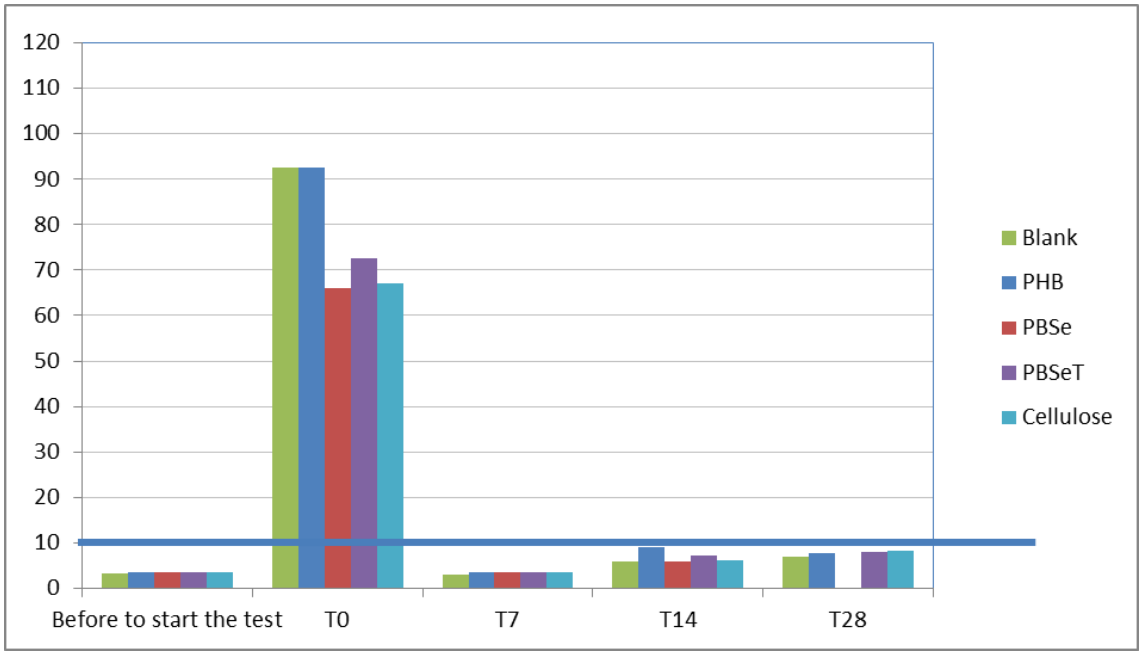

Figure 40. Ammonium-N content (mg/kg soil) – Natural Soil

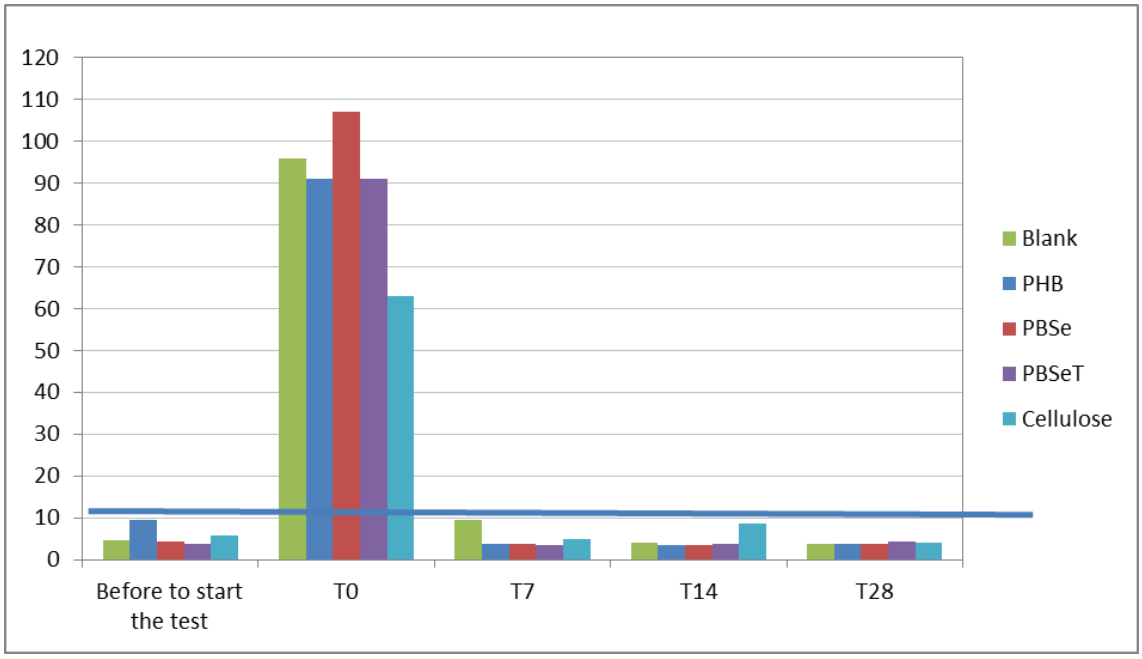

Figure 41. Ammonium-N content (mg/kg soil) – Standard Soil

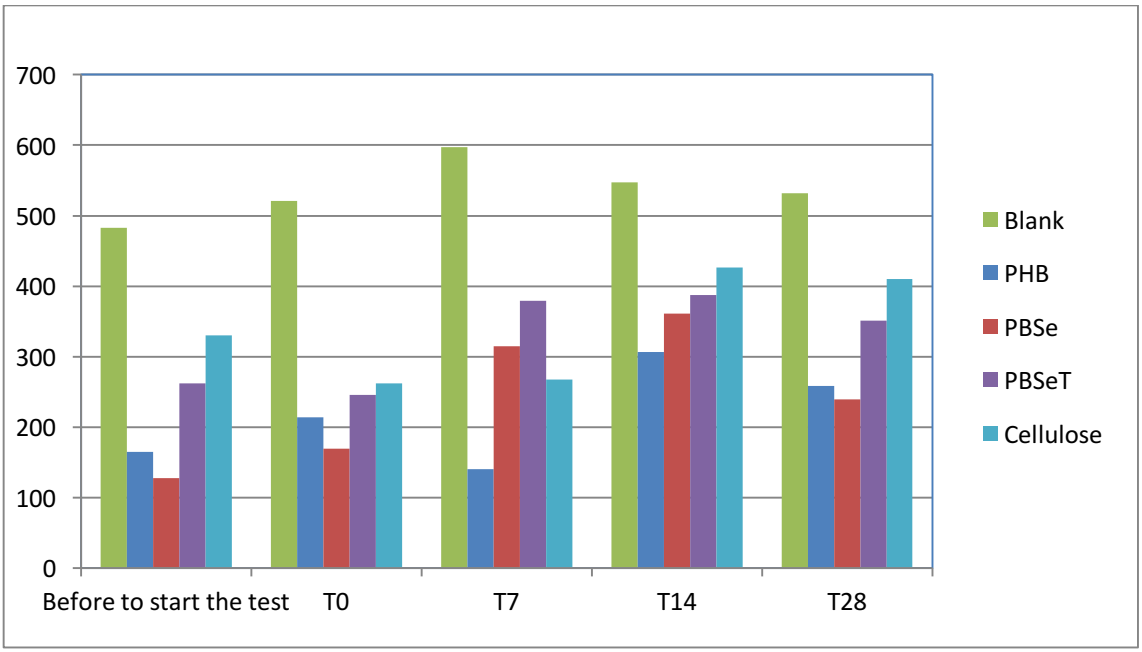

Figure 42. Nitrate-N content (mg/kg soil) – Natural Soil

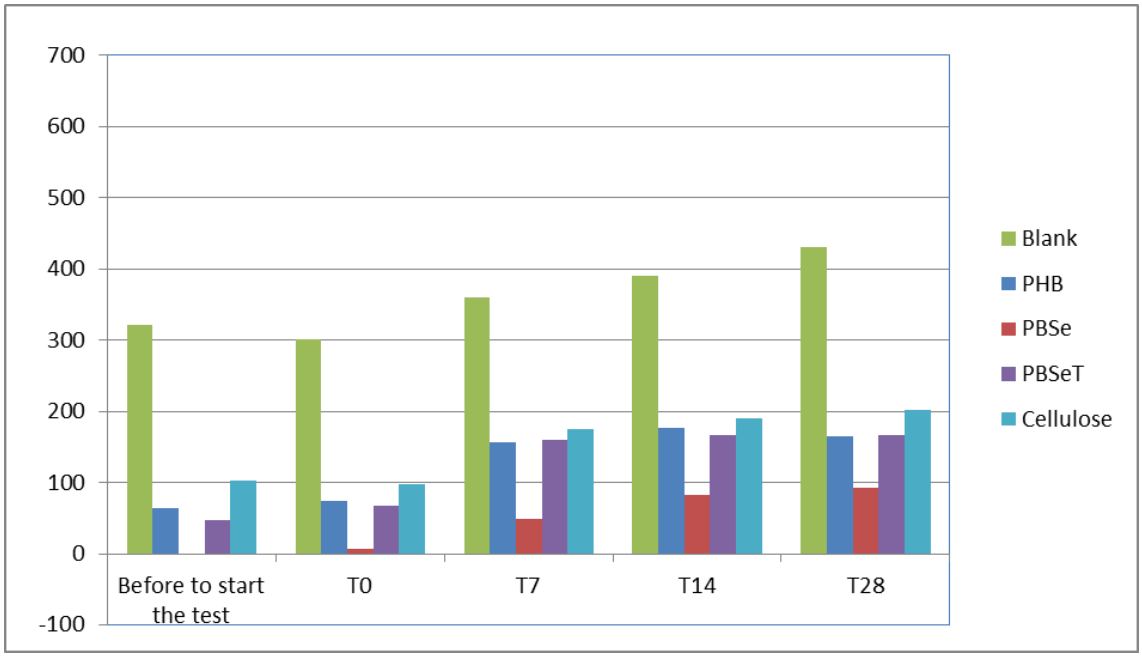

Figure 43. Nitrate-N content (mg/kg soil) – Standard Soil

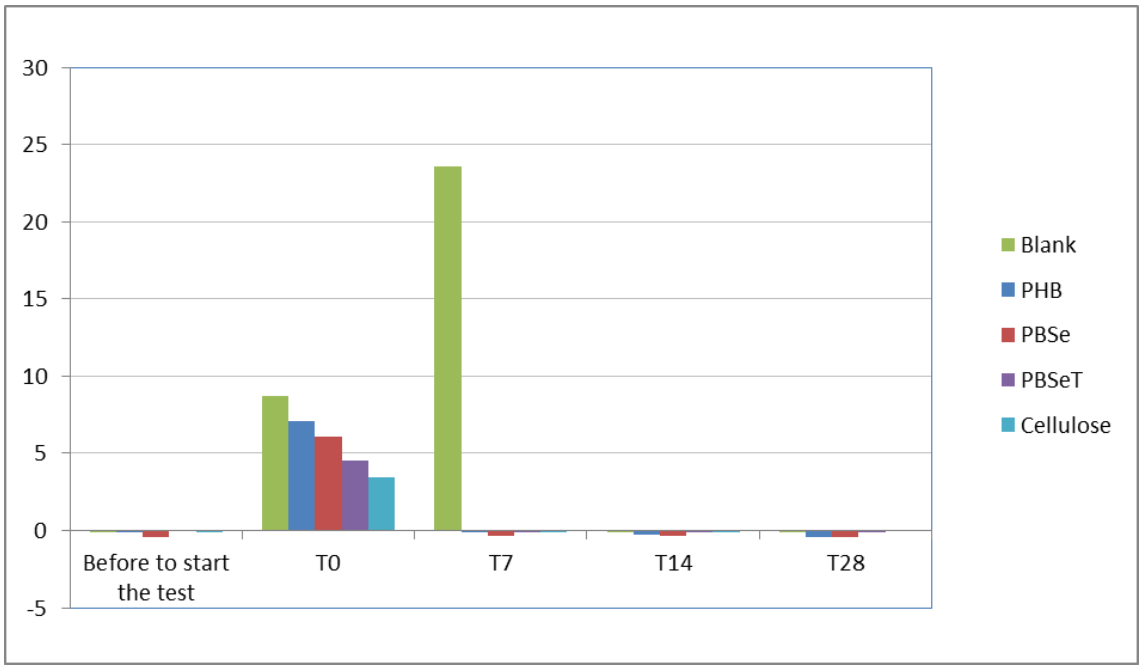

Figure 44. Nitrite-N content (mg/kg soil) – Natural Soil

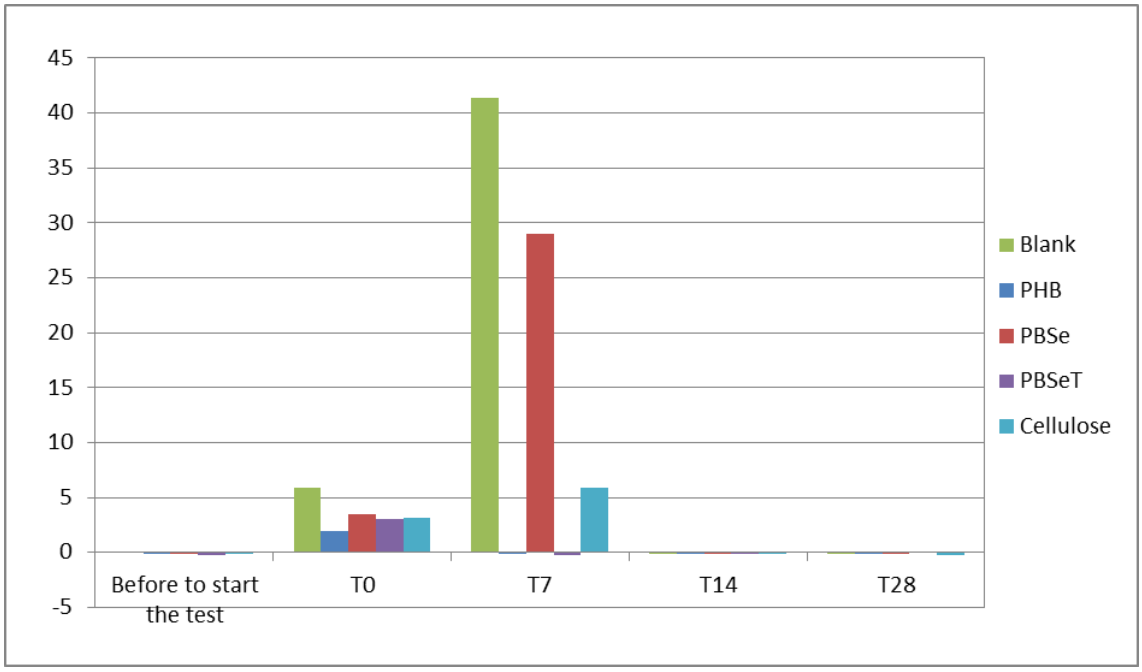

Figure 45. Nitrite-N content (mg/kg soil) – Standard Soil

4.7.2 Carbon transformation test (OECD 217)

4.7.2.1 OWS laboratory - Carbon transformation test with soil of run 2

During the first run (at time 0), only control soil and cellulose soil were tested. In order to measure the background respiration (oxygen consumption) of the soils, also the respiration of control soil without addition of glucose was measured after 7 days. After 14 days and 28 days, the respiration of both soils with and without addition of glucose was measured additionally. Only one reactor was prepared for soils without addition of glucose.

The test method OECD 217 prescribes that glucose-induced respiration shall be measured for 12 hours. In the last test (after 28 days), before starting, the headspace of the reactors was flushed with oxygen to saturate the headspace and the reactors were opened only once (after ± 48 hours). Table 62 up to Table 65 show the test set-up at time 0, after 7 days, 14 days and 28 days.

Table 62. Test set-up of the carbon transformation test at time 0

Soil description	Reactor	Amount of inoculum wet weight (g)	Sand (g)	Amount of glucose (g)
Control soil	RN1	122.95	0.9998	0.3996
Control soil	RN2	123.09	1.0003	0.4000
Control soil	RN3	123.08	1.0001	0.4004
Cellulose soil	RN4	123.01	1.0002	0.4009
Cellulose soil	RN5	123.04	0.9997	0.3995
Cellulose soil	RN6	122.97	0.9995	0.4000

Table 63. Test set-up of the carbon transformation test at time 7 days

Soil description	Reactor	Amount of inoculum wet weight (g)	Sand (g)	Amount of glucose (g)
Control soil	RN1	122.98	1.0000	0.3996
Control soil	RN2	123.01	1.0006	0.4000
Control soil	RN3	122.97	0.9997	0.3997
Cellulose soil	RN4	122.95	1.0002	0.4002
Cellulose soil	RN5	122.98	1.0005	0.3997
Cellulose soil	RN6	122.96	1.0005	0.4007
Control soil without glucose	RN7	123.03	0.9996	-

Table 64.Test set-up of the carbon transformation test at time 14 days

Soil description	Reactor	Amount of inoculum wet weight (g)	Sand (g)	Amount of glucose (g)
Control soil	RN1	122.97	1.0016	0.3988
Control soil	RN2	122.98	1.0018	0.4011
Control soil	RN3	123.08	1.0013	0.4008
Cellulose soil	RN4	123.05	1.0007	0.4006
Cellulose soil	RN5	123.13	1.0019	0.4005
Cellulose soil	RN6	123.02	1.0022	0.4020
Control soil without glucose	RN7	122.94	0.9990	-
Cellulose soil without glucose	RN8	122.97	1.0016	-

Table 65. Test set-up of the carbon transformation test at time 28 days

Soil description	Reactor	Amount of inoculum wet weight (g)	Sand (g)	Amount of glucose (g)
Control soil	RN1	122.9	1.0019	0.4008
Control soil	RN2	122.9	1.0000	0.4008
Control soil	RN3	122.9	1.0008	0.4009
Cellulose soil	RN4	123.1	1.0001	0.4004
Cellulose soil	RN5	123.1	1.0008	0.3999
Cellulose soil	RN6	123.1	1.0026	0.4003
Control soil without glucose	RN7	122.9	1.0013	-
Cellulose soil without glucose	RN8	123.0	1.0020	-

The carbon transformation test was performed after 28 days of pre-incubation (time 0), and then after 7, 14 and 28 days from time 0.

Figure 46 up to Figure 49 show the oxygen consumption during the first 12 hours of the test at the different times. All replicates are shown.

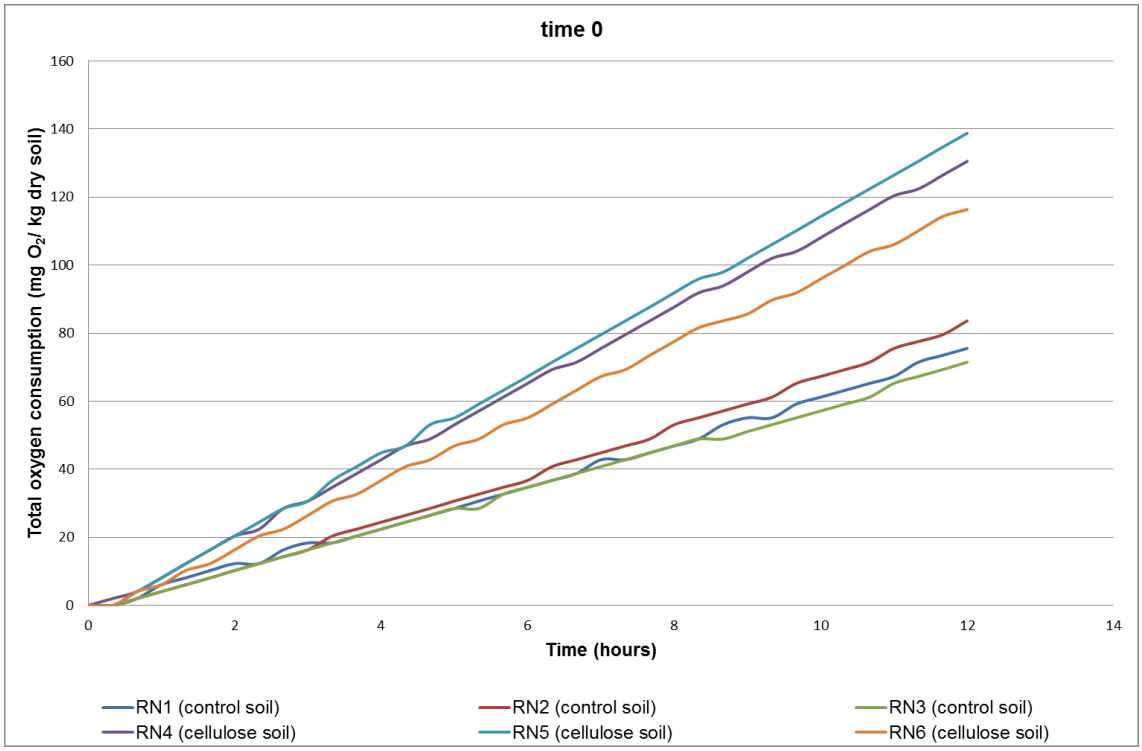

Figure 46. Oxygen consumption (mg O_2/kg dry soil) in the first 12 hours of the test performed at time 0

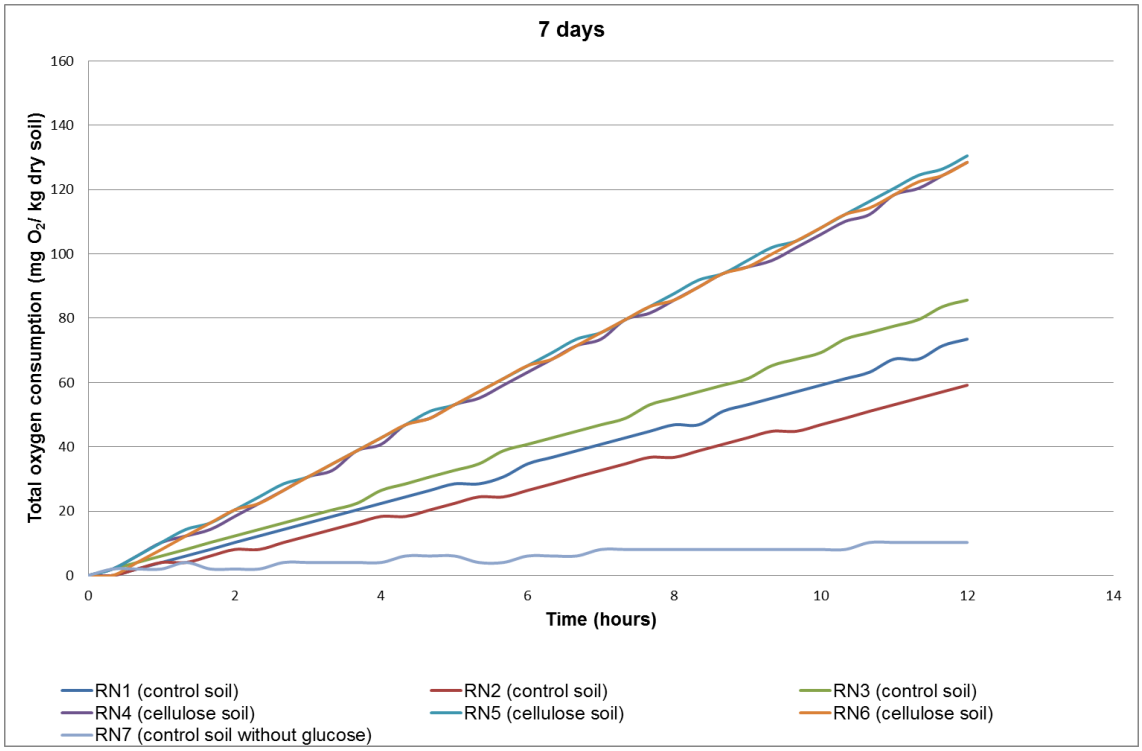

Figure 47. Oxygen consumption (mg O_2/kg dry soil) in the first 12 hours of the test performed after 7 days

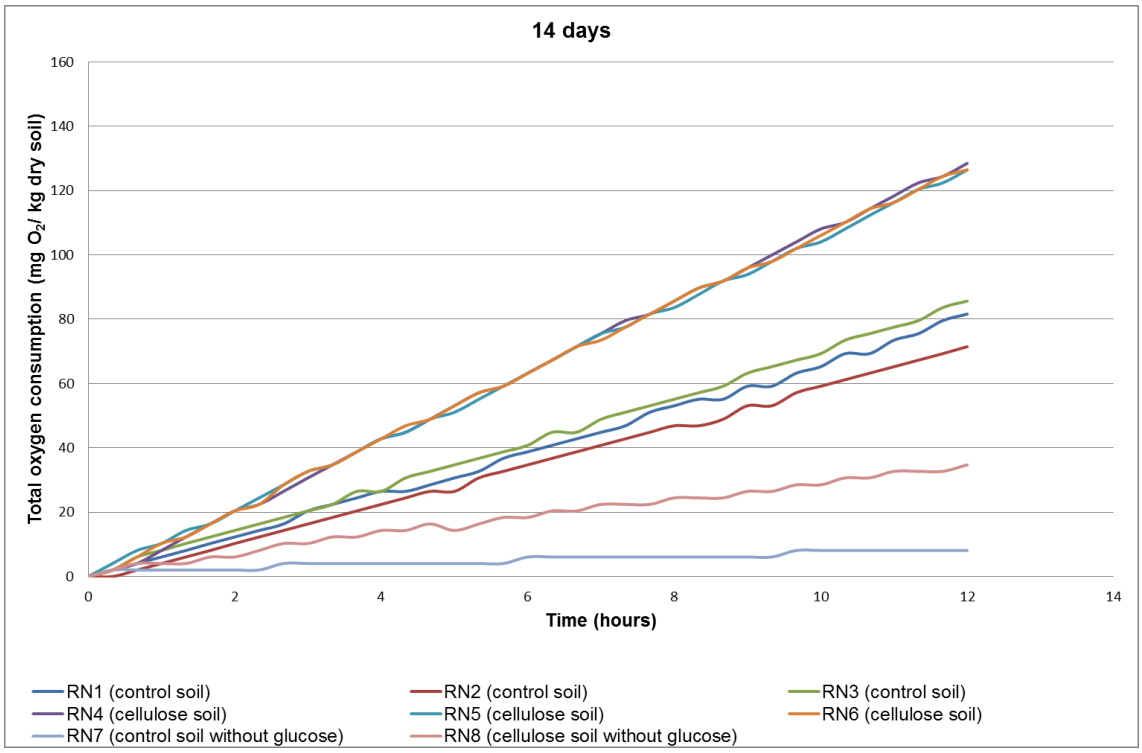

Figure 48. Oxygen consumption (mg O_2/kg dry soil) in the first 12 hours of the test performed after 14 days

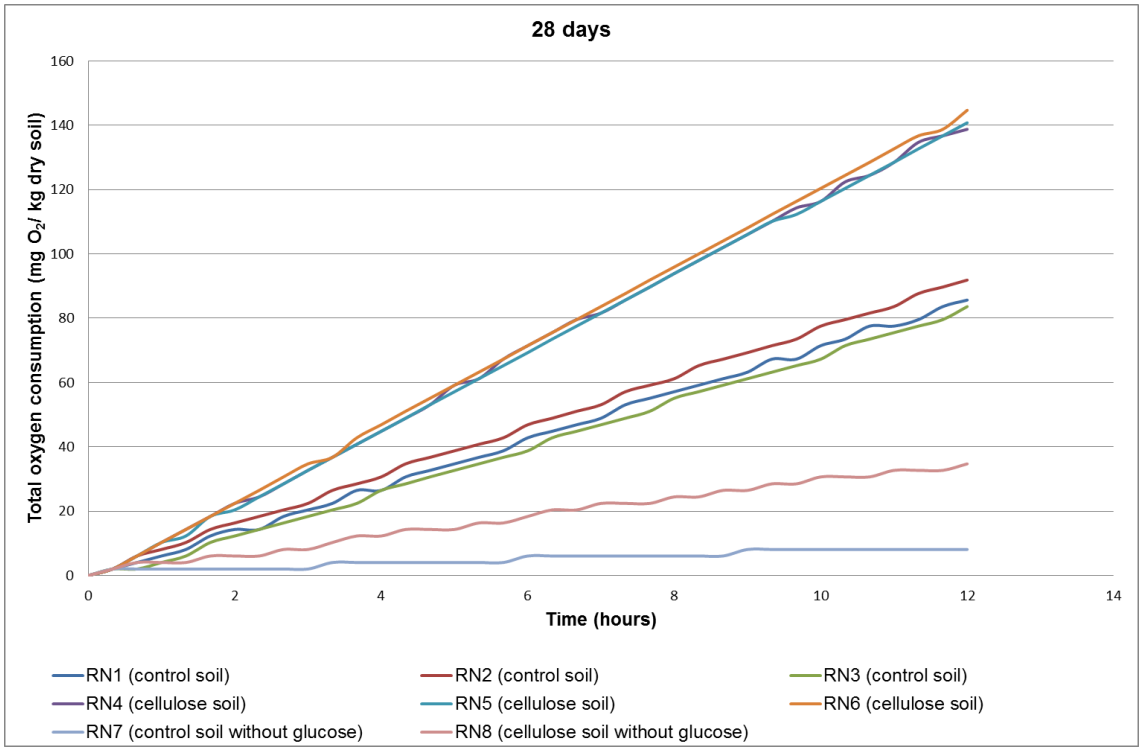

Figure 49. Oxygen consumption (mg O_2/kg dry soil) in the first 12 hours of the test performed after 28 days

The average glucose-induced respiration rate (mg O_2/kg dry soil /h) was also calculated and reported in Table 66 and in Figure 50. The average glucose-induced respiration rate in the cellulose soil is higher when compared to the control soil at the different measurement times (time 0, 7 days, 14 days and 28 days). The standard deviation between the replicates is rather low. Only after 7 days a rather high standard deviation was measured for the control soil (respiration rate: 6.07 mg O_2 / kg dry soil /h ± 1.11 mg O_2 / kg dry soil /h).

Table 66. Respiration rate (mg O_2 / kg dry soil /h) of control soil and cellulose soil measured at the different times of the test

Respiration rate	time 0	7 days	14 days	28 days
Control soil	6.41 ± 0.52	6.07 ± 1.11	6.63 ± 0.61	7.26 ± 0.36
Cellulose soil	10.71 ± 0.95	10.76 ± 0.10	10.60 ± 0.10	11.79 ± 0.25
Control soil without glucose	-	0.86	0.68	0.68
Cellulose soil without glucose	-	-	2.89	2.89

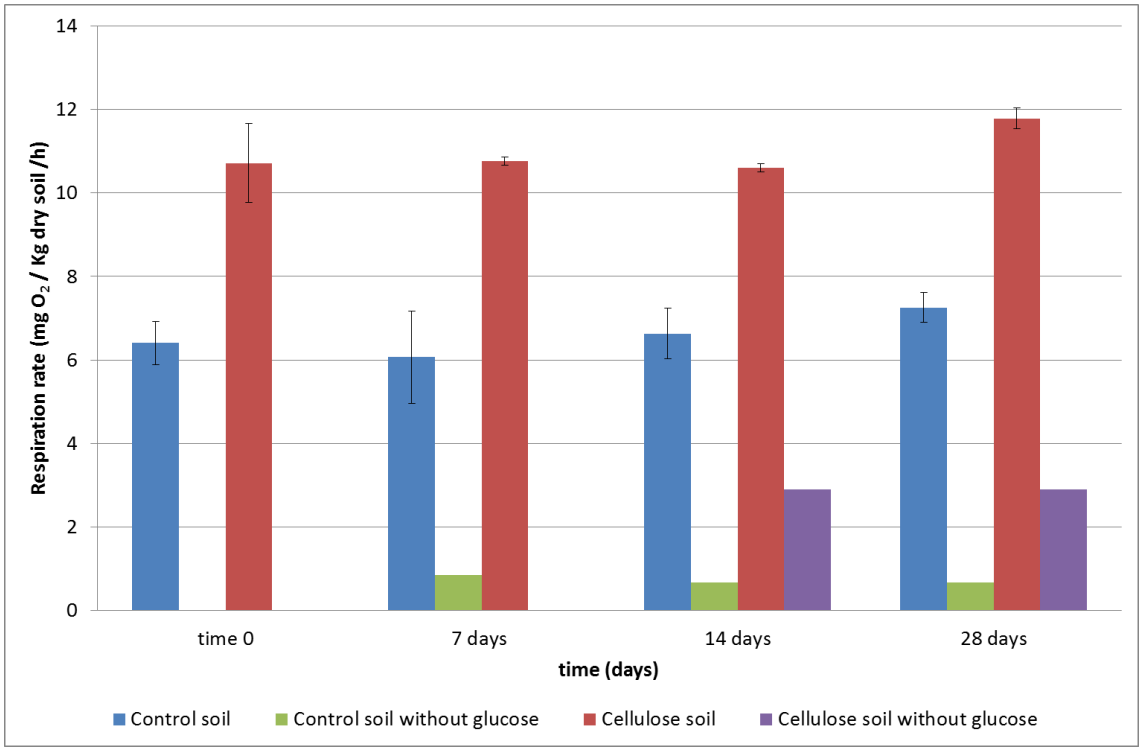

Figure 50. Respiration rate (mg O_2 / kg dry soil /h) of control soil and cellulose soil measured at the different times of the test

As indicated above a correction should be made for the background activity, which is not identical in the control soil and the cellulose filter paper soil (due to the fact that the cellulose filter paper is still degrading). Without such correction, the test set-up would favor the test soil and interpretations could not be made correctly.

Figure 51 shows the glucose-induced respiration rates after a correction for the background activity. The glucose-induced respiration rate in the cellulose filter paper soil is still higher when compared to the control soil, but the difference is less pronounced when compared to the results shown in Figure 50. Consequently, it can be concluded that the C transformation rate is not negatively influenced by the addition of cellulose filter paper at start of the biodegradation phase. The fact that higher respiration rates are measured in the cellulose filter paper soil indicates that more microbial biomass is present in the cellulose soil (due to the biodegradation of the cellulose during the pre-incubation (biodegradation) period).

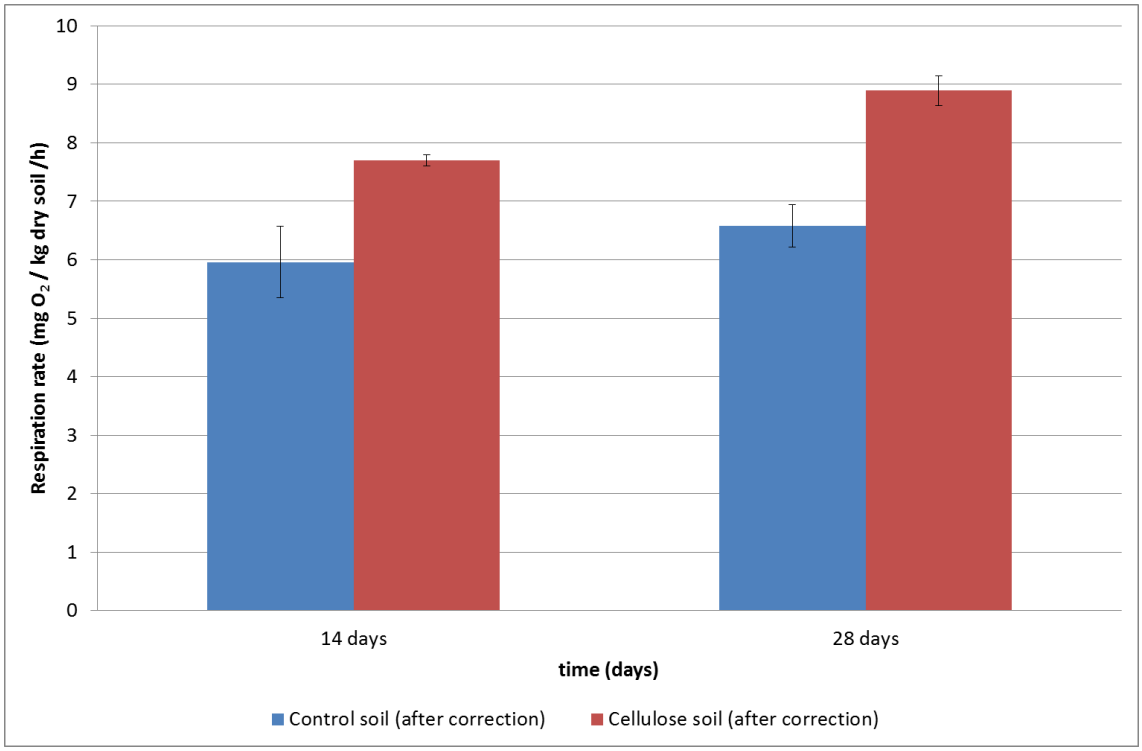

Figure 51. Respiration rate of control soil and cellulose soil obtained after subtracting the respiration rate of the background.

Conclusion: The results of this test indicate that test method OECD 217 "Soil microorganisms: Carbon Transformation Test" is suitable to evaluate the toxicity of residues obtained after the biodegradation phase, but it is advisable to require that also the background activity of the control soil and the test soil are measured and that a correction is made for these background activities. If such correction is not made, the test soils would be favoured.

4.7.2.2 Novamont laboratory - Carbon transformation test at plateau phase

Following soil samples were used for the carbon transformation test:

- Control soil: blank soil without test item from biodegradation test (ISO 17556)
- Test soils: PHB, PBSe, PBSeT and Cellulose (1 g of sample/200g of wet soil) at the end of the biodegradation test (ISO 17556) after 210 days corresponding to the plateau phase

Two days before the start of the test each soil sample was pre-incubated in a beaker covered with a perforated aluminum foil at 22°C ± 2°C. Per glass bottle of 250 ml 50 g soil (dw) and 2.62 g perlite were added. 4000 mg glucose was added per kg soil (dw). The glucose was mixed with 0.5 g quartz sand (particle size <0,5 mm) and homogeneously mixed with the soil. The test was performed in triplicate and additionally also series without glucose addition was tested (for evaluation of background activity). The test was performed at 22°C ± 2°C and the test duration was 12 h. The glucose-induced respiration rate, based on oxygen consumption, was determined with OxyTop device.

The results are shown in Table 67 up to Table 69 and in Figure 52 and Figure 53. A lower respiration rate was observed in the blank reactors (without test materials) indicating no toxic effects for the test materials.

Table 67. Oxygen consumption - soil without glucose

Soil Sample (no) glucose	Replicates mgO_2/kg dry soil			Average mg O_2/kg dry soil without glucose	St. Dev.
	1	2	3		
BLANK	102.24	102.24	112.68	105.7	6.0
PHB	112.68	112.68	112.68	112.7	0.0
PBSe	133.2	133.2	143.28	136.6	5.8
PBSeT	173.88	173.88	184.32	177.4	6.0
Cellulose	122.76	133.2	122.76	126.2	6.0

Table 68. Oxygen consumption - soil with glucose

Soil Sample + glucose	Replicates - mg O_2/kg dry soil			Average mg O2 /kg dry soil with glucose	St. Dev.
	1	2	3		
BLANK	133.2	143.28	133.2	136.6	5.8
PHB	153.72	153.72	153.72	153.7	0.0
PBSe	173.88	184.32	184.32	180.8	6.0
PBSeT	235.44	214.92	225.36	225.2	10.3
Cellulose	173.88	194.4	173.88	180.7	11.8

Table 69. Net Values

Total Oxygen Consumption	NET mg O_2 /kg dry soil			Average
	1	2	3	
BLANK	27.5	37.6	27.5	**30.8**
PHB	41.0	41.0	41.0	**41.0**
PBSe	37.3	47.8	47.8	**44.3**
PBSeT	58.1	37.6	48.0	**47.9**
Cellulose	47.6	68.2	47.6	**54.5**

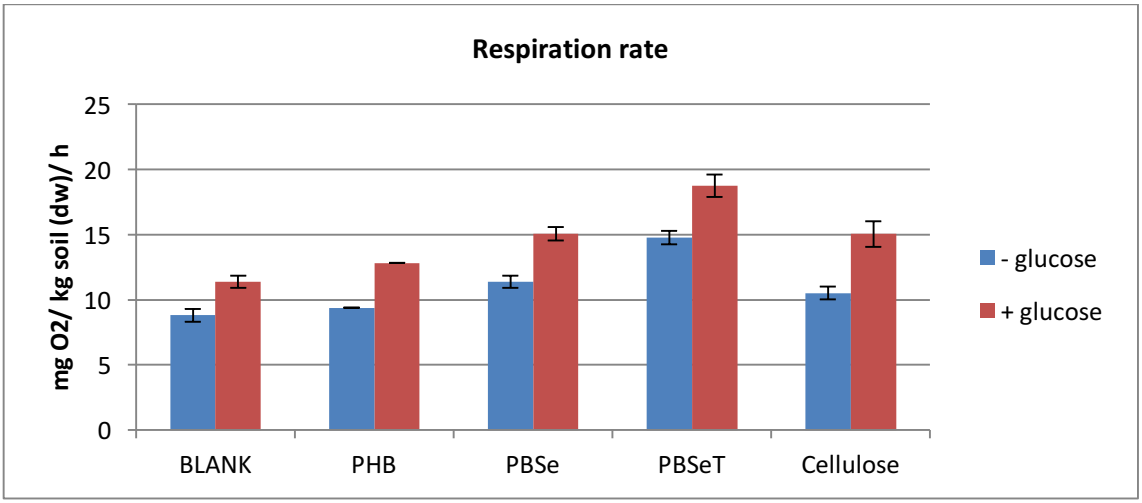

Figure 52. Respiration rate (mg O_2 / kg dry soil/h)

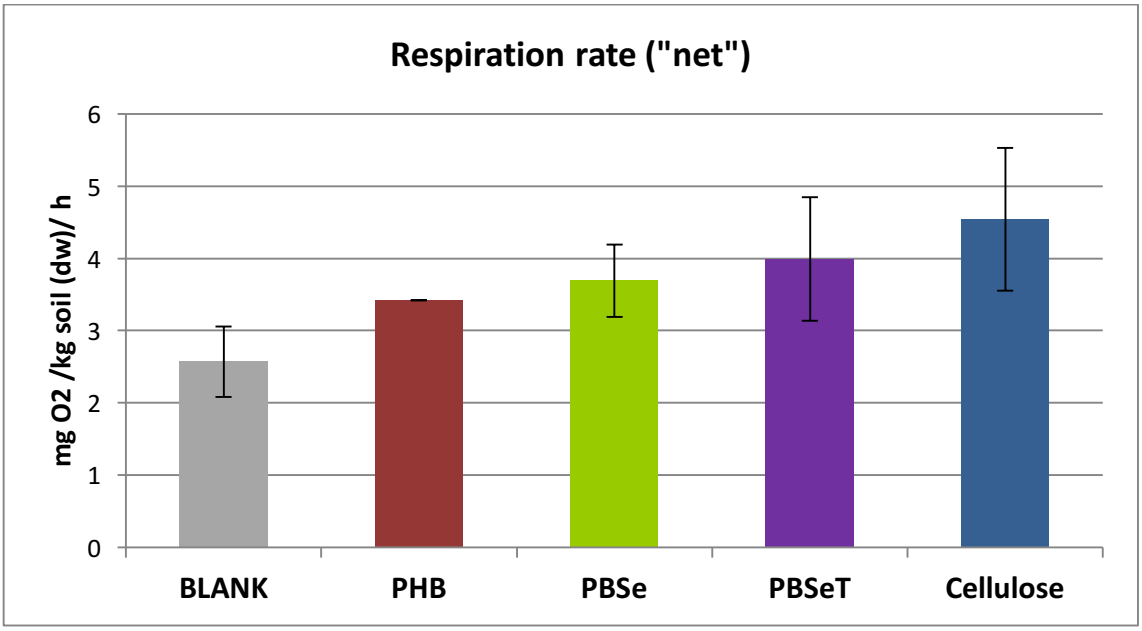

Figure 53. Net respiration rate (mg O_2 / kg dry soil/h)

4.7.3 Ammonium oxidation test (ISO 15685)

4.7.3.1 OWS laboratory - Ammonium oxidation test with soil of run 3

The first time that the ammonification test was executed, interferences with another constitute occurred during the determination of the nitrite content. Additional research was performed and this revealed that KCl caused the interference. Therefore, the procedure was adapted (instead of KCl H_2O was used and the analyses were executed immediately after the sampling in order to avoid that the ammonification further proceeded).

The test was executed after an incubation period of 78 days. The dry matter content of the different soils is given in Table 70. The results of the nitrite content (NO_2^--N) in the suspension (= 50% extract from the test and 50% H_2O), the nitrite content (NO_2^-) per kg dry soil and the nitrite formation rate after 0, 2 and 6 hours is given in Table 71 up to Table 74 for the control soil, the cellulose filter paper soil, the PBSe soil and the LDPE soil.

Table 70. Dry matter content of soils after incubation period

Soil	Dry matter content (%)
Control soil	87.0
Cellulose filter paper soil	86.8
PBSe soil	87.0
LDPE soil	88.8

Table 71. Results ammonification test control soil

Soil	Replicate	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
		0 h	2 h	6 h	0 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
Control soil	1	0.032	0.168	0.555	0.97	5.08	16.77	2.63
	2	0.03	0.164	0.564	0.91	4.95	17.04	2.69
	3	0.025	0.167	0.576	0.76	5.05	17.40	2.77
	MW	0.029	0.166	0.565	0.88	5.03	17.07	2.70
	s	0.00	0.00	0.01	0.11	0.06	0.32	0.07
	cv%	12%	1%	2%	12%	1%	2%	3%

Table 72. Results ammonification test cellulose filter paper soil

Soil	Repl	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
		0 h	2 h	6 h	0 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
Cellulose soil	1	0.022	0.171	0.558	0.67	5.17	16.88	2.70
	2	0.021	0.174	0.555	0.64	5.26	16.79	2.69
	3	0.02	0.166	0.546	0.61	5.02	16.52	2.65
	MW	0.021	0.170	0.553	0.64	5.15	16.73	2.68
	s	0.00	0.00	0.01	0.03	0.12	0.19	0.03
	cv%	5%	2%	1%	5%	2%	1%	1%

Table 73. Results ammonification test PBSe soil

Soil	Replicate	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
		0 h	2 h	6 h	0 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
PBSe soil	1	0.025	0.175	0.614	0.76	5.29	18.54	2.96
	2	0.025	0.174	0.613	0.76	5.25	18.51	2.96
	3	0.022	0.169	0.595	0.66	5.10	17.97	2.88
	MW	0.024	0.173	0.607	0.72	5.21	18.34	2.94
	s	0.00	0.00	0.01	0.05	0.10	0.32	0.05
	cv%	7%	2%	2%	7%	2%	2%	2%

Table 74. Results ammonification test LDPE test

Soil	Replicate	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
		0 h	2 h	6 h	0 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
LDPE soil	1	0.016	0.126	0.46	0.47	3.73	13.61	2.19
	2	0.015	0.119	0.455	0.44	3.52	13.46	2.17
	3	0.015	0.121	0.454	0.44	3.58	13.43	2.16
	MW	0.015	0.122	0.456	0.45	3.61	13.50	2.17
	s	0.00	0.00	0.00	0.02	0.11	0.10	0.01
	cv%	4%	3%	1%	4%	3%	1%	1%

Figure 54 and Figure 55 summarise the results of the ammonification test. It can be concluded that the ammonification test gives nice results. A clear nitrite formation is observed in all soils and little variance is observed between the replicates. The nitrite formation is somewhat higher in the PBSe soil when compared to the control soil (109%).

For LDPE, the nitrite formation is significantly lower when compared to the control soil (only 81%). No explanation can be found for this observation.

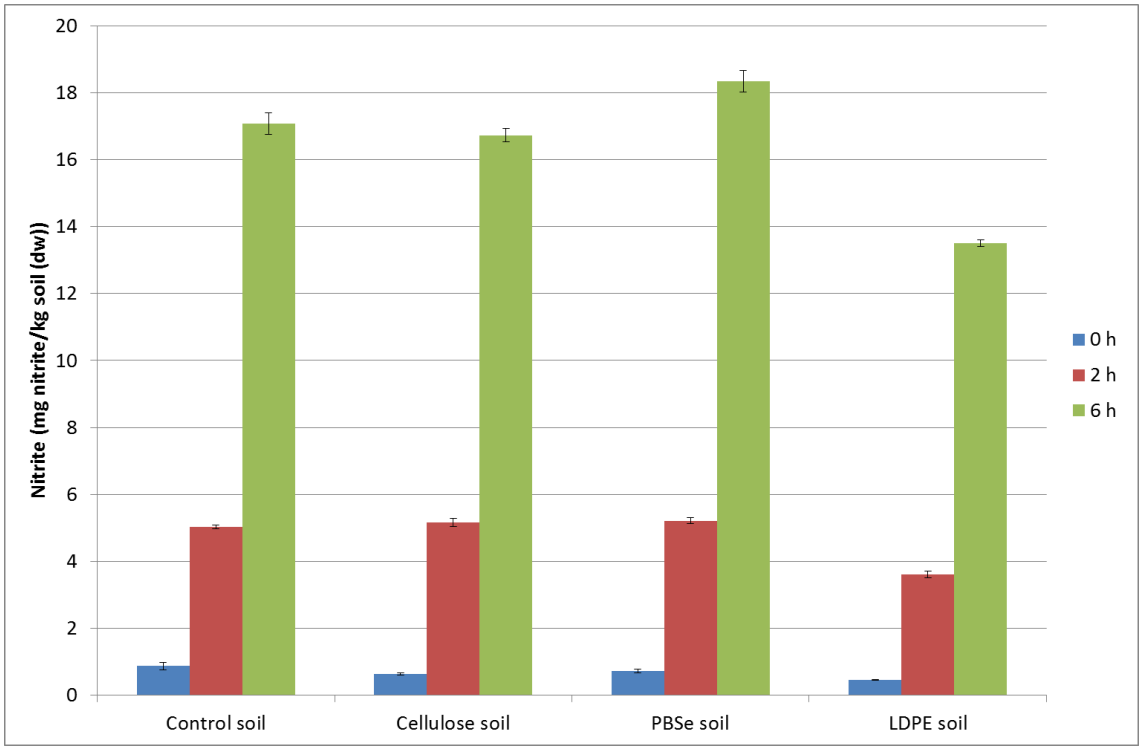

Figure 54. Summary nitrite content during test

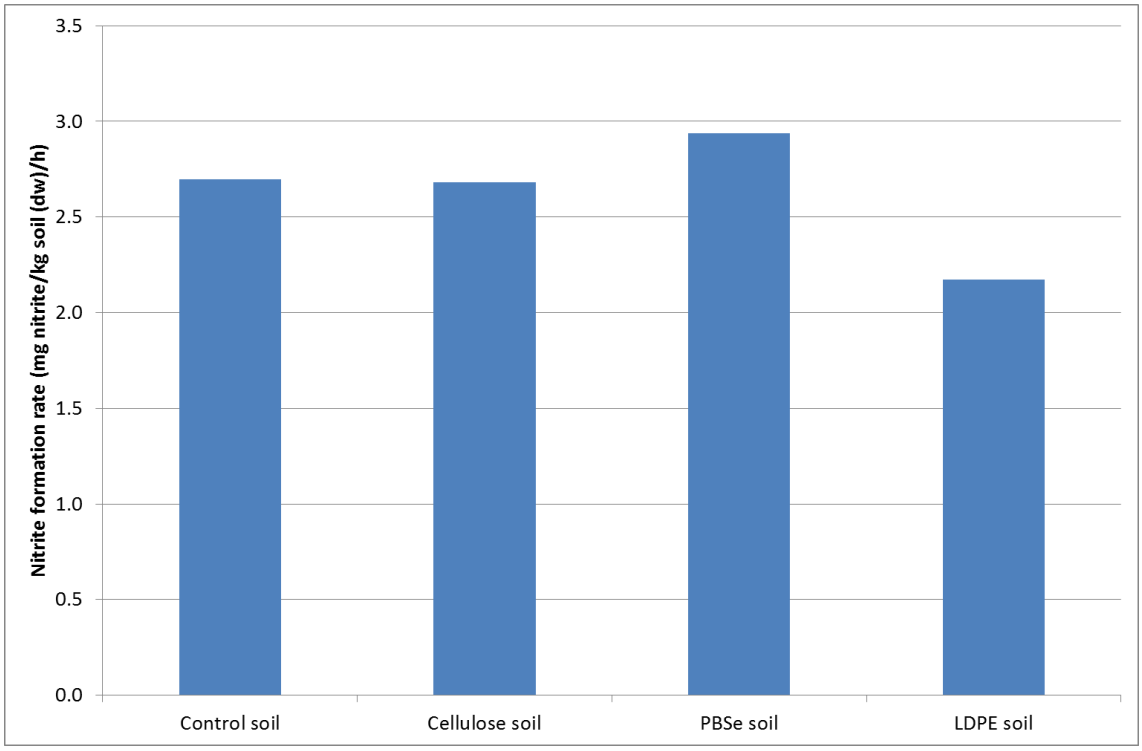

Figure 55. Summary nitrite formation rate

The test was repeated using the same soils (= after an incubation period of 127 days). The results of the dry weight analyses are given in Table 75. The results of the nitrite content (NO_2^--N) in the suspension (= 50% extract from the test and 50% H_2O). the nitrite content (NO_2^-) per kg dry soil and the nitrite formation rate after 0. 2 and 6 hours is given in Table 76 up to Table 80 for the control soil, the cellulose filter paper soil, the PBSe soil, the PBSeT soil and the LDPE soil.

Table 75. Dry matter content of soils after incubation period

Soil	Dry matter content (%)
Control soil	87.8
Cellulose filter paper soil	87.3
PBSe soil	87.6
PBSeT soil	87.3
LDPE soil	91.3

Table 76. Results ammonification test control soil

Soil	Replicate	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
		0 h	2 h	6 h	0 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
Control soil	1	0.028	0.142	0.474	0.84	4.25	14.19	2.23
	2	0.028	0.144	0.479	0.84	4.31	14.34	2.25
	3	0.025	0.144	0.474	0.75	4.31	14.19	2.24
	MW	0.027	0.143	0.476	0.81	4.29	14.24	2.24
	s	0.00	0.00	0.00	0.05	0.03	0.09	0.01
	cv%	6%	1%	1%	6%	1%	1%	1%

Table 77. Results ammonification test cellulose filter paper soil

Soil	Replicate	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate (mg NO_2/kg soil (dw)/h)
		0 h	2 h	6 h	0 h	2 h	6 h	
Cellulose soil	1	0.025	0.162	0.514	0.75	4.87	15.46	2.45
	2	0.025	0.162	0.525	0.75	4.87	15.79	2.51
	3	0.026	0.157	0.517	0.78	4.72	15.55	2.46
	MW	0.025	0.160	0.519	0.76	4.82	15.60	2.47
	s	0.00	0.00	0.01	0.02	0.09	0.17	0.03
	cv%	2%	2%	1%	2%	2%	1%	1%

Table 78. Results ammonification test PBSe soil

Soil	Replicate	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
		0 h	2 h	6 h	0 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
PBSe soil	1	0.014	0.19	0.616	0.42	5.70	18.47	3.01
	2	0.021	0.182	0.604	0.63	5.46	18.11	2.91
	3	0.02	0.173	0.601	0.60	5.19	18.02	2.90
	MW	0.018	0.182	0.607	0.55	5.45	18.20	2.94
	s	0.00	0.01	0.01	0.11	0.26	0.24	0.06
	cv%	21%	5%	1%	21%	5%	1%	2%

Table 79. Results ammonification test PBSeT soil

Soil	Replicate	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate (mg NO_2/kg soil (dw)/h)
		0 h	2 h	6 h	0 h	2 h	6 h	
PBSeT soil	1	0.02	0.162	0.534	0.60	4.87	16.06	2.58
	2	0.018	0.151	0.521	0.54	4.54	15.67	2.52
	3	0.019	0.147	0.524	0.57	4.42	15.76	2.53
	MW	0.019	0.153	0.526	0.57	4.61	15.83	2.54
	s	0.00	0.01	0.01	0.03	0.23	0.20	0.03
	cv%	5%	5%	1%	5%	5%	1%	1%

Table 80. Results ammonification test LDPE test

Soil	Replicate	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
		0 h	2 h	6 h	0 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
LDPE soil	1	0.011	0.095	0.371	0.32	2.74	10.68	1.73
	2	0.009	0.096	0.381	0.26	2.76	10.97	1.79
	3	0.009		0.363	0.26		10.45	1.70
	MW	0.010	0.096	0.372	0.28	2.75	10.70	1.74
	s	0.00	0.00	0.01	0.03	0.02	0.26	0.04
	cv%	12%	1%	2%	12%	1%	2%	3%

Figure 56 and Figure 57 summarise the results of the ammonification test. It can be concluded that the ammonification test gives nice results. A clear nitrite formation is observed in all soils and little variance is observed between the replicates. The nitrite formation in the cellulose filter soil (110%), the PBSe soil (131%) and the PBSeT (114%) soil is higher when compared to the blank soil.

For LDPE, the nitrite formation is significantly lower when compared to the control soil (only 78%). No explanation can be found for this observation. It is only noticed that the totals solids content of the LDPE soil is higher when compared to the other soils.

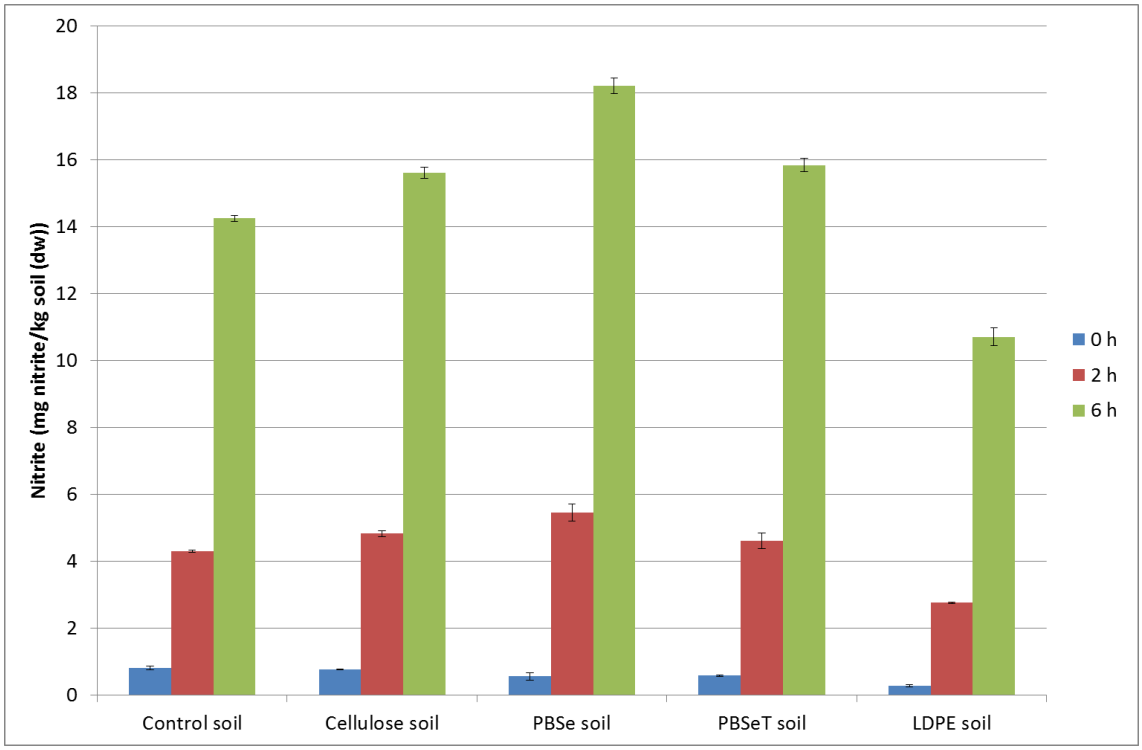

Figure 56. Summary nitrite content during test

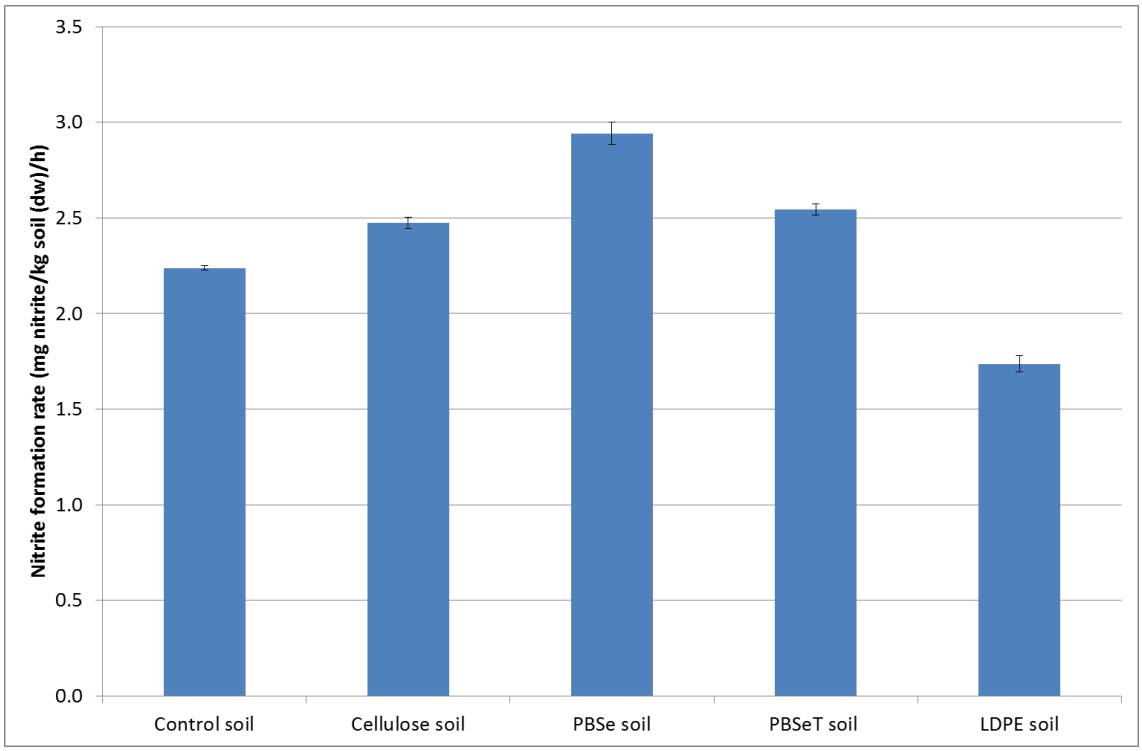

Figure 57. Summary nitrite formation rate

4.7.3.2 OWS laboratory - Ammonium oxidation test with soil of run 4

The **first** test was executed after an incubation period of 33 days. The results of the dry weight analyses are given in Table 81. The results of the nitrite content (NO_2^--N) in the suspension (= 50% extract from the test and 50% H_2O), the nitrite content (NO_2^-) per kg dry soil and the nitrite formation rate after 0, 2 and 6 hours is given in Table 82 up to Table 87 for the control soil, the cellulose filter paper soil, the PHB soil, the PBSe soil, the LDPE soil (source: Open-Bio) and the LDPE soil (source: Aldrich).

Table 81. Dry matter content of soils after incubation period

Soil	Dry matter content (%)
Control soil	79.4
Cellulose filter paper soil	77.7
PHB soil	78.2
PBSe soil	78.3
LDPE soil (source: Open-Bio)	76.9
LDPE soil (source: Aldrich)	79.5

Table 82. Results ammonification test control soil

Soil	Repl.	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate 0-6h (mg NO_2/kg soil (dw)/h)	Nitrite formation rate 2-6h (mg NO_2/kg soil (dw)/h)
		0 h	2 h	6 h	0 h	2 h	6 h		
Control soil	1	0.053	0.244	0.664	1.75	8.08	21.98	3.37	3.48
	2	0.052	0.237	0.649	1.72	7.85	21.49	3.29	3.41
	3	0.049	0.229	0.636	1.62	7.58	21.06	3.24	3.37
	MW	0.051	0.237	0.650	1.70	7.84	21.51	3.30	3.42
	s	0.00	0.01	0.01	0.07	0.25	0.46	0.07	0.05
	cv %	4%	3%	2%	4%	3%	2%	2%	2%

Table 83. Results ammonification test cellulose filter paper soil

Soil	Repl.	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate 0-6h (mg NO_2/kg soil (dw)/h)	Nitrite formation rate 2-6h (mg NO_2/kg soil (dw)/h)
		0 h	2 h	6 h	0 h	2 h	6 h		
Cellulose soil	1	0.049	0.243	0.67	1.66	8.22	22.66	3.50	3.61
	2	0.038	0.236	0.654	1.29	7.98	22.12	3.47	3.53
	3	0.038	0.234	0.66	1.29	7.91	22.32	3.51	3.60
	MW	0.042	0.238	0.661	1.41	8.04	22.37	3.49	3.58
	s	0.01	0.00	0.01	0.21	0.16	0.27	0.02	0.04
	cv %	15%	2%	1%	15%	2%	1%	1%	1%

Table 84. Results ammonification test PHB soil

Soil	Repl.	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate 0-6h (mg NO_2/kg soil (dw)/h)	Nitrite formation rate 2-6h (mg NO_2/kg soil (dw)/h)
		0 h	2 h	6 h	0 h	2 h	6 h		
PHB soil	1	0.042	0.201	0.525	1.41	6.75	17.63	2.70	2.72
	2	0.036	0.198	0.526	1.21	6.65	17.66	2.74	2.75
	3	0.036	0.187	0.523	1.21	6.28	17.56	2.73	2.82
	MW	0.038	0.195	0.525	1.28	6.56	17.62	2.72	2.76
	s	0.00	0.01	0.00	0.12	0.25	0.05	0.02	0.05
	cv %	9%	4%	0%	9%	4%	0%	1%	2%

Table 85. Results ammonification test PBSe soil

Soil	Repl.	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate 0-6h	Nitrite formation rate 2-6h
		0 h	2 h	6 h	0 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)	(mg NO_2/kg soil (dw)/h)
PBSe soil	1	0.026	0.168	0.529	0.87	5.64	17.75	2.81	3.03
	2	0.026	0.166	0.521	0.87	5.57	17.48	2.77	2.98
	3	0.022	0.165	0.522	0.74	5.54	17.51	2.80	2.99
	MW	0.025	0.166	0.524	0.83	5.58	17.58	2.79	3.00
	s	0.00	0.00	0.00	0.08	0.05	0.15	0.02	0.03
	cv %	9%	1%	1%	9%	1%	1%	1%	1%

Table 86. Results ammonification test LDPE (source: Open-Bio) soil

Soil	Repl.	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate 0-6h (mg NO_2/kg soil (dw)/h)	Nitrite formation rate 2-6h (mg NO_2/kg soil (dw)/h)
		0 h	2 h	6 h	0 h	2 h	6 h		
LDPE soil	1	0.032	0.193	0.584	1.09	6.59	19.94	3.14	3.34
	2	0.029	0.188	0.395	0.99	6.42	13.49	2.08	1.77
	3	0.029	0.186	0.556	0.99	6.35	18.99	3.00	3.16
	MW	0.030	0.189	0.512	1.02	6.45	17.47	2.74	2.75
	s	0.00	0.00	0.10	0.06	0.12	3.48	0.57	0.86
	cv %	6%	2%	20%	6%	2%	20%	21%	31%

Table 87. Results ammonification test LDPE (source: Aldrich) soil

Soil	Repl.	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate 0-6h (mg NO_2/kg soil (dw)/h)	Nitrite formation rate 2-6h (mg NO_2/kg soil (dw)/h)
		0 h	2 h	6 h	0 h	2 h	6 h		
LDPE soil (Aldrich)	1	0.027	0.188	0.598	0.89	6.21	19.77	3.15	3.39
	2	0.022	0.19	0.6	0.73	6.28	19.83	3.18	3.39
	3	0.02	0.185	0.586	0.66	6.12	19.37	3.12	3.31
	MW	0.023	0.188	0.595	0.76	6.20	19.66	3.15	3.36
	s	0.00	0.00	0.01	0.12	0.08	0.25	0.03	0.04
	cv %	16%	1%	1%	16%	1%	1%	1%	1%

Figure 58 and Figure 59 summarise the results of the ammonification test. In contrast to the previous executed tests, a negative effect was observed in the PHB and PBSe soil samples. Again a negative effect was observed in the LDPE soil (source: Open-Bio). No negative effect was observed in the Cellulose filter paper soil and the LDPE soil (source: Aldrich) (Table 88).

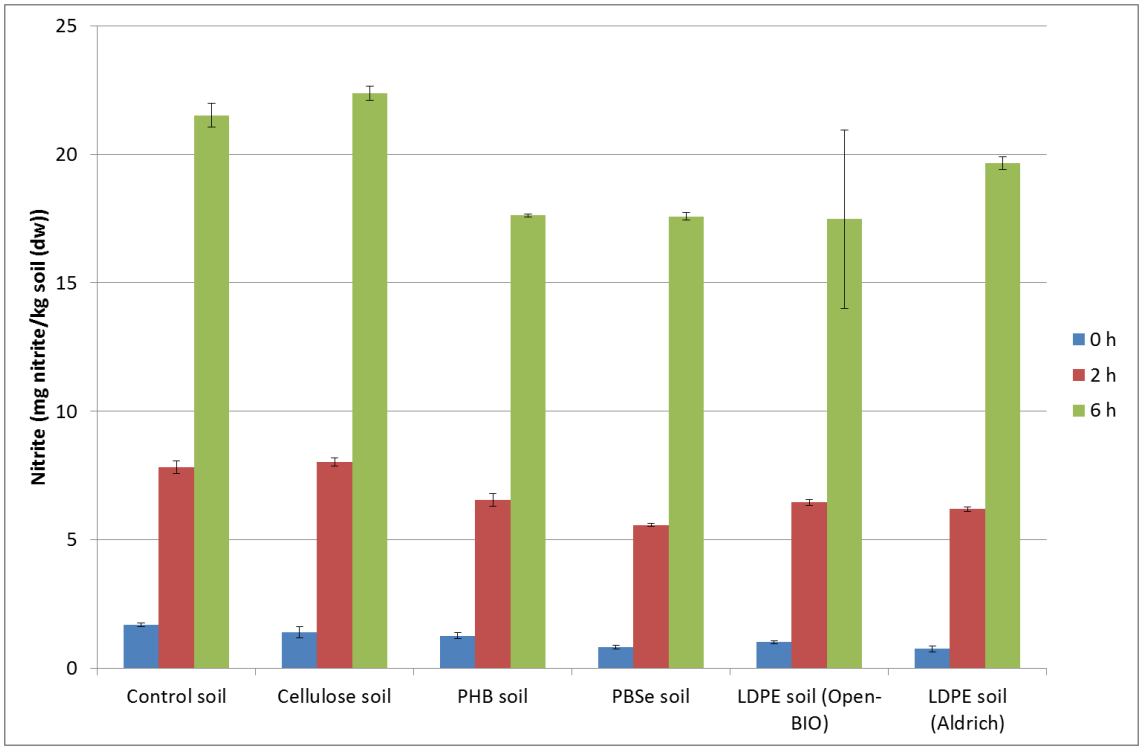

Figure 58. Summary nitrite content during test

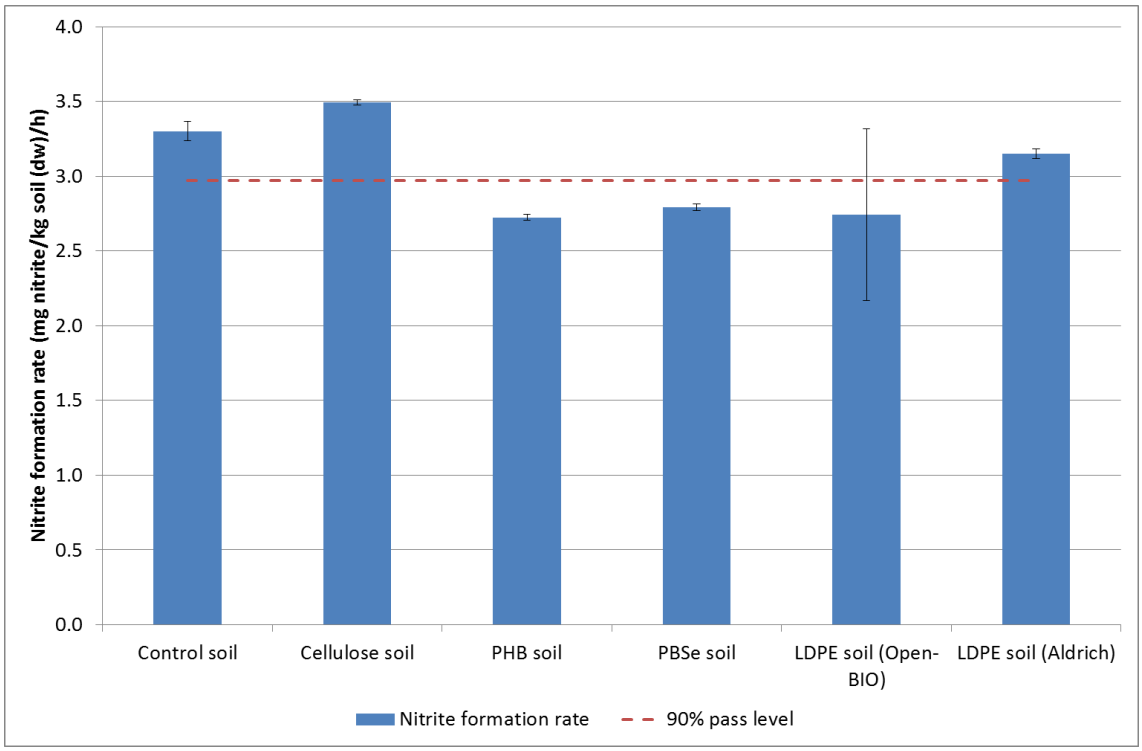

Figure 59. Summary nitrite formation rate (between 0h and 6h)

Table 88. Nitrite formation rate relative to control soil

	Nitrite formation rate (relative to control soil) 0 - 6 hours	Nitrite formation rate (relative to control soil) 2 - 6 hours
Cellulose soil	106%	105%
PHB soil	83%	81%
PBSe soil	85%	88%
LDPE soil (Open-BIO)	83%	81%
LDPE soil (Aldrich)	95%	98%

The second test using the soil of run 4 was executed after an incubation period of 68 days. The results of the dry weight analyses are given in Table 89. The results of the nitrite content (NO_2^--N) in the suspension (= 50% extract from the test and 50% H_2O), the nitrite content (NO_2^-) per kg dry soil and the nitrite formation rate after 0, 2 and 6 hours is given in Table 90 up Table 95 to for the control soil, the cellulose filter paper soil, the PHB soil, the PBSe soil, the LDPE soil (source: Open-Bio) and the LDPE soil (source: Aldrich).

Table 89. Dry matter content of soils after incubation period

Soil	Dry matter content (%)
Control soil	83.6
Cellulose filter paper soil	81.0
PHB soil	80.8
PBSe soil	80.5
LDPE soil (source: Open-Bio)	81.9
LDPE soil (source: Aldrich)	82.4

Table 90. Results ammonification test control soil

Soil	Repl.	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate 0-6h (mg NO_2/kg soil (dw)/h)	Nitrite formation rate 2-6h (mg NO_2/kg soil (dw)/h)
		0 h	2 h	6 h	0 h	2 h	6 h		
Control soil	1	0.071	0.182	0.586	2.23	5.72	18.42	2.70	3.17
	2	0.049	0.156	0.49	1.54	4.90	15.40	2.31	2.62
	3	0.047	0.156	0.549	1.48	4.90	17.25	2.63	3.09
	MW	0.056	0.165	0.542	1.75	5.18	17.02	2.55	2.96
	s	0.01	0.02	0.05	0.42	0.47	1.52	0.21	0.30
	cv %	24%	9%	9%	24%	9%	9%	8%	10%

Table 91. Results ammonification test cellulose filter paper soil

Soil	Repl.	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate 0-6h (mg NO_2/kg soil (dw)/h)	Nitrite formation rate 2-6h (mg NO_2/kg soil (dw)/h)
		0 h	2 h	6 h	0 h	2 h	6 h		
Cellulose soil	1	0.036	0.118	0.518	1.17	3.83	16.80	2.60	3.24
	2	0.039	0.141	0.5	1.26	4.57	16.21	2.49	2.91
	3	0.034	0.121	0.447	1.10	3.92	14.49	2.23	2.64
	MW	0.036	0.127	0.488	1.18	4.11	15.83	2.44	2.93
	s	0.00	0.01	0.04	0.08	0.41	1.20	0.19	0.30
	cv %	7%	10%	8%	7%	10%	8%	8%	10%

Table 92. Results ammonification test PHB soil

Soil	Repl.	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate 0-6h (mg NO_2/kg soil (dw)/h)	Nitrite formation rate 2-6h (mg NO_2/kg soil (dw)/h)
		0 h	2 h	6 h	0 h	2 h	6 h		
PHB soil	1	0.041	0.131	0.491	1.33	4.26	15.96	2.44	2.93
	2	0.039	0.132	0.431	1.27	4.29	14.01	2.12	2.43
	3	0.038	0.126	0.479	1.24	4.10	15.57	2.39	2.87
	MW	0.039	0.130	0.467	1.28	4.21	15.18	2.32	2.74
	s	0.00	0.00	0.03	0.05	0.10	1.03	0.17	0.27
	cv %	4%	2%	7%	4%	2%	7%	7%	10%

Table 93. Results ammonification test PBSe soil

Soil	Repl.	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate 0-6h (mg NO_2/kg soil (dw)/h)	Nitrite formation rate 2-6h (mg NO_2/kg soil (dw)/h)
		0 h	2 h	6 h	0 h	2 h	6 h		
PBSe soil	1	0.026	0.087	0.311	0.85	2.84	10.14	1.55	1.83
	2	0.024	0.09	0.338	0.78	2.94	11.03	1.71	2.02
	3	0.032	0.085	0.345	1.04	2.77	11.25	1.70	2.12
	MW	0.027	0.087	0.331	0.89	2.85	10.81	1.65	1.99
	s	0.00	0.00	0.02	0.14	0.08	0.59	0.09	0.15
	cv %	15%	3%	5%	15%	3%	5%	5%	8%

Table 94. Results ammonification test LDPE (source: Open-Bio) soil

Soil	Repl.	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate 0-6h (mg NO_2/kg soil (dw)/h)	Nitrite formation rate 2-6h (mg NO_2/kg soil (dw)/h)
		0 h	2 h	6 h	0 h	2 h	6 h		
LDPE soil (Open-BIO)	1	0.027	0.116	0.441	0.87	3.72	14.14	2.21	2.61
	2	0.025	0.112	0.466	0.80	3.59	14.94	2.36	2.84
	3	0.026	0.114	0.433	0.83	3.66	13.88	2.18	2.56
	MW	0.026	0.114	0.447	0.83	3.66	14.32	2.25	2.67
	s	0.00	0.00	0.02	0.03	0.06	0.55	0.10	0.15
	cv %	4%	2%	4%	4%	2%	4%	4%	6%

Table 95. Results ammonification test LDPE (source: Aldrich) soil

Soil	Repl.	Nitrite-N (mg NO_2-N/l suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate 0-6h (mg NO_2/kg soil (dw)/h)	Nitrite formation rate 2-6h (mg NO_2/kg soil (dw)/h)
		0 h	2 h	6 h	0 h	2 h	6 h		
LDPE soil (Aldrich)	1	0.032	0.201	0.498	1.02	6.41	15.87	2.48	2.37
	2	0.025	0.116	0.449	0.80	3.70	14.31	2.25	2.65
	3	0.022	0.107	0.491	0.70	3.41	15.65	2.49	3.06
	MW	0.026	0.141	0.479	0.84	4.51	15.28	2.41	2.69
	s	0.01	0.05	0.03	0.16	1.65	0.84	0.13	0.35
	cv %	19%	37%	6%	19%	37%	6%	6%	13%

Figure 60 up to Figure 62 summarise the results of the ammonification test. Comparable to the first run, no negative effect was observed in the Cellulose filter paper soil and the LDPE soil (source: Aldrich). Moreover, results of the PHB are (in contrast to the first run) also characterised by no negative effect. Most probably the longer stabilisation period is the reason for this difference. Results remain negative for the PBSe soil. This might be caused by the fact that PBSe is characterised by a somewhat slower biodegradation when compared to PHB. The LDPE soil (Open-Bio source) more than 90% was reached when compared to the blank soil taken into account the measurements between 2 and 6 hours. while this was not the case when taken into account the measurements between 0 and 6 hours (Table 96).

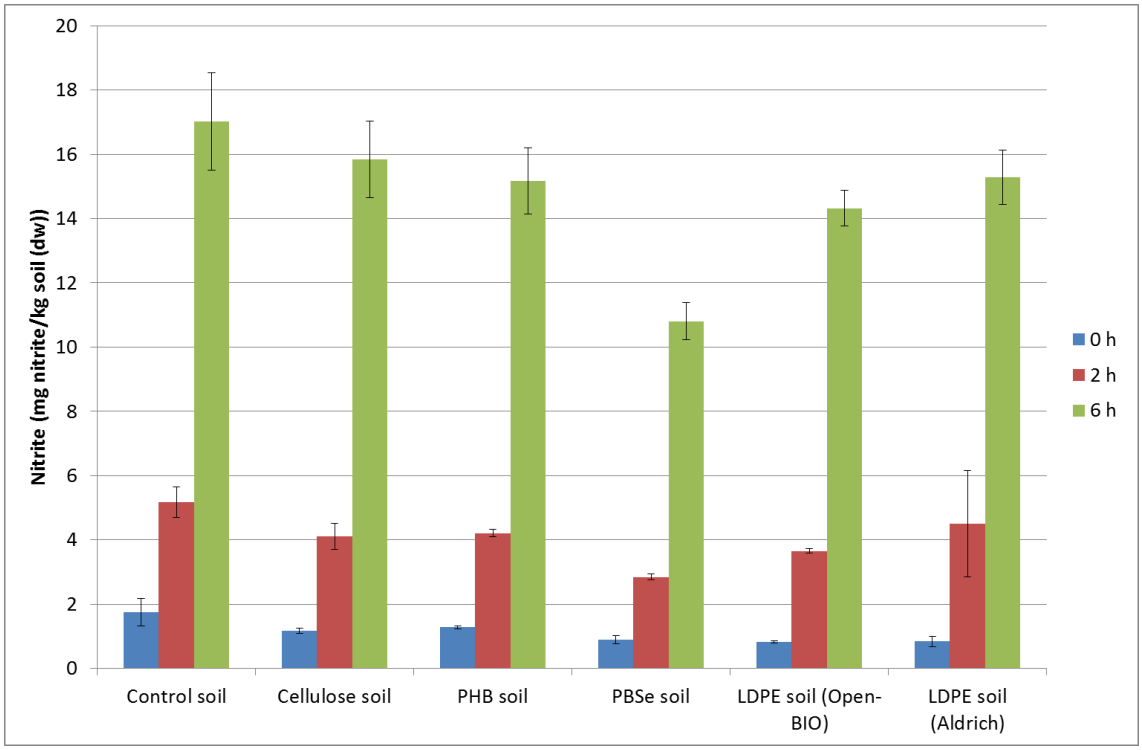

Figure 60. Summary nitrite content during test

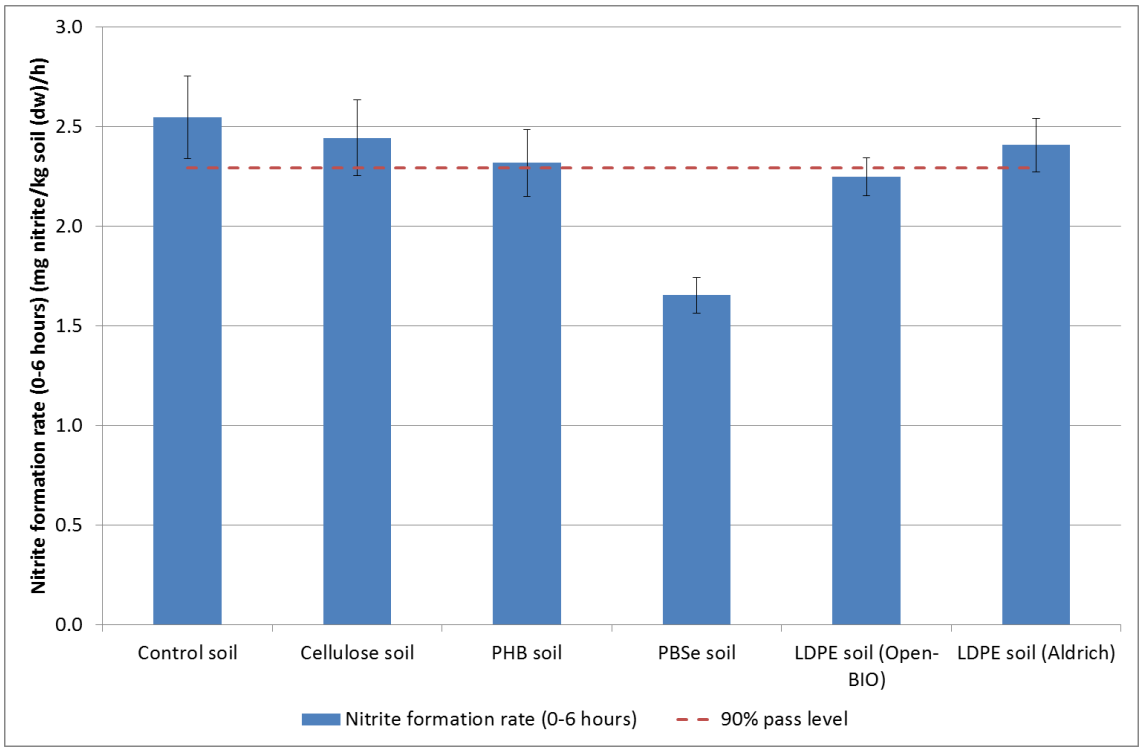

Figure 61. Summary nitrite formation rate (between 0h and 6h)

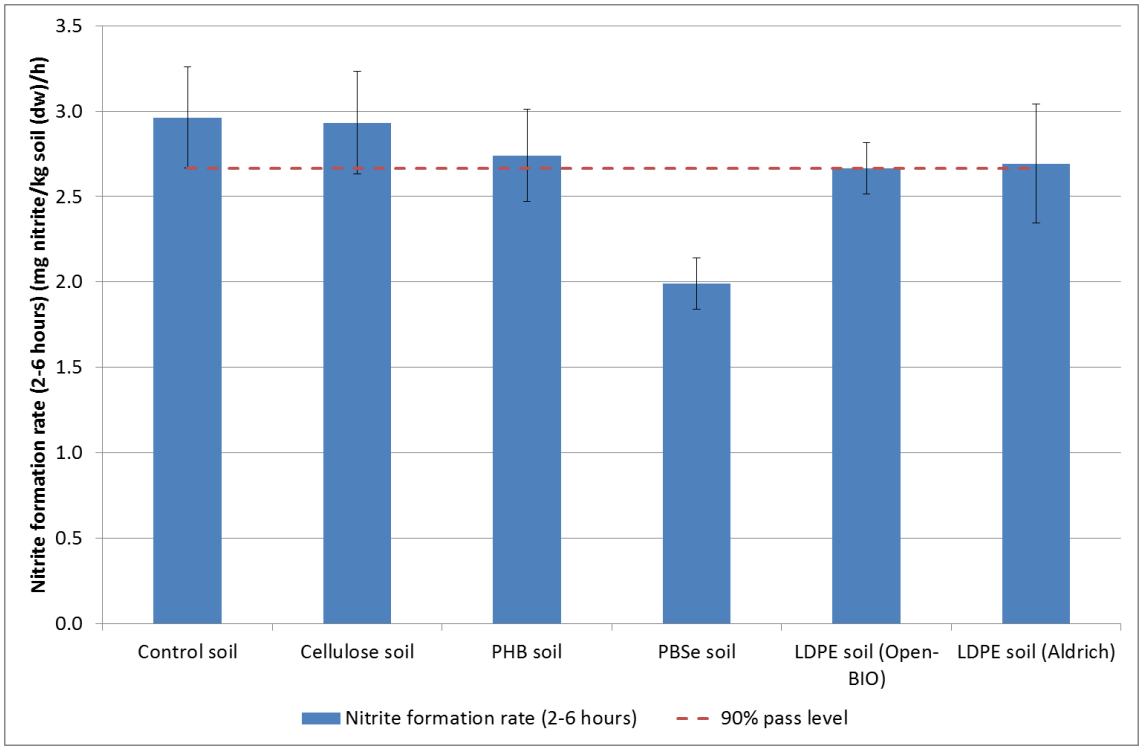

Figure 62. Summary nitrite formation rate (between 0h and 6h)

Table 96. Nitrite formation rate relative to control soil

	Nitrite formation rate (relative to control soil) 0 - 6 hours	Nitrite formation rate (relative to control soil) 2 - 6 hours
Cellulose soil	96%	99%
PHB soil	91%	93%
PBSe soil	65%	67%
LDPE soil (Open-Bio)	88%	90%
LDPE soil (Aldrich)	95%	91%

4.7.3.3 Novamont laboratory - Ammonium oxidation test during active biodegradation phase

Following samples were used:

- Blank soil: blank soil without test item
- Test soil: PHB, PBSe, LDPE soil (concentration: 1%) after 34 days of incubation; PBSeT soil (concentration: 1%) after 63 days of incubation corresponding to the active phase of biodegradation when material reached 50-60% of biodegradation.

A description of the parameters is given below:
Amount of soil: 25 g soil (ww) in 250 ml Erlenmeyer flask

Extraction volume (ml): 100 ml

Volume of the test medium ISO15685*: 100 ml – water content (ml) of soil
* Test medium (pH 7.2) composition: 10 ml stock solution A, 15 ml $NaClO_3$ 0.5 mol/l, 0.198 g $(NH_4)_2SO_4$ up to 1000 ml with distilled water.

Replicates: 3 for each soil sample

Incubation: 25°C ± 1°C in an orbital shaking incubator at 175 rpm

Test duration: 6 h

Sampling: 2 ml of soil slurry after 40 minutes (reported in tables as 0-1h), 2h and 6h of incubation + 2 ml KCl (4 mol/l). Samples were centrifuged at 3000g for 10 minutes.

Analysis: spectrophotometric determination of nitrite concentration at 543 nm after reaction with sulfanilamide and *N*-(1-naphthyl)ethylenediamine.

The test set-up is given in Table 97.

Table 97. Test set-up (active phase of biodegradation)

Test Soil	Days of incubation	Replicate	Water Content %	Soil ww (g)	Extraction volume (ml)	Test medium (ml)
Blank	63	3	14.6	25	100	96.36
PBSeT	63	3	14.8	25	100	96.36
Blank	34	3	14.6	25	100	96.36
PHB	34	3	15.2	25	100	96.21
PBSe	34	3	13.8	25	100	96.56
LDPE	34	3	14.9	25	100	96.27

The nitrite-nitrogen content in the suspension (N-NO_2 in mg/l) and the nitrite content per kg dry soil (NO_2 in mg/kg) after (0), 2 and 6 hours and the nitrite formation rate (NO_2 in mg/kg/h) of each soil tested in the active phase of biodegradation are reported in Table 98 up to Table 104.

Table 98. Nitrite-nitrogen and nitrite formation in blank soil

Blank soil	N-NO_2/L (mg N-NO_2/L suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
Replicate	0 h	2 h	6 h	0 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
1	0.015	0.090	0.253	0.470	2.756	7.777	1.26
2	0.017	0.069	0.270	0.513	2.126	8.300	1.54
3	0.019	0.078	0.257	0.598	2.414	7.894	1.37
MW	0.017	0.079	0.260	0.527	2.432	7.990	1.390
Dev. st	0.002	0.010	0.009	0.065	0.316	0.275	0.145
cv%	12	13	3	12	13	3	10

Table 99. Nitrite-nitrogen and nitrite formation in PBSeT soil

PBSeT Soil	N-NO_2/L (mg N-NO_2/L suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
Replicate	0 h	2 h	6 h	0 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
1	0.013	0.109	0.269	0.396	3.351	8.309	1.24
2	0.031	0.093	0.291	0.964	2.859	8.962	1.53
3	0.035	0.092	0.276	1.071	2.848	8.502	1.41
MW	0.026	0.098	0.279	0.810	3.019	8.591	1.393
Dev. st	0.012	0.009	0.011	0.363	0.288	0.336	0.144
cv%	45	10	4	45	10	4	10

Prior to performing the rapid nitrification test with the second series of sample in the active phase, the nitrite content of soil samples was determined following ISO 14238 (2013): nitrite was extracted by shaking samples with 1M KCl solution for 60 minutes at 150 rpm (Table 100).

Table 100. Nitrite content of soil samples after an 34 days of incubation (prior to perform rapid nitrification test), extraction with KCl 1M

Soil Sample	Nitrite (mg NO_2/kg soil (dw))	
	Average	dev st
Blank	1.12	0.07
PHB	2.60	0.02
PBSe*	43.32	1.35
LDPE	0.52	0.03

* Nitrite content of PBSe soil was also checked after 49 days of incubation following the extraction procedure of ISO14238 (2013). The nitrite level was 0,048 ± 0,009 mg/kg soil (dw).

Table 101. Nitrite-nitrogen and nitrite formation in blank soil

Blank	$N\text{-}NO_2/L$ (mg $N\text{-}NO_2/L$ suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
Replicate	0-1 h	2 h	6 h	0-1 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
1	0.044	0.062	0.244	1.356	1.901	7.498	1.399
2	0.040	0.064	0.249	1.239	1.965	7.648	1.421
3	0.036	0.063	0.253	1.122	1.944	7.786	1.461
MW	0.040	0.063	0.248	1.239	1.937	7.644	1.427
Dev. st	0.004	0.001	0.005	0.117	0.033	0.144	0.031
cv%	9	2	2	9	2	2	2

Table 102. Nitrite-nitrogen and nitrite formation in PHB soil

PHB Soil	$N\text{-}NO_2/L$ (mg $N\text{-}NO_2/L$ suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
Replicate	0-1 h	2 h	6 h	0-1 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
1	0.038	0.053	0.194	1.161	1.645	6.011	1.091
2	0.047	0.077	0.245	1.441	2.387	7.591	1.301
3	0.038	0.058	0.217	1.161	1.796	6.731	1.234
MW	0.041	0.063	0.219	1.254	1.943	6.778	1.209
Dev. st	0.005	0.013	0.026	0.160	0.392	0.791	0.107
cv%	13	20	12	13	20	12	9

Table 103. Nitrite-nitrogen and nitrite formation in PBSe soil

PBSe Soil	$N\text{-}NO_2/L$ (mg $N\text{-}NO_2/L$ suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
Replicate	0-1 h	2 h	6 h	0-1 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
1	1.719	1.684	2.035	52.368	51.310	61.996	2.671
2	1.938	1.757	2.142	59.033	53.532	65.275	2.936
3	1.691	1.813	2.066	51.522	55.225	62.948	1.931
MW	1.782	1.751	2.081	54.308	53.356	63.406	2.513
Dev. st	0.135	0.064	0.055	4.114	1.963	1.687	0.521
cv%	8	4	3	8	4	3	21

Table 104. Nitrite-nitrogen and nitrite formation in LDPE soil

LDPE	$N\text{-}NO_2/L$ (mg $N\text{-}NO_2/L$ suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
Replicate	0-1 h	2 h	6 h	0-1 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
1	0.040	0.076	0.282	1.222	2.337	8.717	1.595
2	0.042	0.080	0.279	1.287	2.477	8.621	1.536
3	0.045	0.084	0.293	1.383	2.584	9.050	1.616
MW	0.042	0.080	0.285	1.297	2.466	8.796	1.582
Dev. st	0.003	0.004	0.007	0.081	0.124	0.225	0.042
cv%	6	5	3	6	5	3	3

In Table 105 and Figure 63 the nitrite formation rate expressed as mg NO_2 /kg of soil dry weight and as percentage relative to blank soil are reported. The rate of nitrite formation is similar for blank and PBSeT soil. The highest nitrite formation rate was observed in the PBSe soil (176 ± 36 %) and the lowest in the PHB soil sample (85 ± 7%). The initial NO_2 concentration measured in the PBSe soil (43.3 mg/kg), prior to performing the nitrification rapid test, was much higher when compared to the content of other soil samples analyzed. The content was lowered to 0.048 mg/kg after an additional period of 15 days. These high differences in NO_2 content are probably caused by the different biological activities that could be found during the active phase of biodegradation of materials. No negative effect was observed in the LDPE soil (111±3%).

Table 105. Nitrite formation rate (2-6 hours)

Soil sample	Nitrite formation rate (mg NO_2/kg soil (dw)/h)		Nitrite formation rate relative to blank soil (%)	
	Average	dev st	Average	dev st
Blank	1.39	0.14	100	10.1
PBSeT	1.39	0.14	100	10.1
Blank	1.43	0.03	100	2.2
PHB	1.21	0.11	85	7.5
PBSe	2.51	0.52	176	36.5
LDPE	1.58	0.04	111	2.9

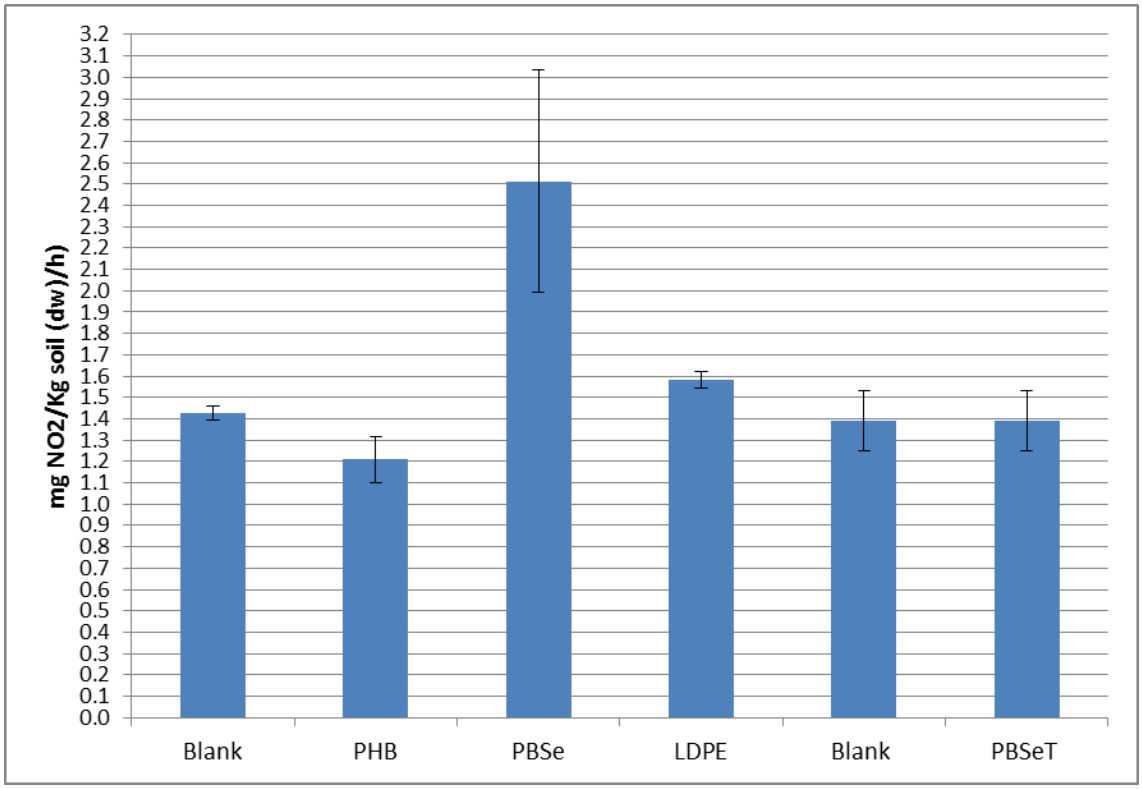

Figure 63. Nitrite formation rate (NO_2 mg/kg soil (dw)/h)

4.7.3.4 Novamont laboratory - Ammonium oxidation test at plateau phase

Following soil samples were used:

- Blank soil: blank soil without test item
- Test soils: PHB, PBSe, PBSeT and Cellulose soil (concentration: 1%) at the end of the biodegradation test corresponding to plateau phase after 103 days

Note: the soil samples were stored at 4°C during 7 months before starting the test

A description of the parameters is given below:
Pre- incubation: two days before test start each soil samples were pre-incubated in a beaker covered with a perforated aluminum foil at 22°C ± 2°C

Amount of soil: 10 g soil (ww) in 100 ml Erlenmeyer flask

Extraction volume (ml): 40 ml

Volume of the test medium ISO15685*: 40 ml – water content (ml) of soil
* Test medium (pH 7.2) composition: 10 ml stock solution A, 15 ml $NaClO_3$ 0.5 mol/l, 0.198 g $(NH_4)_2SO_4$ up to 1000 ml with distilled water.

Replicates: 3 for each soil sample

Incubation: 25°C ± 1°C in an orbital shaking incubator at 175 rpm

Test duration: 6 h

Sampling: 2 ml of soil slurry after 2h and 6h of incubation + 2 ml KCl (4 mol/l). Samples were centrifuged at 3000g for 10 minutes.

Analysis: spectrophotometric determination of nitrite concentration at 543 nm after reaction with sulfanilamide and *N*-(1-naphthyl)ethylenediamine.

The test set-up is given in Table 106.

Table 106. Test set-up

Test Soil	Days of incubation	Replicate	Water Content %	Soil ww (g)	Extraction volume (ml)	Test medium (ml)
Blank	103	3	14.6	10	40	38.5
PHB	103	3	15.5	10	40	38.5
PBSe	103	3	15.2	10	40	38.5
PBSeT	103	3	16.1	10	40	38.4
Cellulose	103	3	15.5	10	40	38.5

The nitrite-nitrogen content in the suspension (N-NO_2 in mg/l) and the nitrite content per kg dry soil (NO_2 in mg/kg) after 2 and 6 hours and the nitrite formation rate (NO_2 in mg/kg/h) of each soil tested at the plateau phase of biodegradation are reported in Table 107 up to Table 111.

Table 107. Nitrite-nitrogen and nitrite formation in blank soil

Blank	N-NO_2/L (mg N-NO_2/L suspension)		Nitrite (mg NO_2/kg soil (dw))		Nitrite formation rate (mg NO_2/kg soil (dw)/h)
Replicate	2 h	6 h	2 h	6 h	
1	0.019	0.081	0.577	2.500	0.481
2	0.017	0.083	0.523	2.553	0.507
3	0.028	0.080	0.865	2.457	0.398
MW	0.021	0.081	0.655	2.504	0.462
Dev. St	0.006	0.002	0.184	0.048	0.057
cv%	28	2	28	2	12

Table 108. Nitrite-nitrogen and nitrite formation in PHB soil

PHB Soil	N-NO_2/L (mg N-NO_2/L suspension)		Nitrite (mg NO_2/kg soil (dw))		Nitrite formation rate (mg NO_2/kg soil (dw)/h)
Replicate	2 h	6 h	2 h	6 h	
1	0.000	0.041	0.000	1.274	0.319
2	0.000	0.045	0.000	1.393	0.348
3	0.000	0.039	0.000	1.220	0.305
MW	0.000	0.042	0.000	1.296	0.324
Dev. St	0.000	0.003	0.000	0.088	0.022
cv%	0	7	0	7	7

Table 109. Nitrite-nitrogen and nitrite formation in PBSe soil

PBSe Soil	N-NO_2/L (mg N-NO_2/L suspension)		Nitrite (mg NO_2/kg soil (dw))		Nitrite formation rate (mg NO_2/kg soil (dw)/h)
Replicate	2 h	6 h	2 h	6 h	
1	0.007	0.089	0.215	2.751	0.634
2	0.004	0.075	0.118	2.310	0.548
3	0.008	0.080	0.258	2.461	0.551
MW	0.006	0.081	0.197	2.507	0.578
Dev. St	0.002	0.007	0.072	0.224	0.049
cv%	36	9	36	9	8

Table 110. Nitrite-nitrogen and nitrite formation in PBSeT soil

PBSeT Soil Replicate	N-NO_2/L (mg N-NO_2/L suspension) 2 h	6 h	Nitrite (mg NO_2/kg soil (dw)) 2 h	6 h	Nitrite formation rate (mg NO_2/kg soil (dw)/h)
1	0.034	0.112	1.077	3.502	0.606
2	0.029	0.123	0.903	3.860	0.739
3	0.036	0.121	1.120	3.795	0.669
MW	0.033	0.119	1.033	3.719	0.672
Dev. St	0.004	0.006	0.115	0.191	0.067
cv%	11	5	11	5	10

Table 111. Nitrite-nitrogen and nitrite formation in Celllose soil

Cellulose Soil Replicate	N-NO_2/L (mg N-NO_2/L suspension) 2 h	6 h	Nitrite (mg NO_2/kg soil (dw)) 2 h	6 h	Nitrite formation rate (mg NO_2/kg soil (dw)/h)
1	0.007	0.066	0.227	2.051	0.456
2	0.009	0.077	0.292	2.397	0.526
3	0.016	0.083	0.507	2.581	0.518
MW	0.011	0.075	0.342	2.343	0.500
Dev. St	0.005	0.009	0.147	0.269	0.038
cv%	43	11	43	11	8

In Table 112 and Figure 64 the nitrite formation rate expressed as mg NO_2 /kg of soil dry weight and as percentage relative to blank soil are reported. The nitrite formation rate is similar for the blank and the cellulose soil, while it is higher in the PBSe soil (125±11%) and PBSeT soil (145±14%). The lowest rate was measured in the PHB soil (70±5%). For PHB, the value was even lower than the 90% threshold.

Table 112. Nitrite formation rate (2-6 hours)

Soil Sample	Nitrite formation rate (mg NO_2/kg soil (dw)/h) Average	dev st	Nitrite formation rate relative to blank soil (%) Average	dev st
Blank	0.462	0.057	100	12.4
PHB	0.324	0.022	70	4.8
PBSe	0.578	0.049	125	10.6
PBSeT	0.672	0.067	145	14.4
Cellulose	0.500	0.038	108	8.3

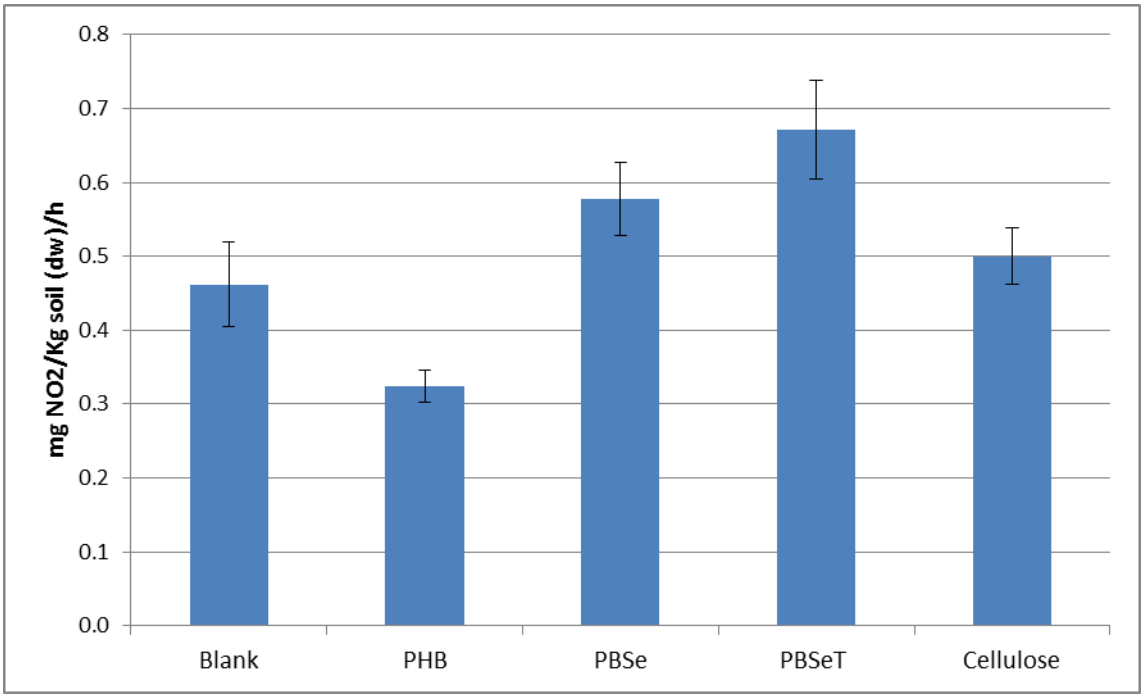

Figure 64. Nitrite formation rate (NO_2 mg/kg soil (dw)/h)

The test was repeated after a longer stabilisation phase.

Following soil samples were used:

- Blank soil: blank soil without test item
- Test soil: the same test soils analyzed in active phase of biodegradation (after 34 days of incubation), PHB, PBSe, LDPE soil (concentration: 1%), are analyzed also after 174 days of incubation corresponding to the **plateau phase** of biodegradation.

A description of the parameters is given below:

Amount of soil: 25 g soil (ww) in 250 ml Erlenmeyer flask

Extraction volume (ml): 100 ml

Volume of the test medium ISO15685: 100 ml – water content (ml) of soil

Replicates: 3 for each soil sample

Incubation: 25°C ± 1°C in an orbital shaking incubator at 175 rpm.

Test duration: 6 h

Sampling: 2 ml of soil slurry after 40 minutes (reported in tables as 0-1h), 2h and 6h of incubation + 2ml KCl (4 mol/l). Samples were centrifuged at 3000g for 10 minutes.

Analysis: spectrophotometric determination of nitrite concentration at 543 nm after reaction with sulfanilamide and *N*-(1-naphthyl)ethylenediamine.

The test set-up is given in Table 113

Table 113. Test set-up

Test Soil	Days of incubation	Replicate	Water content %	Soil ww (g)	Soil dw (g)	Extraction volume (ml)	Test medium (ml)
Blank	174	3	16.5	25	20.88	100	95.88
PHB	174	3	16.8	25	20.80	100	95.80
PBSe	174	3	17.1	25	20.74	100	95.74
LDPE	174	3	15.3	25	21.18	100	96.18

The nitrite-nitrogen content in the suspension (N-NO_2 in mg/l) and the nitrite content per kg dry soil (NO_2 in mg/kg) after 2 and 6 hours and the nitrite formation rate (NO_2 in mg/kg/h) of each soil tested at the plateau phase of biodegradation are reported in Table 114 up to Table 117.

Table 114. Nitrite-nitrogen and nitrite formation in blank soil

Blank	$N\text{-}NO_2/L$ (mg $N\text{-}NO_2/L$ suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
Replicate	0-1 h	2 h	6 h	0-1 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
1	0.022	0.057	0.124	0.699	1.781	3.911	0.533
2	0.027	0.037	0.119	0.852	1.169	3.747	0.645
3	0.025	0.034	0.113	0.776	1.071	3.550	0.620
MW	0.025	0.043	0.119	0.776	1.340	3.736	**0.599**
Dev. st	0.002	0.012	0.006	0.076	0.385	0.180	0.059
cv%	10	29	5	10	29	5	10

Table 115. Nitrite-nitrogen and nitrite formation in PHB soil

PHB Soil	$N\text{-}NO_2/L$ (mg $N\text{-}NO_2/L$ suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
Replicate	0-1 h	2 h	6 h	0-1 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
1	0.006	0.010	0.043	0.186	0.307	1.360	0.263
2	0.008	0.031	0.042	0.263	0.976	1.327	0.088
3	0.008	0.030	0.044	0.252	0.932	1.393	0.115
MW	0.007	0.023	0.043	0.234	0.738	1.360	**0.155**
Dev. st	0.001	0.012	0.001	0.042	0.374	0.033	0.094
cv%	18	51	2	18	51	2	61

Table 116. Nitrite-nitrogen and nitrite formation in PBSe soil

PBSe Soil	$N\text{-}NO_2/L$ (mg $N\text{-}NO_2/L$ suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
Replicate	0-1 h	2 h	6 h	0-1 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
1	0.025	0.053	0.152	0.792	1.672	4.807	0.784
2	0.020	0.040	0.148	0.638	1.276	4.686	0.852
3	0.021	0.050	0.146	0.671	1.584	4.631	0.762
MW	0.022	0.048	0.149	0.700	1.511	4.708	**0.799**
Dev. st	0.003	0.007	0.003	0.081	0.208	0.090	0.047
cv%	12	14	2	12	14	2	6

Table 117. Nitrite-nitrogen and nitrite formation in LDPE soil

LDPE	N-NO_2/L (mg N-NO_2/L suspension)			Nitrite (mg NO_2/kg soil (dw))			Nitrite formation rate
Replicate	0-1 h	2 h	6 h	0-1 h	2 h	6 h	(mg NO_2/kg soil (dw)/h)
1	0.023	0.052	0.126	0.711	1.605	3.899	0.574
2	0.023	0.043	0.124	0.700	1.325	3.834	0.627
3	0.025	0.071	0.123	0.775	2.208	3.813	0.401
MW	0.023	0.055	0.124	0.729	1.713	3.849	**0.534**
Dev. st	0.001	0.015	0.001	0.041	0.451	0.045	0.118
cv%	6	26	1	6	26	1	22

The nitrite formation rate is shown in Table 118 and in Figure 65. In the ammonification test performed in the plateau phase at the end of polymers biodegradation (after 174 days of incubation), the highest nitrite formation rate was observed in the PBSe soil (133 ± 8% relative to blank soil) and the lowest in the PHB soil sample (26 ± 16% relative to blank soil), as observed in the test conducted during the active phase. For LDPE, the value was slightly lower than the 90% threshold (89± 20%).

Table 118. Nitrite formation rate in mg/kg soil (dw)/h and relative to blank soil (%)

Soil sample	Nitrite formation rate (mg NO_2/kg soil (dw)/h)		Nitrite formation rate relative to blank soil (%)	
	Average	dev st	Average	dev st
Blank	0.60	0.06	100	9.8
PHB	0.16	0.09	26	15.8
PBSe	0.80	0.05	133	7.9
LDPE	0.53	0.12	89	19.7

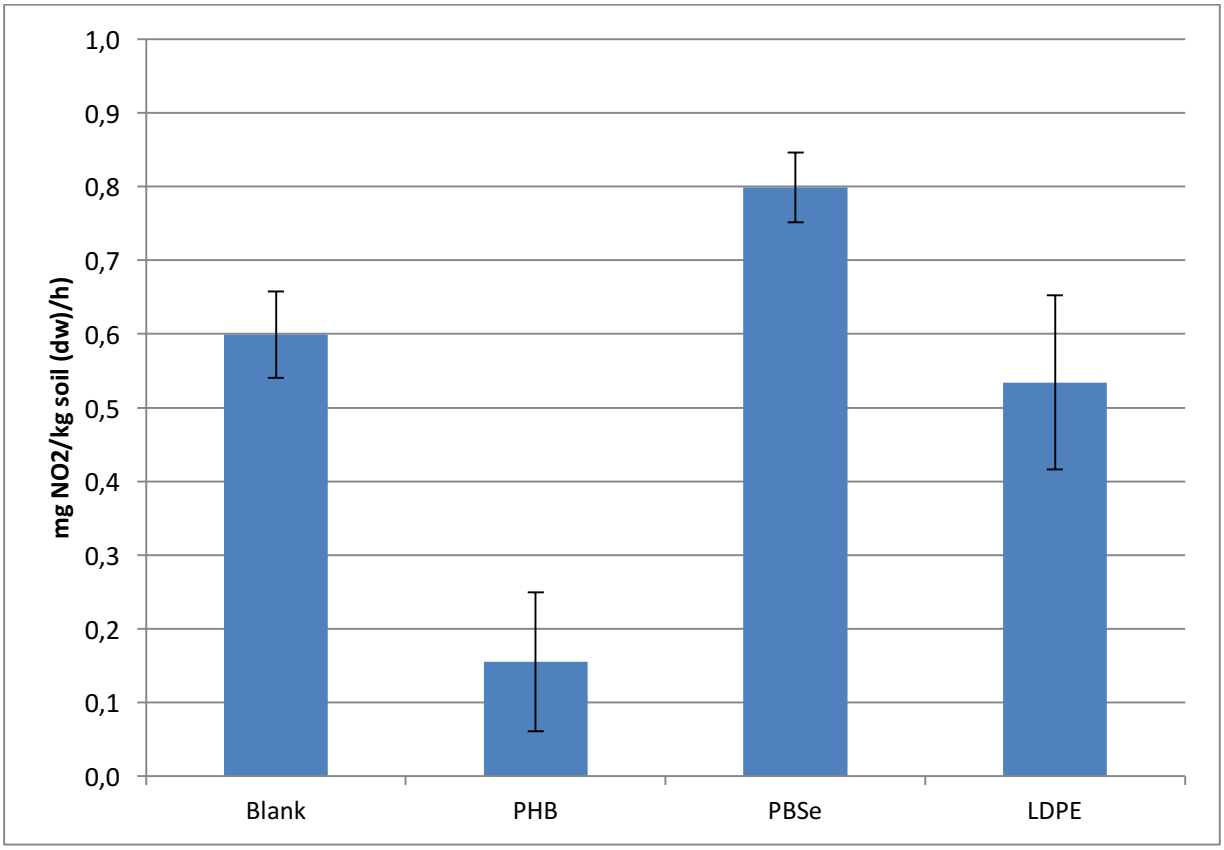

Figure 65. Nitrite formation rate (NO_2 mg/kg soil (dw)/h)

5 Conclusion

The objective of this research was to investigate the applicability of toxicity test methods for chemicals (= direct toxicity test method) as toxicity test methods for polymer residuals obtained after a biodegradation phase in soil. This research has been used as pre-standardisation work during the development of prEN 17033 *Plastics - biodegradable mulch films for use in agriculture and horticulture - requirements and test methods* (CEN/TC 249 Plastics/WG7 Thermoplastic films for use in agriculture/TG 1 Biodegradable mulch films).

When a biodegradable substance is added to soil, the soil characteristics change (at least temporarily). During biodegradation, organic carbon of the sample is converted to carbon dioxide by means of micro-organisms. Not all carbon is immediately converted to carbon dioxide. Part of the carbon is also converted to microbial biomass. In order to produce microbial biomass, the microorganisms also need nitrogen. Therefore, the nitrogen content (ammonium and/or nitrate) and consequently also the electrical conductivity, which is representative for the salt content, of the soil both decrease during the biodegradation phase. Such changes can strongly influence the results of plant toxicity tests (as ammonium and nitrate are fertilisers that influence the plant biomass), earthworm toxicity tests (as earthworms are sensitive for high salt contents) and microbial toxicity tests that monitor the carbon and nitrogen transformation in soil.

Following toxicity tests were investigated:

- Toxicity towards higher plants
  - OECD 208 *Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test*
- Toxicity towards earthworms
  - ISO 11268-1 *Soil quality — Effects of pollutants on earthworms — Part 1: Determination of acute toxicity to Eisenia fetida/Eisenia andrei* and/or OECD 207 *Earthworm, acute toxicity test*
- Toxicity towards soil micro-organisms
  - ISO 14238 *Soil quality – Biological methods – Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes*
  - OECD 217 *Soil microorganisms: Carbon Transformation Test*
  - ISO 15685 *Soil quality - Determination of potential nitrification and inhibition of nitrification - Rapid test by ammonium oxidation*

In order to discover the weaknesses of the above mentioned test methods towards testing of biodegradation residuals of polymers, several polymers with varying biodegradability (LDPE, cellulose, PHB, PBSe and PBSeT) were added in a 1% concentration to soil. Two types of soil were used: natural soil and standard soil as prescribed by ISO 17556. During the active biodegradation phase and/or in the plateau phase, the obtained soils were used for the toxicity tests.

From the **soil biodegradation test**, it can be concluded that test item LDPE is a not biodegradable polymer, cellulose is a positive reference material and PHB, PBSe and PBSeT are polymers that are biodegradable in soil. PHB, PBSe and PBSeT biodegraded at a similar rate at Novamont laboratory, while PBSeT was characterised by a lower biodegradation rate at OWS laboraratory (which used natural soil to which no nutrients were added).

The performed **plants toxicity tests** when using natural soil (without addition of nutrients) clearly illustrate that the nutrient content of the soil after the biodegradation phase should be carefully monitored before starting the plant toxicity tests. Due to the biodegradation of the test item that was added in a 1% concentration at start of the incubation phase, the nutrient content in the test soil decreases. Consequently, a significantly lower plant yield is measured for the plants in the test soil when compared to the blank soil. This lower plant yield is also observed in the cellulose soil (= positive reference). The lower plant yield in the cellulose soil illustrates that the lower plant yield is not caused by a toxic effect, but by a fertilizing effect. In order to evaluate the toxicity of the test item in a correct way, it is therefore recommended to compared the germination and plant yield also with a positive reference soil. Moreover, it is recommended to measure the nitrogen content before the plant toxicity tests. In case it is observed that the nitrate content in the test soil is indeed lower than the blank soil, the fertilising effect can be solved by adding a fertilizer till a similar nitrogen content is obtained as in the blank soil.

When using standard soil (to which a nutrient solution has been added) or natural soil to which nutrients were added, it is observed that the nutrient content in the blank soil can also be too high to allow normal plant germination and plant growth. Several tests (OWS standard soil and Novamont) illustrated that the validity criterion was not reached (< 70% germination in blank soil) due to the high nutrient levels in the blank soil. When the nutrient content in the blank was too high, it is observed that the germination and the plant biomass in the test soils is higher than the blank soil. This can be explained by the fact that the nutrient content in the test soils is lower when compared to the blank soil (and in this case more optimal for the plant germination and growth). It is recommended to avoid the use of standard soil or natural soil to which a lot of nutrients were added. In case standard soil would be used, the concentration of the salt solution as prescribed by ISO 17556 should be significantly reduced to avoid invalid toxicity tests. Moreover, in case of effects on blank soil the use of reference soil (after cellulose degradation) is recommended to interpret the results of the toxicity test correctly.

The performed **earthworm toxicity tests** when using natural soil (without addition of nutrients) does not reveal problems. Earthworm weight is even generally higher when biodegradable polymers are added. However, when using natural soil to which nutrients are added or standard soil as prescribed by ISO 17556, results can become very difficult to interpret. The high nutrient content in the blank soil can result in total mortality of the earthworms and invalid results. When 100% mortality was observed in the nutrient rich blank soil, it was also observed that the survival in the test soils was significantly higher (due to the fact that the

nutrient and salt content has decreased due to the biodegradation). It is recommended to avoid the use of standard soil or natural soil to which a lot of nutrients were added. In case standard soil would be used, the concentration of the salt solution as prescribed by ISO 17556 should be significantly reduced to avoid invalid toxicity tests. Moreover, in case of effects on blank soil the use of reference soil (after cellulose degradation) is recommended to interpret the results of the toxicity test correctly.

The **long term nitrification test (ISO 14238)** was evaluated by means of the addition of Luzerne meal and ammonium sulfate. Both nitrogen source gave comparable results. Results in natural soil to which no nutrients were added showed no nitrate formation in the test soils. This was most probably caused by the fact that nitrogen had become limiting and that the microorganisms immediately consumed the ammonium instead of converting it to nitrate. When performing the test on the standard soil series, a nitrate formation was observed which was in most cases even higher than the nitrate formation in the blank soil. From these results, it can be concluded that it is important that nitrogen does not become limiting during the biodegradation phase. Besides requiring that the nitrate formation should be at least 90% when compared to the blank soil or positive reference soil, the pass criteria of this test could be expanded by also requiring that (1) the trend of $N\text{-}NH_4$ decrease should be similar of blank soil (or reference soil) and after 28 days the $N\text{-}NH_4$ content should be less than 10 mg/kg and (2) after 28 days no nitrite should be measurable in the soil.

The **carbon transformation test (OECD 217)** can be used to evaluate the toxicity of biodegradation residuals, but the performed tests showed that it is important to test in parallel also the soil as such (without addition of glucose). The results should be corrected by means of the background activity measured in the series without glucose. It must be noted that the biodegradation test (ISO 17556) is in fact also a kind of carbon transformation test. Therefore, it can be argued that this test might be superfluous. Moreover, in our opinion the carbon transformation test is not very sensitive for the evaluation of toxicity of biodegradation residuals of polymers as glucose is easily biodegradable and only in presence of strong toxicity this test might be useful.

The short term **rapid ammonification test** (ISO 15685) has been performed several times by both laboratories. Most of the results seem rather promising, but still some problems were detected for which no clear explanation was found (e.g. lower nitrite formation in LDPE series and PHB series). Using this test method for the evaluation of the toxicity of biodegradation residuals of polymers after an incubation period is soil, seems not (yet) possible based on the performed research. Additional research is needed in order to demonstrate and confirm the suitability of this test method for the evaluation of toxicity towards soil microorganisms of biodegradation residuals of polymers.

In general to perform ecotoxicity tests during the active biodegradation phase could be a risk due to the fact that a lot of processes are taking place at the same moment. In fact different “strange” results were obtained when performing toxicity tests during the active biodegrada-

tion phase. The biodegradation of a material is a transitory phenomenon and it is probably better to determine the effects on the soil at the end of the biodegradation process.

Finally, it can be concluded that the direct toxicity test methods can be suitable to evaluate also the toxicity of biodegradation residuals of polymers. Especially the toxicity test with higher plants and the toxicity test with earthworms are suitable to use as test method for the evaluation of toxicity of biodegradation residuals of polymers. More problems were observed for the toxicity tests with soil micro-organisms (most probably caused by the fact that the soil characteristics change due to the addition of biodegradable substances) and therefore caution is needed when interpreting the results of such toxicity tests. During the research activity some false positive results were obtained for the toxicity tests with soil micro-organisms, this fact makes this kind of test not yet ready for standardization. All performed tests clearly illustrate that it is useful to determine the soil characteristics (pH, nutrients, etc.) at least before the toxicity tests (and even during the incubation period to monitor if nutrients do not become limiting) and it is recommended to positive (cellulose) reference soil as “control” to calculate the eventual effects. The use of only blank soil could underestimate the effects.